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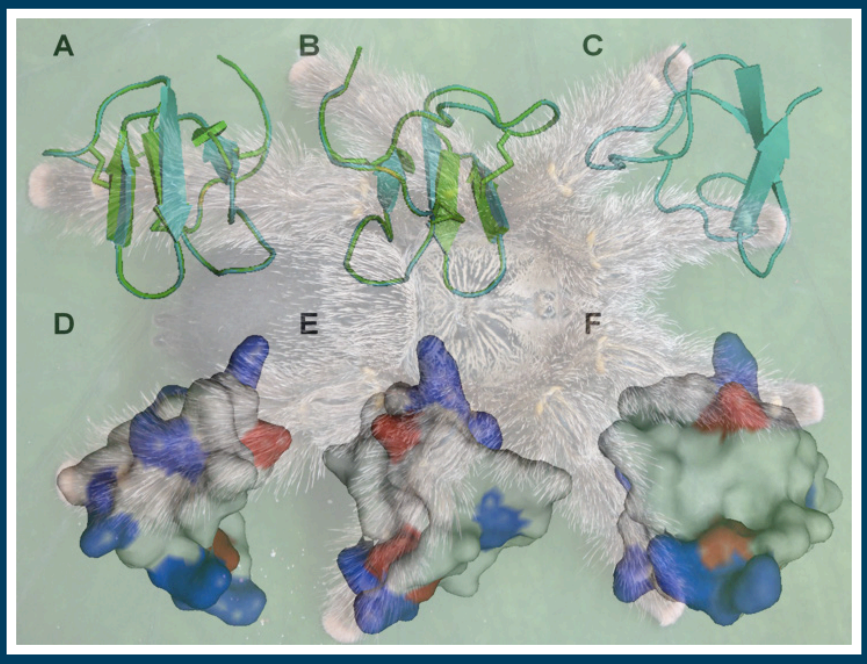
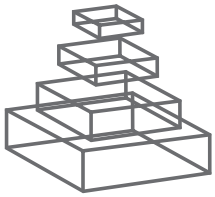


ANTIMICROBIAL COMPOUNDS FROM NATURAL SOURCES

Topic Editors
Mirian A. Hayashi, Fernando C. Bizerra and Pedro Ismael Da Silva Jr

frontiers in MICROBIOLOGY



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ANTIMICROBIAL COMPOUNDS FROM NATURAL SOURCES

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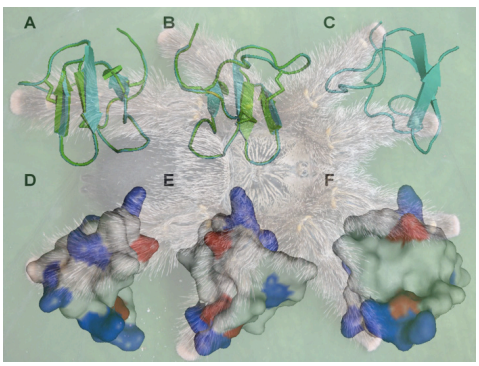

Structural model for Juruin, an antifungal peptide from the venom of the Amazonian Pink Toe spider, which contains the inhibitory cystine knot motif.
Comparison of the structures of Juruin from Avicularia juruensis and U1-theraphotoxin-Ba1a (PDB ID: 2KGH) from Brachypelma ruhnaui, after homology modeling of Juruin.
Images taken from: Ayroza G, Ferreira ILC, Sayegh RSR, Tashima AK and da Silva PI (2012) Juruin: an antifungal peptide from the venom of the Amazonian Pink Toe spider, Avicularia juruensis, which contains the inhibitory cystine knot motif. *Front. Microbio.* 3:324. doi: 10.3389/fmicb.2012.00324

The nature is a generous source of a number of compounds with potential application for the treatment of several diseases including the infectious diseases, which is of utmost concern for the modern medicine due to the observed striding antimicrobial resistance. A number of sources of natural compounds with valuable and clinical antimicrobial activity can be listed, comprising medicinal plants, marine and terrestrial organisms, which includes fungi and bacteria. Nevertheless, there is still a vast fauna and flora that, once systematically explored, could provide additional antimicrobial leads and drugs.

Investigators were invited to contribute with original research and/or review articles on this area, specifically with studies exploiting the mechanism of action and the structure-activity aspects of natural compounds with antimicrobial activity that provides insights on potential ways to overcome the antimicrobial resistance.

Therefore, thanks to the contribution of active researchers in the field, several scientific studies mainly focused on natural products with antimicrobial activity are presented in this Research Topic Ebook.

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EDITORIAL
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Antimicrobial compounds from natural sources

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Infectious diseases are one of the main causes of morbidity and mortality worldwide. Nowadays many infections are often caused by multi-resistant microorganisms resulting in difficult to treat diseases and, consequently, substantial increases in healthcare costs. The relative easy access to the antimicrobials and also the massive employment of these compounds for industrial purposes, including food production, have both strongly contributed to the progressive increase of resistant microorganisms. As a result, these multi-resistant microorganisms are reasserting themselves as worldwide threats.

Research into natural products has demonstrated significant progress in the discovery of new compounds with antimicrobial activity. In fact, nature is a generous source of compounds with the potential to treat diseases, including infectious diseases. Among the known sources of natural compounds with valuable antimicrobial activity, we highlighted the medicinal plants and marine and terrestrial organisms, including fungi and bacteria. Nevertheless, there is still a vast fauna and flora that once systematically explored, could provide additional antimicrobial leads and new drugs.

Thousands of natural products with the potential to act as antimicrobial compounds or as a structural lead compound still await further investigation.

In this Research Topic Ebook, we present several scientific studies mainly focused on natural products with antimicrobial activity, which are the case of the natural antimicrobial peptides (AMPs) and host defense peptides (HDPs). This topic also includes recent studies on the roles of honey hydrogen peroxide in antimicrobial activity against resistant microbial strains, as well as the use of essential oils for food preservation. Such a wide and interesting topic also gave us an opportunity to include diverse sources, including plants, terrestrial and sea animals. Not to mention the interesting and unusual sources such as coal or lignite, which may provide future antimicrobial compounds candidates. The recent development of a patented process to GMP standards (PA107470/GB), rendering the obtainment of carbohydrate derived fulvic acid (CHD-FA), stimulated Sherry et al. (2012) to study and describe for the first time a highly effective novel antiseptic effect of fulvic acid with exquisite biofilm activity that acts by disrupting cell membranes. The antifungic peptide from Amazonian Pink Toe spider Juruin, described by Ayroza et al. (2012) is another outstanding example of the potential contribution of a systematic exploration of nature aiming to provide additional antimicrobial leads and drugs.

In other words, nature is a generous source of compounds, with the potential to treat diseases, including infectious diseases. Studies exploiting the mechanism of action and the structure-activity aspects of these natural compounds may provide both additional antimicrobial leads and drugs, and also significant insight into potential possibilities to overcome the antimicrobial resistance.

ACKNOWLEDGMENTS

We are grateful to FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the continuous support.

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Re-examining the role of hydrogen peroxide in bacteriostatic and bactericidal activities of honey

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The aim of this study was to critically analyze the effects of hydrogen peroxide on growth and survival of bacterial cells in order to prove or disprove its purported role as a main component responsible for the antibacterial activity of honey. Using the sensitive peroxide/peroxidase assay, broth microdilution assay and DNA degradation assays, the quantitative relationships between the content of H_2O_2 and honey's antibacterial activity was established. The results showed that: (A) the average H_2O_2 content in honey was over 900-fold lower than that observed in disinfectants that kills bacteria on contact. (B) A supplementation of bacterial cultures with H_2O_2 inhibited *E. coli* and *B. subtilis* growth in a concentration-dependent manner, with minimal inhibitory concentrations (MIC_{90}) values of 1.25 mM/10^7 cfu/ml and 2.5 mM/10^7 cfu/ml for *E. coli* and *B. subtilis*, respectively. In contrast, the MIC_{90} of honey against *E. coli* correlated with honey H_2O_2 content of 2.5 mM, and growth inhibition of *B. subtilis* by honey did not correlate with honey H_2O_2 levels at all. (C) A supplementation of bacterial cultures with H_2O_2 caused a concentration-dependent degradation of bacterial DNA, with the minimum DNA degrading concentration occurring at 2.5 mM H_2O_2. DNA degradation by honey occurred at lower than $\leq$2.5 mM concentration of honey H_2O_2 suggested an enhancing effect of other honey components. (D) Honeys with low H_2O_2 content were unable to cleave DNA but the addition of H_2O_2 restored this activity. The DNase-like activity was heat-resistant but catalase-sensitive indicating that H_2O_2 participated in the oxidative DNA damage. We concluded that the honey H_2O_2 was involved in oxidative damage causing bacterial growth inhibition and DNA degradation, but these effects were modulated by other honey components.

Keywords: oxidative stress, hydrogen peroxide, bacteriostatic activity, honey, DNA degradation

INTRODUCTION

Hydrogen peroxide is generally thought to be the main compound responsible for the antibacterial action of honey (White et al., 1963; Weston, 2000; Brudzynski, 2006). Hydrogen peroxide in honey is produced mainly during glucose oxidation catalyzed by the bee enzyme, glucose oxidase (FAD-oxidoreductase, EC 1.1.3.4; White et al., 1963). The levels of hydrogen peroxide in honey are determined by the difference between the rate of its production and its destruction by catalases. Glucose oxidase is introduced to honey during nectar harvesting by bees. This enzyme is found in all honeys but its concentration may differ from honey to honey depending on the age and health status of the foraging bees (Pernal and Currie, 2000) as well as the richness and diversity of the foraged diet (Alaux et al., 2010). Catalases on the other hand, are of pollen origin. Catalase efficiently hydrolyzes hydrogen peroxide to oxygen and water due to its high turnover numbers. The total concentration of catalase depends on the amount of pollen grains in honey (Weston, 2000), and consequently, the hydrogen peroxide levels in different honeys may vary considerably (Brudzynski, 2006).

A substantial correlation has been found between the level of endogenous hydrogen peroxide and the extent of inhibition of bacterial growth by honey (White et al., 1963; Brudzynski, 2006). We have observed that in honeys with a high content of this oxidizing compound, bacteria cannot respond normally to proliferative signals and their growth remains arrested even at high honey dilutions. Pre-treatment of honey with catalase restored, to a certain extent, the bacterial growth, thus suggesting that endogenous H_2O_2 was implicated in the growth inhibition (Brudzynski, 2006).

Most of the conclusions on the H_2O_2 oxidizing action on bacteria are drawn from the simplified *in vitro* models, where direct effects of hydrogen peroxide on bacterial cells were analyzed. In contrast, honey represent complex chemical milieu consisting of over 100 different compounds (including antioxidants and traces of transition metals), where the interaction between these components and hydrogen peroxide may influence its oxidative action. We have recently unraveled that honey is a dynamic reaction mixture which facilitates and propagates the Maillard reaction (Brudzynski and Miotto, 2011b). The Maillard reaction which initially involves reaction between amino groups of amino acids or proteins with carbonyl groups of reducing sugars leads to a cascade of redox reactions in which several bioactive molecules are continuously formed and lost due to their cross-linking to

other molecules (gain or loss of function; Brudzynski and Miotto, 2011b). We have shown that polyphenol-based melanoidins are a major group of Maillard reaction products possessing radical-scavenging activity (Brudzynski and Miotto, 2011a,b). These compounds are likely to interact with hydrogen peroxide and, depending of their concentration and redox capacity, either enhanced or diminished the oxidative activity of honey's H_2O_2. In view of these facts, we hypothesized that the oxidizing action of honey's hydrogen peroxide on bacterial cells may be modulated by the presence of other bioactive molecules in honey and therefore, may differ from the action of hydrogen peroxide alone.

Hydrogen peroxide is commonly used to disinfect and sanitize medical equipment in hospitals. For this purpose, the high concentrations of H_2O_2 in these disinfectants have to be maintained to overwhelmed defense systems of bacteria. At high concentrations, ranging from 3 to 30% (0.8 to 8 M), its bactericidal effectiveness has been demonstrated against several microorganisms including *Staphylococcus*-, *Streptococcus*-, *Pseudomonas*-species, and *Bacillus* spores (Rutala et al., 2008).Under these conditions, the bacterial cell death results from the accumulation of irreversible oxidative damages to the membrane layers, proteins, enzymes, and DNA (**?**Davies, 2000; Rutala et al., 2008; Finnegan et al., 2010).

However, the hydrogen peroxide content in honey is about 900-fold lower (Brudzynski, 2006). Moreover, the literature data indicate that the cell death of cultured mammalian, yeast, and bacterial cells required H_2O_2 concentrations higher than 50 mM and was associated with chromosomal DNA degradation (Imlay and Linn, 1987a,b; Brandi et al., 1989; Davies, 1999; Bai and Konat, 2003; Ribeiro et al., 2006), which is still five to 10-fold higher then that observed in honeys. Therefore, we have undertaken this study to re-examined the role of hydrogen peroxide in antibacterial activity of honeys.

The hydrogen peroxide efficacy as an oxidative biocide is related to the bacterial sensitivity to peroxide stress. Defense mechanisms to oxidative stress varies between bacterial species such as Gram-negative *E. coli* and Gram-positive *B. subtilis* used in this study and depend on the growth phase (exponential- versus stationary-phase of growth), and on the adaptive and survival mechanisms (non-spore forming versus spore-forming bacteria; Dowds et al., 1987; Chen et al., 1995; Storz and Imlay, 1999; Cabiscol et al., 2000). In honey, the effects of H_2O_2 on the growth and survival of microorganisms may be mitigated or enhanced due to the presence of honey compounds. On one hand, a high content of sugars in honey that abstracts free water molecules from milieu inhibits bacterial growth and proliferation, but honey dilutions may create growth-supportive conditions due to the abundance of sugars as a carbon source for the growing cells. Hydrogen peroxide has deleterious effects on the growth and survival of bacterial cells but honey antioxidants such as catalases, polyphenols, Maillard reaction products, and ascorbic acid may lower the oxidative stress to cells and may have a protective effect against endogenous H_2O_2 (Brudzynski, 2006). Even less information exists on the mechanism of bactericidal action of honey's hydrogen peroxide. The most fundamental and unsolved questions concerns the molecular targets of H_2O_2 cytotoxicity: does molecular hydrogen peroxide at concentrations present in honey cause DNA degradation?

During last decades, several honey compounds were identified as those implicated in honey antibacterial activity (for review, Irish et al., 2011). Despite this knowledge, the mechanisms by which these compounds lead to bacterial growth inhibition and bacterial death have never been explained or proven in biochemical terms. Since there is a persistent view that hydrogen peroxide is a main player in these events, the aim of this study was to critically analyze the effects of hydrogen peroxide on growth and survival of bacterial cells in order to prove or disprove its purported role as a main component responsible for the antibacterial activity of honey.

MATERIALS AND METHODS

HONEY SAMPLES

Honey samples included raw, unpasteurized honeys donated by Canadian beekeepers and two samples of commercial Active Manuka honey (Honey New Zealand Ltd., New Zealand, UMF 20+, and 25+; M and M2, respectively; **Table 1**) that were used as a reference in this study. During the study, honey samples were kept in the original packaging, at room temperature ($22 \pm 2^{\circ}C$) and in the dark.

A stock solution of 50% (w/v) honey was prepared by dissolving 1.35 g honey (average density 1.35 g/ml) in 1 ml of sterile, distilled water warmed at 37°C. The stock solution was prepared immediately before conducting the antibacterial assays.

PREPARATION OF ARTIFICIAL HONEY

Artificial honey was prepared by dissolving 76.8 g of fructose and 60.6 g of glucose separately in 100 ml of sterile, deionized water, and by mixing these two solutions in a 1:1 ratio. The osmolarity of the artificial honey was adjusted to that of the honey samples (BRIX) using refractometric measurements.

BACTERIAL STRAINS

Standard strains of *Bacillus subtilis* (ATCC 6633) and *Escherichia coli* (ATCC 14948; Thermo Fisher Scientific Remel Products, Lenexa, KS 66215) were grown in Mueller–Hinton broth (MHB; Difco Laboratories) overnight in a shaking water-bath at 37°C.

Overnight cultures were diluted with broth to the equivalent of the 0.5 McFarland Standard (approx.10^8 cfu/ml) which was measured spectrophotometrically at $A_{600\,nm}$.

ANTIBACTERIAL ASSAY

The antibacterial activity of honeys was determined using a broth microdilution assay using a 96-well microplate format. Serial twofold dilutions of honey were prepared by mixing and transferring 110 µl of honey with 110 µl of inoculated broth (10^6 cfu/ml final concentrations for each microorganism) from row A to row H of a microplate. Row G contained only inoculum and served as a positive control and row H contained sterile MHB and served as a blank.

After overnight incubation of plates at 37°C in a shaking water-bath, bacterial growth was measured at $A_{595\,nm}$ using the Synergy HT multi-detection microplate reader (Synergy HT, Bio-Tek Instruments, Winooski, VT, USA).

The contribution of color of honeys to the absorption was corrected by subtracting the absorbance values before (zero time) incubation from the values obtained after overnight incubation.

Table 1 | Hydrogen peroxide concentrations in different honeys. Relationship between antibacterial activities of honey and hydrogen peroxide concentrations.

Honey sample	Plant source	Hydrogen peroxide concentration (mM/l)*	*E. coli* MIC dilution (concentration)	*B. subtilis* MIC dilution (concentration)
M2	Manuka (UMF 25)	1.04 ± 0.17	16 (6.25%)	16 (6.25%)
H58	Buckwheat	2.68 ± 0.04	16 (6.25%)	8 (12.5%)
H23	Buckwheat	2.12 ± 0.22	8 (12.5%)	4 (25%)
H20	Sweet clover	2.37 ± 0.03	8 (12.5%)	4 (25%)
H11	Wildflower/clover	2.49 ± 0.03	8 (12.5%)	8 (12.5%)
H56	Blueberry	0.52 ± 0.11	4 (25%)	2 (50%)
H60	Clover blend	0.67 ± 0.11	4 (25%)	2 (50%)
M	Manuka (UMF 20)	0.72 ± 0.02	4 (25%)	4 (25%)
H200	Buckwheat	0.248 ± 0.02	2 (50%)	2 (50%)
H203	Buckwheat	0.744 ± 0.01	4 (25%)	4 (25%)
H204	Buckwheat	1.168 ± 0.05	4 (25%)	4 (25%)
H205	Buckwheat/alfalfa	1.112 ± 0.02	4 (25%)	4 (25%)

**Hydrogen peroxide concentration was measured at honey dilution of 8× (25% v/v) and represent an average of three experimental trials, where each honey was tested in triplicate.*

The absorbance readings obtained from the dose–response curves were used to construct growth inhibition profiles (GIPs). The minimal inhibitory concentrations (MIC) were determined from the GIPs and represented the lowest concentration of honey that inhibited the bacterial growth. The MIC end point in our experiments was honey concentration at which 90% bacterial growth reduction was observed as measured by the absorbance at $A_{595\,nm}$.

Statistical analysis and dose response curves were obtained using KC4 software (Synergy HT, Bio-Tek Instruments, Winooski, VT, USA).

HYDROGEN PEROXIDE ASSAY

Hydrogen peroxide concentration in honeys was determined using the hydrogen peroxide/peroxidase assay kit (Amplex Red, Molecular Probes, Invitrogen, Burlington, ON, Canada). The assay was conducted in the 96-well microplates according to the manufacturer's instruction. The fluorescence of the formed product, resorufin, was measured at 530 nm excitation and a 590 nm emission using the Synergy HT (Molecular Devices, BioTek Instruments, Winooski, VT, USA) multi-detection microplate reader, and the dose–response curves were generated using the KC4™data reduction software.

To calculate the hydrogen peroxide concentrations of the honeys, a standard curve was run alongside the honey serial dilutions. The standard curve was prepared from the 200 μM H_2O_2 stock solution. Each of the honey samples, and the standard curve, were tested in triplicate.

CATALASE-TREATMENT OF HONEYS

Honey were treated with catalase (13 800 U/mg solid; Sigma-Aldrich, Canada) at ratio of 1000 units per 1 ml of 50% honey solution in sterile water for 2 h at room temperature.

INCUBATION OF BACTERIAL CULTURES WITH HONEY OR HYDROGEN PEROXIDE

Overnight cultures of *E. coli* and *B. subtilis* (1.5 ml, adjusted to 10^7 cfu/ml in MHB) were treated with either the 50% honey solution in a 1:1 ratio (v/v), an artificial honey solution, or with hydrogen peroxide solutions containing 5, 2.5, 1.2, 0.62, and 0.3125 mM (final concentrations) H_2O_2 prepared from the 20 mM stock solution. After overnight incubation at 37°C with continuous shaking, the cells were harvested by centrifugation at 3,000 × *g* (Eppendorf) for 30 s and then their DNA was isolated.

DNA ISOLATION

The total genomic bacterial DNA was isolated from the untreated, control cells and from the honey- or hydrogen peroxide-treated cells using a DNA isolation kit (Norgen Biotek Corporation, St. Catharines, ON., Canada), according to the manufacturer's instructions.

AGAROSE GEL ELECTROPHORESIS

Agarose gel (1.3%) electrophoresis was carried out in 1× TAE buffer containing ethidium bromide (0.1 μg/ml w/v). Ten microliters of DNA isolated from the untreated and treated bacterial cells was mixed with 5X loading dye (0.25% bromophenol blue, 0.25% xylene xyanol, 40% sucrose) and loaded into the gel. The DNA molecular weight markers selected were the HighRanger 1 kb DNA Ladder, MidRanger 1 kb DNA Ladder, and PCRSizer 100 bp DNA Ladder from Norgen Biotek (Thorold, Ontario). The gels were run at 85 V for 1 h and then visualized and photographed using the Gel Doc 1000 system and the Quantity One 1-D Analysis software (version 4.6.2 Basic) from Bio-Rad.

RESULTS

DETERMINATION OF THE HYDROGEN PEROXIDE CONCENTRATIONS IN HONEYS

Formation of H_2O_2 depends on the honey dilution since glucose oxidase is inactive in undiluted honey (White et al., 1963; Brudzynski, 2006). Honeys used in this study required a four to 16-fold dilution for the maximal production of hydrogen peroxide to be observed (**Figure 1**). At the peak, H_2O_2 concentrations ranged from 2.68 ± 0.04 to 0.248 ± 0.02 mM in the different honeys (**Table 1**), as measured by a sensitive, high-throughput hydrogen peroxide/peroxidase assay (Amplex Red assay).

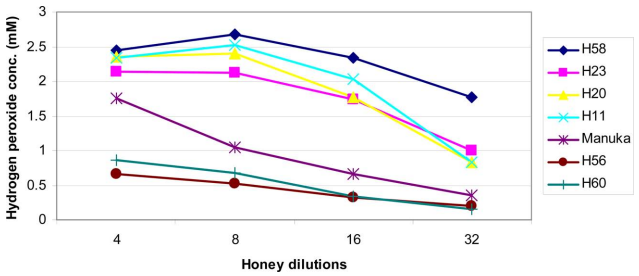

FIGURE 1 | Effect of honey dilutions on the production of hydrogen peroxide. Honeys of buckwheat origin, H58 and H23, together with sweet clover (H20), and wildflower/clover (H11) produced distinctively higher amounts of H_2O_2 than Manuka (M2) or honey blends (H56 and H60). The H_2O_2 content was measured in twofold serially diluted honeys, the *x* axis represents a log2 values.

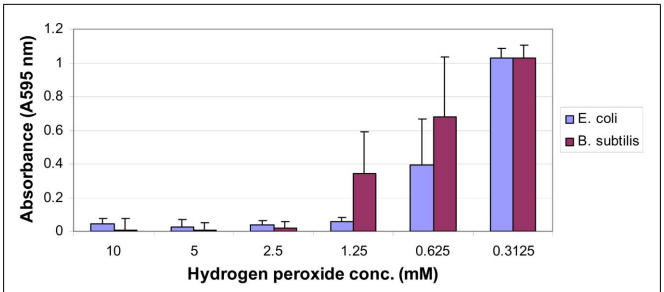

FIGURE 2 | Effect of increasing the concentration of exogenous hydrogen peroxide on the growth of *E. coli* (blue line) and *B. subtilis* (red line). Each point represents the mean and SD of three separate experiments conducted in triplicate.

CONCENTRATION-DEPENDENT EFFECT OF HYDROGEN PEROXIDE ON BACTERIAL GROWTH INHIBITION

Throughout this study, we used terms: endogenous hydrogen peroxide to describe H_2O_2 produced in honey by glucose oxidase and exogenous hydrogen peroxide, which has been added as a supplement to the bacterial cultures. These terms were introduced in order to differentiate between the effects of honey's endogenous H_2O_2 whose action on bacterial cells could be modulated/obscured by other honey components as opposed to true, well-defined action of exogenous hydrogen peroxide directly added to bacterial culture.

In agreement with previous reports (Brudzynski, 2006), we found a strong correlation between the content of honey hydrogen peroxide and the growth inhibitory action of Canadian honeys; honeys with high MIC_{90} values (6.25 to 12.5% v/v) corresponding to16 to 8× dilution) also possessed a high content of H_2O_2 (**Table 1**). Since the minimum inhibitory concentration values and the hydrogen peroxide peak were both observed at the 4 to 16× honey dilutions, we hypothesized that the maximal hydrogen peroxide production is required to achieve the bacteriostatic activity of honey at the MIC_{90} level. To test this assumption, we first examined the dose–response relationship between the concentration of exogenous hydrogen peroxide, ranging from 10 to 0.312 mM, and its growth inhibitory activity against *E. coli* and *B. subtilis*. The dose–response curves and growth inhibitory profiles revealed very reproducibly that H_2O_2 concentrations of 1.25 mM (1.25 μmoles/10^7 cfu/ml) and 2.5 mM (2.5 μmoles/10^7 cfu/ml) were required to inhibit the growth of *E. coli* and *B. subtilis* by 90%, respectively (**Figure 2**).

RELATIONSHIP BETWEEN THE ENDOGENOUS H_2O_2 CONTENT AND THE GROWTH INHIBITORY ACTIVITY OF HONEYS

To investigate whether the content of honey H_2O_2 influences honey's bacteriostatic potency in a similar manner to that of exogenous H_2O_2, each honey was analyzed for growth inhibitory activity and the production of hydrogen peroxide in the same range of honey dilutions. When the profiles of hydrogen peroxide production were superimposed on the growth inhibitory profiles of honeys against *E. coli*, it appeared that almost all of the bacteriostatic activity of honeys could be assigned to the effects of this compound (**Figure 3A**). In honeys, the endogenous H_2O_2 of 2.5 mM was of critical importance for the growth inhibition of *E. coli*; the dilutions that reduced H_2O_2 concentrations below this value showed a loss of honey potency to inhibit bacterial growth at the MIC_{90} level (**Figure 3A**). These data suggest that upon honey dilution, endogenous H_2O_2 mediates growth inhibition of *E. coli*. However, the concentrations required to reach MIC_{90} were twofold higher than that found for exogenous hydrogen peroxide (2.5 versus 1.25 mM, respectively; **Figure 3A**).

In contrast to *E. coli*, the inhibition of growth of *B. subtilis* seemed not to be due to the effect of the levels of honey H_2O_2 (**Figure 3B**). A rapid increase of *B. subtilis* growth with honey dilutions occurred despite the presence of high levels of H_2O_2 (honeys H58, H23, H20, and H11, **Figure 3B**). While exposure of the *B. subtilis* culture to exogenous H_2O_2 resulted in a concentration-dependent growth inhibition with MIC_{90} at 2.5 mM (**Figure 2**), comparable concentrations of H_2O_2 in honeys were ineffective. This indicated that other honey compounds/physical features were responsible for the growth inhibition, such as honey's high osmolarity. Moreover, higher honey dilutions, beyond 16-fold, had a stimulatory effect on *B. subtilis* growth (**Figure 3B**).

Thus, our results demonstrated for the first time that bacteriostatic effects of endogenous versus exogenous hydrogen peroxide are markedly different due to the presence of other honey components and, more importantly, that the effects of honey H_2O_2 on bacterial growth are markedly different in *E. coli* and *B. subtilis*.

COMPARISON OF EFFECTS OF HONEY AND HYDROGEN PEROXIDE ON DNA DEGRADATION IN BACTERIAL CELLS

To exert effectively its oxidative biocide action, the concentrations of hydrogen peroxide in various disinfectants are high ranging from 3 to 30% (0.8 to 8 M). In contrast, we have established that the average content of H_2O_2 in tested honeys ranged from 0.5 to 2.7 mM (**Table 1**). The concentrations of H_2O_2 measured in honeys, therefore was about 260–1600-fold lower than the effective bactericidal dose of H_2O_2 in disinfectants. Therefore, we asked the question whether hydrogen peroxide at concentrations present in honey can cause DNA degradation and ultimately bacterial cell death.

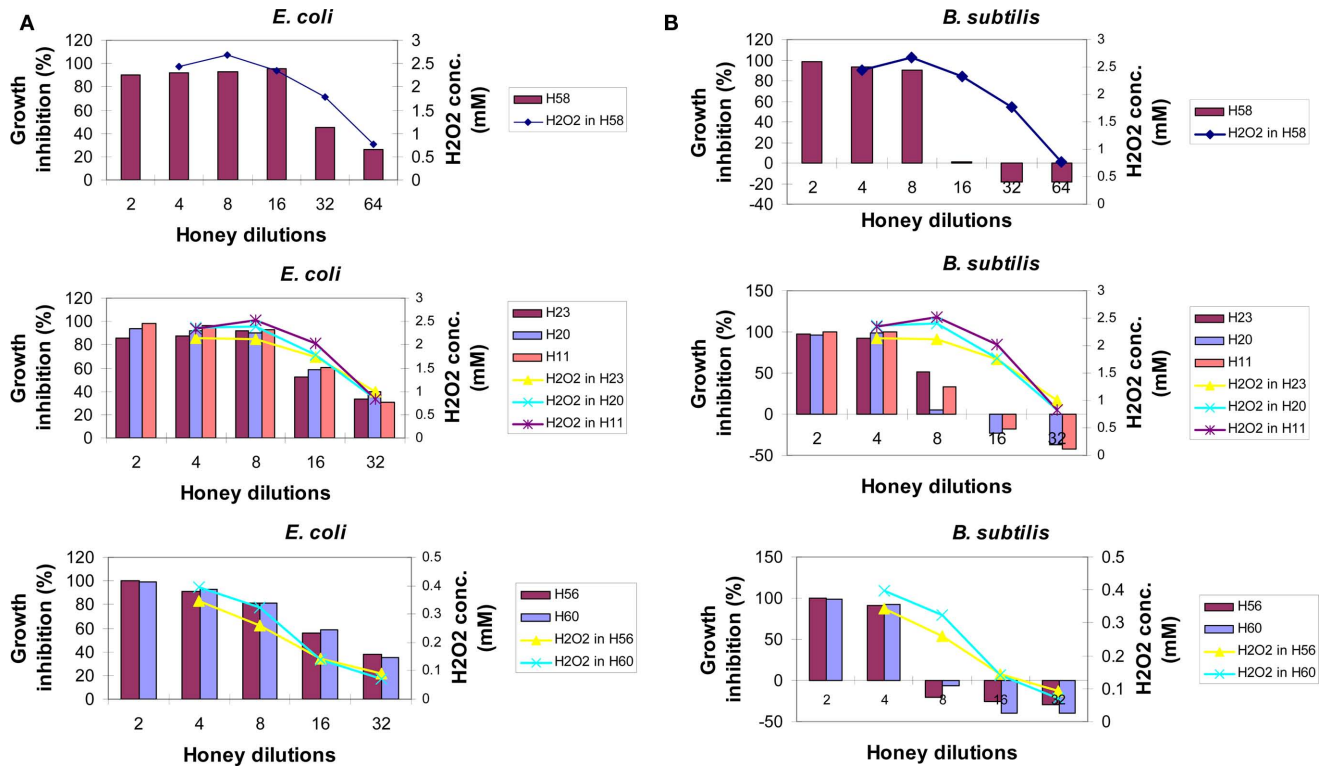

FIGURE 3 | The relationship between bacteriostatic effect of honey and the content of en-H2O2 on *E. coli* (A) or *B. subtilis* cultures (B) Growth inhibition profiles were determined for different honeys using the broth microdilution assay (columns). The content of honey H_2O_2 at each honey dilution was determined using the peroxide/peroxidase assay, as described in the Section "Materials and Methods." Of note: growth inhibition profiles of artificial honey of osmolarity equal to that of natural honey provided MIC_{90} values of 25% (v/v) against both *E. coli* and *B. subtilis*. Each point or column represents the mean values of three separate experiments run in triplicate.

To examine the effects of honey and hydrogen peroxide on the integrity of bacterial DNA, *E. coli* cultures (10^7 cfu/ml) were exposed to increasing concentrations of exogenous H_2O_2 (5–0.3125 mM) or to honeys containing known amounts of H_2O_2. After 24 h incubation at 37°C, bacterial DNA was isolated and its integrity examined on agarose gels. **Figure 4** shows that the exposure of *E. coli* cultures to hydrogen peroxide at concentrations of 5 and 2.5 mM caused DNA degradation, while H_2O_2 concentrations lower than 2.5 mM were ineffective.

In contrast, honeys of relatively high H_2O_2 concentrations but below 2.5 mM (H203, 204, 205; **Table 1**) exerted DNA degrading activity (**Figure 4**). The ability of honeys H_2O_2 to degrade DNA appeared to be concentration-dependent. Honey H200 containing 0.25 mM H_2O_2 was unable to cleave DNA (**Figure 5**). The differences in the concentrations of H_2O_2 between exogenous and honey's hydrogen peroxide that were required to effectively degrade chromosomal DNA may indicate that the action of honey H_2O_2 is enhanced by other honey components.

Manuka honey also possessed low concentration of H_2O_2 (0.72 mM), but efficiently degraded DNA (**Figures 4** and **5**). The antibacterial activity of manuka honey however is not regulated by the honey H_2O_2 content (Molan and Russell, 1988; Allen et al., 1991).

DNA DEGRADATION IN *E. COLI* CELLS EXPOSED TO CATALASE-TREATED OR HEAT-TREATED HONEYS

To gain more insight into the role of H_2O_2 in chromosomal DNA degradation, *E. coli* cultures were exposed to honeys which were treated with catalase. Removal of H_2O_2 by catalase abolished DNA degrading activity of honey H205 and had a protective effect on bacterial DNA (**Figure 5**). The short incubation of catalase-treated honey H204 with DNA (8 h instead of 24 h) also prevented DNA degradation (**Figure 5**). Inactive honey H200 remained unable to degrade DNA after catalase-treatment (**Figure 5**). However, when honey H200 was supplemented with 2 mM H_2O_2, and then incubated with *E. coli* culture at 37°C for 8 h, it became active in degrading DNA and the extent of DNA degradation was comparable to that of honey H204 (**Figure 5**). On the other hand, catalase–treatment of manuka honey did not prevent DNA degradation, consistent with the notion, that manuka honey antibacterial activity is hydrogen peroxide-independent (Molan and Russell, 1988; Allen et al., 1991).

To investigate the potential involvement of DNases in DNA degradation, honeys were heat-treated under conditions which inactivate DNase activity (75°C for 10 min). Unheated and heat-treated honeys were then incubated with *E. coli* cultures at 37°C for 8 h, followed by DNA isolation and its analysis on agarose gels. Heat-treatment of active honeys H205 and H23 did not prevent

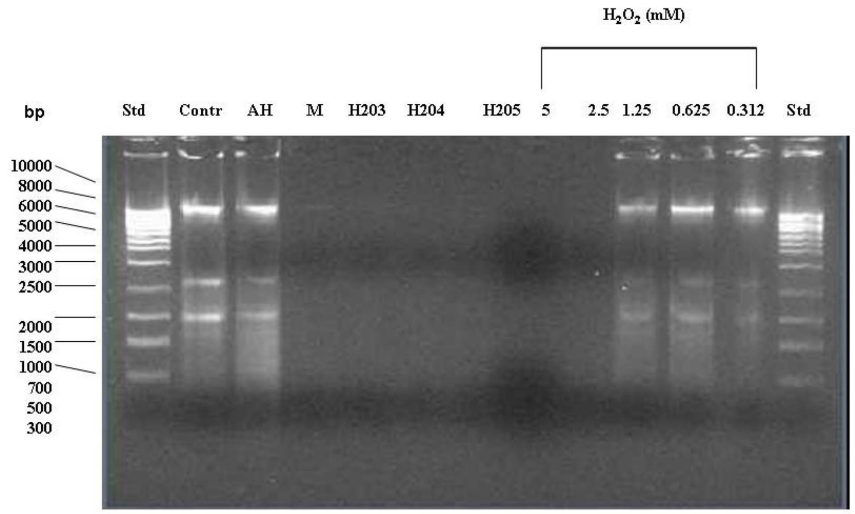

FIGURE 4 | Effect of exposure of *E. coli* cultures to honey or exogenous hydrogen peroxide on the integrity of bacterial DNA. The cells were treated with honeys (manuka, buckwheat honeys H203, H204, and H205) or with increasing concentrations of exogenous H_2O_2 (5–0.312 mM). Untreated cells and cells treated with the sugar solution (artificial honey, AH) served as the controls. The integrity of DNA was analyzed on agarose gels.

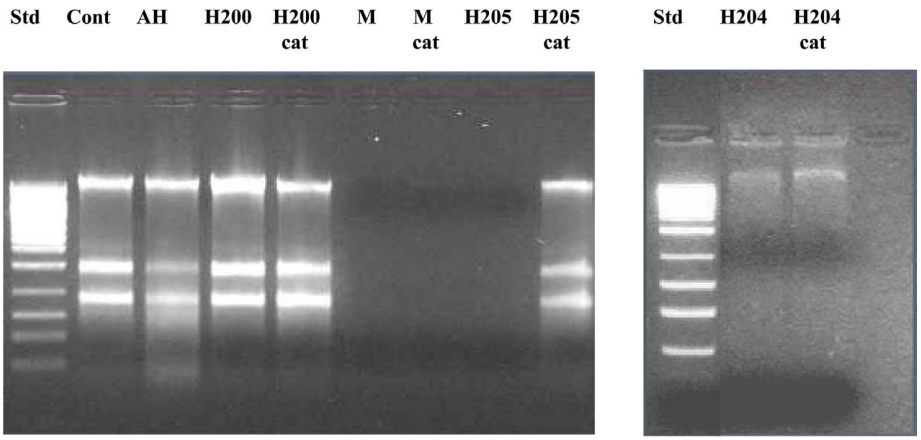

FIGURE 5 | Effect of exposure of *E. coli* cultures to honeys untreated and treated with catalase (cat) on the integrity of chromosomal DNA. "Cont" represents DNA isolated from untreated *E. coli* cells, AH-cell treated with artificial honey, or buckwheat honeys H200, H205, and M-manuka honey after 24 h incubation and H204 after 8 h incubation.

DNA degradation suggesting against the involvement of DNase in this process (**Figures 6** and **7**). Moreover, the fact that some honeys displayed DNA degrading activity (H23 or H205) in bacterial culture while others did not (H200 and H60) makes it unlikely that this process was mainly due to the contamination of honeys with DNases. On the other hand, inability of honeys H200 and H60 to degrade DNA was closely related to the very low concentration of H_2O_2 in these honeys.

Together, these results provided a strong support for the role of H_2O_2 in DNA degradation.

DISCUSSION

The findings described in this study revise the old views and provide novel information on the role of hydrogen peroxide in the regulation of bacteriostatic and bactericidal activities of honey.

Firstly, we found that the exponentially growing *E. coli* and *B. subtilis* cells were inhibited in a concentration-dependent manner by exogenous H_2O_2 reaching MIC_{90} at 1.25 mM (1.25 μmoles/10^7 cfu/ml) and 2.5 mM (2.5 μmoles/10^7 cfu/ml), respectively. The bacteriostatic efficacy of H_2O_2 however differed significantly from that of honey H_2O_2. The main factors that contributed to these differences were (a) bacterial susceptibility/resistance to the oxidative action of hydrogen peroxide and (b) interference from other honey components.

Endogenous H_2O_2 inhibited the growth of *E. coli* in a concentration-dependent manner, but its MIC_{90} was twofold higher than those of exogenous H_2O_2 (2.5 versus 1.25 mM, respectively). The honeys MIC_{90} levels against *E. coli* coincided with the dilutions at which a peak of hydrogen peroxide production occurred. Treatment of honeys with catalase led to a

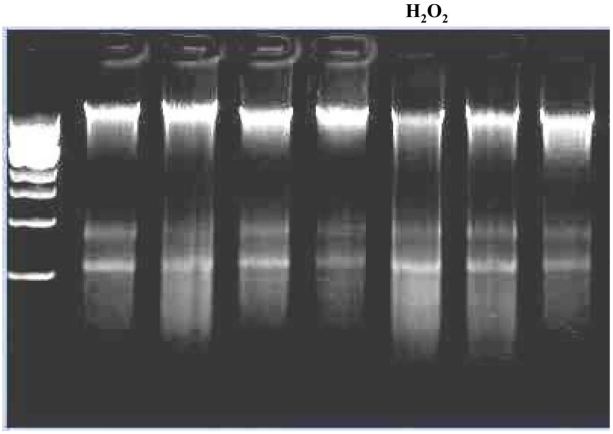

FIGURE 6 | Effect of exposure of *E. coli* cultures to heat-treated (h), catalase-treated (cat), and untreated (un) honeys on the integrity of chromosomal DNA. AH-cell treated with artificial honey, or buckwheat honeys H200, and H205. Lane "H200 2 mM H_2O_2" represents the effect of honey H200 supplementation with hydrogen peroxide on the *E. coli* DNA integrity.

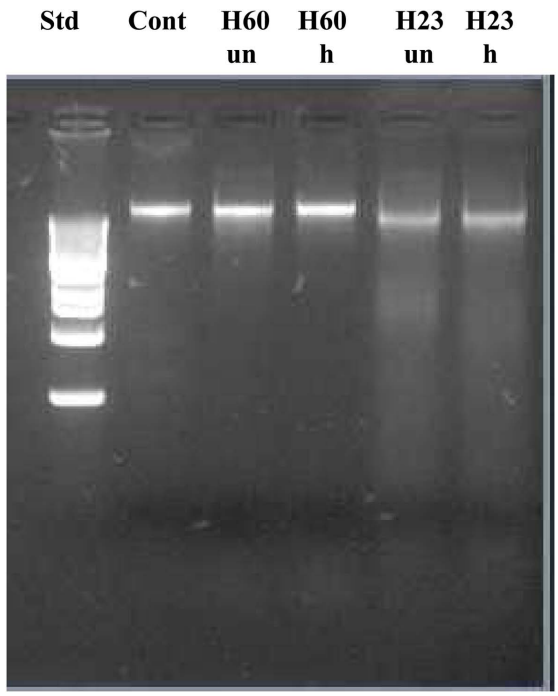

FIGURE 7 | Effect of exposure of *E. coli* naked DNA to unheated and heat-treated honeys H60 and H23.

significant reduction in their bacteriostatic activity (Brudzynski, 2006). Together, these data provide direct evidence that *E. coli* growth is sensitive to oxidative action of honey H_2O_2.

In contrast, growth inhibition of *B. subtilis* was not due to the action of honey H_2O_2. While exposure of *B. subtilis* cultures to H_2O_2 resulted in a concentration-dependent growth inhibition, the comparable concentrations of honey H_2O_2 in honey were ineffective in arresting *B. subtilis* growth. The rapid decrease in bacteriostatic activity of honey upon dilution was observed even in the presence of high concentrations of honey H_2O_2. These results suggest that other honey compounds were responsible for the inhibition of *B. subtilis* growth. As a consequence of growth arrest, the change in sensitivity of *B. subtilis* to honey H_2O_2 occurred. Instead of growth inhibition, we observed growth stimulation of *B. subtilis* at high honey dilutions (16-fold and over) and in the presence of high levels of H_2O_2. Literature data provides compelling evidence that transition from the exponential-phase growth to the stationary-phase growth evokes *B. subiltis* sporulation and with it, increased resistance to hydrogen peroxide. The transition to stationary-phase growth activates RNA polymerase σ^s transcriptional factor which regulates a stationary-phase gene expression of *rpoS* regulon. The expression of σ^s factor in *B. subtilis* evokes spore-formation to enhance bacterial survival (Dowds et al., 1987; Dowds, 1994; Loewen et al., 1998; Zheng et al., 1999; Chen and Schellhorn, 2003). Dowds et al. (1987) have shown that stationary-phase cultures of *B. subtilis* displayed viability even at the 10 mM concentration of H_2O_2. These data may explain, at least in part, an apparent insensitivity of *B. subtilis* to high levels of hydrogen peroxide in honey.

These results revealed significant differences in the sensitivities of *E. coli* and *B. subtilis* to oxidative stress caused by honey H_2O_2. As aerobic bacteria, both *E. coli* and *B. subtilis* are equipped with molecular machinery to cope with oxidative stress by activating several stress genes under *oxyR*- or *perR*-regulons, in *E. coli* and *B. subtilis* respectively (Dowds et al., 1987; Christman et al., 1989; Dowds, 1994; Bsat et al., 1998; Storz and Imlay, 1999). The *oxyR* and *perR* genes control expression of inducible forms of *katG* (catalase hydroperoxidase I, HP1), *ahpCF* (alkylhydroperoxide reductase) that function to reduce hydrogen peroxide to levels that are not harmful to growing cells (Hassan and Fridovich, 1978; Loewen and Switala, 1987; Storz et al., 1990; Seaver and Imlay, 2001). While these responses are similar in both bacteria, the main difference concerns their adaptive and survival mechanisms to oxidative stress.

Relatively little is known about the contribution of honey's hydrogen peroxide to bacterial cell death. The most important result obtained in this work is the demonstration that honey H_2O_2 participated in bacterial DNA degradation. Several lines of evidence support this finding. Firstly, the treatment of exponential-phase *E. coli* cultures with increasing concentrations of exogenous hydrogen peroxide (5–0.3125 mM) or honeys of different content of endogenous H_2O_2 led to a concentration-dependent DNA degradation. While the minimum DNA degrading activity of exogenous H_2O_2 occurred at 2.5 mM (2.5 μmoles/10^7 cfu/ml), in contrast, honeys possessing H_2O_2 concentrations lower than 2.5 mM were still active in this process. Secondly, DNA degradation by active honeys was abolished by removal of H_2O_2 by catalase. Thirdly, honeys with the low content of H_2O_2 were unable to degrade DNA but the supplementation with 2 mM of hydrogen peroxide caused the appearance of this activity. The extent of DNA degradation by honey, which was supplemented with H_2O_2, was comparable to that of active honeys.

Heat-treatment of active honeys prior to incubation with *E. coli* cultures did not prevent DNA degradation, suggesting against the involvement of DNase in this process. Moreover, not all tested honeys displayed DNA degrading activity on *E. coli* cells. Given that bacterial cells are impermeable to DNase, the DNA degradation by honeys observed in this study could not be simply explained by the DNase contaminations. Rather, the close relationship between DNA degradation and H_2O_2 content in honeys advocates for the role of H_2O_2 in the mechanism of DNA cleavage.

DNA degradation is a lethal event which ultimately kills the cell. Literature data indicate that the concentration of hydrogen peroxide plays a decisive role in the type of cell death that follows H_2O_2 exposure. In simplified *in vitro* models, where direct effects of hydrogen peroxide on bacterial cells were analyzed, two separate modes of killing were observed for *E. coli*. At low concentrations of H_2O_2 ($\leq$2.5 mM), *E. coli* cells were dying because of DNA damage inflicted on the metabolically active cells (Imlay and Linn, 1986; Imlay and Linn, 1987a,b; Brandi et al., 1989). At H_2O_2 concentrations of 10–50 mM, cell death resulted from cytotoxic effects due to hydroxyl radicals formed from hydrogen peroxide (Imlay and Linn, 1987a,b; Brandi et al., 1989). In a full agreement with these data, we established that the minimum DNA degrading activity of exogenous H_2O_2 on *E. coli* cells was 2.5 mM (2.5 μmoles/10^7 cfu/ml). In contrast to exogenous H_2O_2, the minimum DNA degrading activity of honey H_2O_2 was below 2.5 mM. The lower concentrations of honeys H_2O_2 required to effectively degrade chromosomal DNA strongly suggest that the oxidizing effect of H_2O_2 was augmented by other honey components such as transition metals (Fe, Cu) commonly present in honeys. In support of this notion, the recent literature evidence indicates that it is the hydroxyl radical (HO) that is produced in the metal-catalyzed Fenton reaction from H_2O_2 rather than molecular hydrogen peroxide that causes the oxidative damage to membrane structures, proteins, and DNA (Imlay et al., 1988; Storz and Imlay, 1999; Cabiscol et al., 2000; Imlay, 2003).

In conclusion, our study demonstrated that honey H_2O_2 exerted bacteriostatic and DNA degrading activities to bacterial cells. The extent of damaging effects of honey H_2O_2 was strongly influenced by the bacterial sensitivity to oxidative stress, the growth phase and their survival strategy (non-spore forming versus spore forming species) as well as by the modulation of other honey compounds.

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Exploring the pharmacological potential of promiscuous host-defense peptides: from natural screenings to biotechnological applications

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In the last few years, the number of bacteria with enhanced resistance to conventional antibiotics has dramatically increased. Most of such bacteria belong to regular microbial flora, becoming a real challenge, especially for immune-depressed patients. Since the treatment is sometimes extremely expensive, and in some circumstances completely inefficient for the most severe cases, researchers are still determined to discover novel compounds. Among them, host-defense peptides (HDPs) have been found as the first natural barrier against microorganisms in nearly all living groups. This molecular class has been gaining attention every day for multiple reasons. For decades, it was believed that these defense peptides had been involved only with the permeation of the lipid bilayer in pathogen membranes, their main target. Currently, it is known that these peptides can bind to numerous targets, as well as lipids including proteins and carbohydrates, from the surface to deep within the cell. Moreover, by using *in vivo* models, it was shown that HDPs could act both in pathogens and cognate hosts, improving immunological functions as well as acting through multiple pathways to control infections. This review focuses on structural and functional properties of HDP peptides and the additional strategies used to select them. Furthermore, strategies to avoid problems in large-scale manufacture by using molecular and biochemical techniques will also be explored. In summary, this review intends to construct a bridge between academic research and pharmaceutical industry, providing novel insights into the utilization of HDPs against resistant bacterial strains that cause infections in humans.

Keywords: host-defense peptides, innate immunity, microbial infections, antimicrobials

INTRODUCTION

Infectious diseases caused by fungi and bacteria have affected humanity since the early days of civilization. Nevertheless, the discovery of penicillin by Fleming (1929) provided a potent defense in mammalian survival against pathogens. Based on penicillin, several other molecules and different antibiotic classes have been developed. However, all these agents have lost efficiency and are becoming useless against resistant bacterial strains.

Antimicrobial peptides (AMPs) have arisen as an alternative strategy for the treatment of infections caused by super bugs (Arias and Murray, 2009). AMPs are natural antibiotics found in microorganisms, plants, and animals (Hancock and Chapple, 1999). They can be structurally classified, being formed by α-helices, β-sheets, extended structures, or disordered loops. Moreover, these peptides could also be classified by physical–chemical properties: cationic, anionic, and amphipathic (Peters et al., 2010). Mostly, cationic AMPs show their antimicrobial activity as a result of lipid bilayer disruption. Nevertheless they may also act on various cell targets, in some cases being considered promiscuous molecules (Huang et al., 2010). In the last few years the conventional idea that peptides possess an unconditional structure directly related to a particular function clashes with the peptide's ability to change and develop new functions. Considering these contrasting ideas, the knowledge of peptide promiscuity, in which multiple functions may be associated with a sole structure, has been gaining consideration in several research fields including the development of antibiotics. Indeed, several AMPs have shown their wide range of functions that are able to control numerous target pathogens simultaneously and in different conditions (as reviewed by Franco, 2011). Moreover, some peptides, in addition to activity against pathogens, also have shown multiple activities related to host innate immunity, called host-defense peptides (HDPs; Hancock et al., 2006).

Host-defense peptides are relatively small compounds, with 12–50 amino acid residues, positive net charge (+2 to +9), and are isolated from single-celled microorganisms, invertebrates, plants, amphibians, birds, fishes, and mammals including humans (Hancock and Sahl, 2006). Furthermore, HDPs are classified

into various groups according to a three-dimensional structure arrangement that includes α-helices (magainin, cecropin, and cathelicidin), β-sheets (hepcidin, human α-defensin 1), a mixture of α-helices/β-sheets (human β-defensins 1), cyclic (cyclotides and catestatin), as well as extended and flexible loops (e.g., indolicidin; **Figure 1**; Hancock et al., 2006). In addition to their direct action against microorganisms, as previously described, HDPs also present activities related to innate immunity. These include the induction or modulation of pro-inflammatory cytokine and chemokine production, chemotaxis, apoptosis, inflammatory response inhibition, recruitment, and stimulation of proliferation of macrophages, neutrophils, eosinophils, T lymphocyte activation, and differentiation of dendritic cells (DCs; Bowdish et al., 2005; Nijnik et al., 2009). One property that makes HDPs extremely attractive molecules for therapeutic use is that they are, in general, non-toxic to mammalian cells. The basis for this selectivity appears to be related to the lipid composition of the target membrane (fluidity, negative charge, and the absence/presence of cholesterol; Nicolas, 2009). The negative charge of a bacterial outer membrane is an example of a typical HDP target. In contrast, zwitterionic membranes, commonly found in plants and animals are not normally accessible to HDPs (Matsuzaki, 1999; Zasloff, 2002). Furthermore, the presence of cholesterol in the membrane may usually reduces HDP activity, since cholesterol helps in lipid bilayer stabilization, thus reducing membrane fluidity and flexibility (Matsuzaki, 1999).

This concept, in addition to peptide promiscuity, adds remarkable value to peptide antibiotic compounds, which have been shown to be much more useful for an organism's protection than merely membrane disruptors. The phenomenon of protein promiscuity, in which several functions are associated with a single peptide structure, has gained attention in several research fields, especially in the area of drug design (Franco, 2011). From this perspective, this review focuses on these compounds from various sources in order to shed some light on the structure and the observed mechanism of action. Furthermore, biotechnological and pharmaceutical potential will also be evaluated and discussed, as well as isolation and production in large-scale.

PLANT HOST-DEFENSE PEPTIDES

Plants have an arsenal of peptides in their own primitive innate immune system that is completely different from that of animal systems. Since plants are unable to synthesize antibodies, these peptides form an efficient barrier against bacteria, fungi, and insects. Furthermore, along with these peptides, plants have a set of physical and chemical barriers that act together to prevent infection (Sels et al., 2008). The plant cell wall is the first level of defense, formed by a complex network of proteins and polysaccharides. Even during a pathogen attack, plants are able to deposit callose, otherwise known as the polysaccharide β-1,3-glucan, at the infection site and further synthesize lignin-like polymers, thus reinforcing the wall (Hématy et al., 2009). Callose is a polysaccharide that acts in response to multiple biotic and abiotic stresses, as well as during a variety of processes in plant development (Chen and Kim, 2009). When a pathogen crosses through the cell wall, plants can induce a hypersensitive response (HR) or synthesize antibacterial and antifungal compounds for infection control. HR is characterized by an induced cell death at the point of pathogen contact and can be related to resistance (Mur et al., 2008). The antimicrobials produced are: ROS, secondary metabolites, proteins, and peptides were found (Benko-Iseppon et al., 2010).

In addition to providing a primary defense function, several plant peptides have shown a side biological property that could be exploited for biotechnological applications. This ability, in which a single structure provides multiple functions, has been denominated peptide promiscuity (Franco, 2011). Several plant peptides that show medicinal properties include defensins, thionins, and cyclotides. These peptides have shown a wide diversity of simultaneous activities, in addition to antibacterial and antifungal properties, which includes anti-HIV (Wong and Ng, 2006), antitumor (Lin et al., 2010), leishmanicidal (Berrocal-Lobo et al., 2009), uterotonic (Gran, 1973), anthelmintic (Colgrave et al., 2009), and neurotensin blocking activities (Witherup et al., 1994). However, no plant peptide was found to have both simultaneous antimicrobial and immunomodulatory activities in mammals, clearly limiting the classification of HDPs into the plant kingdom. Nevertheless, we believe that this lack of immunomodulatory activity is related to the low quantity of studies in this field and not to the intrinsic properties of these promiscuous peptides in plants.

Plant AMPs show common properties that are characterized by a low molecular mass (around 10 kDa), cationic charge, and the presence of cysteine residues that are linked through disulfide bonds, thus stabilizing the molecular structure (Padovan et al., 2010). However, defensins, cyclotides, and thionins have specific and typical structures that will be described below.

Defensins are ubiquitous peptides in plant species, showing several nuanced functions (Padovan et al., 2010). Defensins are cationic peptides with pI values around 9.0 and are 45–55 amino acids residues long, with molecular masses ranging between 5 and 7 kDa (Carvalho Ade and Gomes, 2009). The common tertiary structure consists of a triple-stranded antiparallel β-sheet, and one α-helix which in most cases is stabilized by either three, four, or five disulfide bonds (**Figure 2A**; Garcia-Olmedo et al., 1998). The primary sequence is low conserved between different defensins, and this may explain the diverse biological activities reported (Padovan et al., 2010). Some of the defensins can be classified as promiscuous peptides (Franco, 2011) having multiple-biological functions (**Table 1**). In addition to defensins, thionins are peptides with low molecular masses (5 kDa), 45–47 amino acid residues in length and can present three or four conserved disulfide bonds that stabilize the structural fold (Padovan et al., 2010). Due to their sequences and disulfide bonds patterns, thionins are classified into five structural types (I–V). The conserved structure is formed by two antiparallel α-helices, one β-turn, and one antiparallel β-sheet (**Figure 2B**; Padovan et al., 2010). Some thionins demonstrated unusual biological functions (**Table 1**).

Finally, cyclotides are small peptides characterized by 28–37 amino acids long, presenting three disulfide bonds, and a cyclic backbone formed through the linking of amino and carboxyl-terminus (Craik, 2010). This structure confers to the cyclotides a strong and stable molecular structure (**Figure 2C**). Although *M. cochinensis* trypsin inhibitors have been classified as cyclotides due

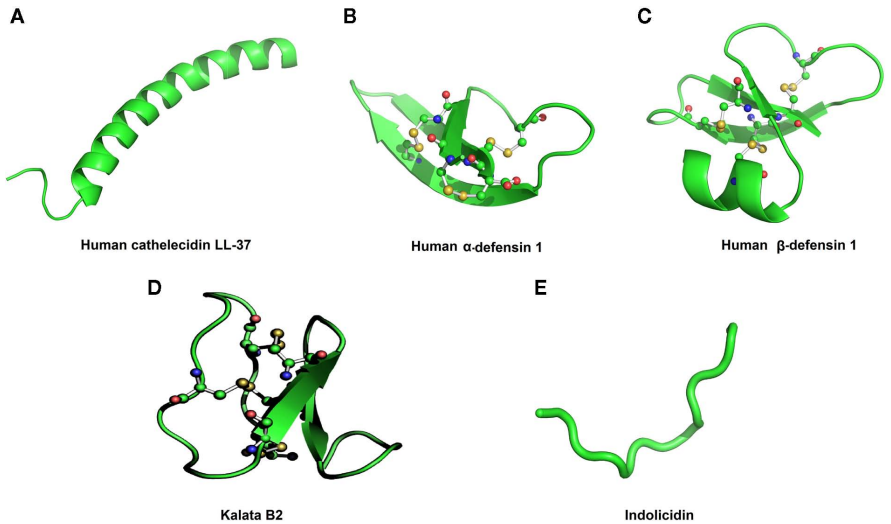

FIGURE 1 | An overview of the major structural classes of host-defense peptides including (A) α-helices, (B) β-sheets, (C) a mixture of α-helices/β-sheets structures, (D) cyclic, and (E) extended structures. Disulfide bonds are represented in ball and stick.

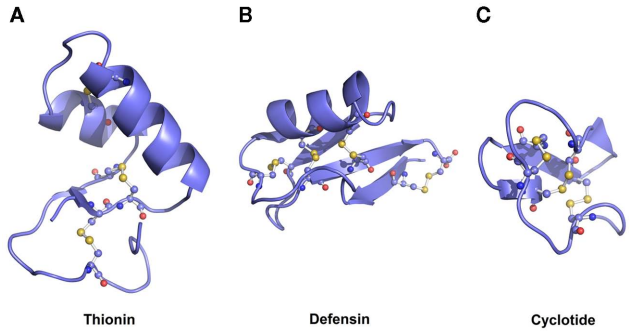

FIGURE 2 | Three-dimensional structures of plant antimicrobial peptides. (A) Defensin – *Vigna radiata* defensin-2, **(B)** Thionin – *Viscum album* viscotoxin A3, and **(C)** Cyclotide – *Viola odorata* violacin A. Disulfide bonds are represented in ball and stick.

to their structural similarities, they do not display sequence homology to the previously identified plant cyclotides (Felizmenio-Quimio et al., 2001). Until now, cyclotides were found in Violaceae, Rubiaceae, Apocynaceae, Cucurbitaceae, Poaceae, and Fabaceae families (Craik, 2010). Despite their conserved molecular structures, these cyclic peptides have shown diverse biological functions in addition to insecticidal activity (**Table 1**).

HOST-DEFENSE PEPTIDES AND THEIR MULTIPLE ROLES IN ANIMAL IMMUNE DEFENSE

Previously, it was believed that the direct antimicrobial activity of AMPs was essential for their role in innate immunity and host-defense. However, some studies have shown that peptide concentrations found in various body parts, such as mucous for example, are relatively low when compared with those used in *in vitro* studies (Hirsch et al., 2008). It has been known that, in addition to this activity against pathogens, HDPs may also have an indirect antimicrobial effect, as previously cited (Auvynet and Rosenstein, 2009). The human cathelicidin LL-37 (hCAP18), a well-characterized multifunctional HDP, is constitutively produced by leukocytes and induced in barrier organs upon inflammation and infection, thus being essential for immune response to injury and tissue infection (Bowdish et al., 2005). Another well-characterized HDP is the human β-defensin-2 (hBD2). It is known that this peptide plays an important role in immune response at different mucosal surfaces and also in the skin barrier (Ganz, 2003). Among the LL-37 and hBD2 wide activities spectrum, the induction of histamine and prostaglandin D2 released by mast cells after stimulating HDPs have important roles in microbial invasion response (Niyonsaba et al., 2001). Concomitantly to these activities, LL-37 induces keratinocytes to release IL-1β, IL-8, TNF-α, IL-6, colony-stimulating factor and granulocyte–macrophage (GM-CSF), and immature DCs to release TNF-α and IL-6 (Braff et al., 2005). However, the peptide response is dependent on the cell type that is studied. For example, in human peripheral blood mononuclear cells (PBMCs), LL-37 inhibits the expression of pro-inflammatory molecules, such as TNF-α and IL-6, and nuclear translocation of NFκB p50/p65 induced by toll-like receptor (TLR)-2 and TLR-4 in response to lipoic acid (LA) and lipopolysaccharide (LPS), respectively. This inhibition is an important event in severe microbial infections, such as sepsis (Mookherjee et al., 2006). In addition, the hBD2 also acts on TLRs, functioning as an endogenous TLR-4 ligand, further activating immature DCs, and triggering a strong Th1 response (Biragyn et al., 2002).

Moreover, other not-so-well-studied peptides also act significantly on the mammal immune system. Human α-defensins (HNP-1, HNP-2, and HNP-3) and β-defensins (hBD3 and hBD4) are involved in neutrophil and monocyte recruitment, while mast cells may be attracted to the infection site by LL-37, HNP-1–3, and hBD2 (Chen et al., 2007). Besides, human α-defensins and β-defensins are chemotactic for immature DCs and memory T cells

Table 1 | Plant HDPs and their multiple activities related to host-protection.

Peptide name	Source	Activity	Possible application	Reference
DEFENSINS				
–	*Phaseolus angularis*	Inhibit tumor cell lines L121 and MBL2	Antitumor therapy	Ma et al. (2009)
Limenin	*P. limensis*	Inhibit myeloma (M1) and leukemia (L1210) cell lines and inhibit HIV-1 reverse transcriptase	Antitumor and antiviral therapy	Wong and Ng (2006)
Lunatusin	*P. lunatus*	Inhibit breast cancer cell line (MCF-7), the activity of HIV-1 reverse transcriptase, and the translation in a cell-free rabbit reticulocyte lysate system	Antitumor and antiviral therapy	Wong and Ng (2005)
–	*P. vulgaris*	Inhibit hepatom (HepG2), breast (MCF-7), colon (HT29), and cervical cancer (SiHa) cells, and inhibit HIV-1 reverse transcriptase activity	Antitumor and antiviral therapy	Lin et al. (2010)
THIONINS				
Without name	*Triticum aestivum*	Inhibit the proliferation of *Leishmania donovani*	Leishmanicidal therapy	Berrocal-Lobo et al. (2009)
PTH1	*Solanum tuberosum*	Inhibit the proliferation of *L. donovani*	Leishmanicidal therapy	
CYCLOTIDES				
Kalata B1	*Oldenlandia affinis*	Uterotonic, anti-HIV activities, and anthelmintic activity against parasites of sheep, humans, and dogs	Anthelmintic, antiviral, and uterotonic therapy	Gran (1973), Colgrave et al. (2008), Colgrave et al. (2009)
Kalata B6	*O. affinis*	Anthelmintic activity against gastrointestinal parasites of sheep, humans, and dogs	Anthelmintic therapy	Colgrave et al. (2008), Colgrave et al. (2009)
CirA	*Chassalia parvifolia*	Anti-HIV activity	Antiviral therapy	Gustafson et al. (1994)
CirB	*C. parvifolia*	Anti-HIV activity	Antiviral therapy	
Cyclopsychotride	*Psychotria longipes*	Ability to block neuropeptide neurotensin	Antipsychotic therapy	Witherup et al. (1994)
Cycloviolacin O2	*Viola biflora*	*In vitro* antitumor activity	Antitumor therapy	Gerlach et al. (2010)

(Yang et al., 2002), where human α-defensins selectively induce the migration of human cells $CD4^+$, $CD45^+$, and $CD8^+$ naive, suggesting that HDPs play an important role in mobilizing and amplifying the innate and adaptive immunity against microbial invasions (Yang et al., 2001). Indolicidin, for example, inhibits TNF-α secretion by macrophages in response to LPS treatment (Bowdish et al., 2005). Similar to the effects of LL-37, indolicidin induces the production of IL-8 in human bronchial epithelial cells $16HBE14o^-$. They also have demonstrated that the peptide bactenecin 2A, a member of bactenecins family, presented chemotactic activity over TH1 cells. Dermcidin-1L, another HDP example, stimulates keratinocytes to secrete TNF-α, IL-8, CXCL10, and CCL20 (Niyonsaba et al., 2009). Another group of peptides with strong antimicrobial activity and immunomodulatory activity are protegrins. Protegrins-1 and -3 promote rapid and efficient mature IL-1 beta release. Both peptides also promote modification in the morphology of monocytes and a loss of latency of the cell membranes (Perregaux et al., 2002).

Recent data have shown that HDPs are multifunctional molecules at different tissue levels, acting in the neuroendocrine system. Cathelicidins and defensin mRNA were widely found in the brain (Su et al., 2010), suggesting that HDPs could be involved in innate immune brain defense. Recently, it has been discovered that some neuropeptides play an important role in antimicrobial activity. Enkelytin, a pro-enkephalin-derived peptide, has shown strong activity against Gram-positive bacteria and also has acted as a link of communication and interaction between nervous, endocrine, and immune systems (Goumon et al., 1996). Met5-enkephalin peptide stimulated the proliferation of natural killer cells, B- and T-lymphocytes, migration of monocytes, lymphocytes, and neutrophils, and also stimulated the secretion of IL-6 by monocytes (Kamphuis et al., 1998). Recently, Shan et al. (2011) suggested that Met5-enkephalin appeared to be involved in the regulation between the neuroendocrine and immune systems, modulating several cell functions related to innate and adaptive immunity. They also have found that Met5-enkephalin activated CD4 + T cells by increasing the expression of delta receptors. Moreover, this peptide also induced DC maturation through the expression of surface molecules (MHC class II, CD86, CD40), stimulated the production of IL-12, and down-regulated the intracellular acid phosphatases (ACP) in DCs. In addition, Met5-enkephalin (alone or in combination with either IL-2 or IFN-γ) up-regulated the proliferation of CD4 + T cells and increased production of interferon-γ in these cells. The peptide leucine-enkephalin stimulated the production of T helper cells and cytotoxic T cells (Sizemore et al., 1991).

Among peptides with antimicrobial activity described in the neuroendocrine system, those derived from chromogranins have been highlighted. They are members of the granin family, which are acidic proteins present in secretory vesicles in the endocrine and immune systems (Shooshtarizadeh et al., 2010). Chromogranins have shown multiple-biological activities including neuroendocrine activity, modulation of homeostatic processes such as regulation of calcium metabolism and glucose, gastrointestinal

motility, nociception, strong activity in tissue repair, and inflammatory response mediation. More recently, peptides derived from chromogranins could act as defense agents during infections of bacteria and fungi. Moreover, chromogranin-derived peptides have shown activity in innate immunity, being able to activate polymorphonuclear neutrophils, mediate the monocyte chemotaxis, and induce microglial cell activation (Shooshtarizadeh et al., 2010). Peptides derived from chromogranins have shown special activity against pathogens commonly found in the skin, revealing that the expression of these peptides may be performed by keratinocytes. In these cells, chromogranin A fragments generated catestatin: a broad-spectrum antimicrobial peptide. These fragments induce monocyte chemotaxis and act on the production of cytokines and chemokines by mast cells (granulocyte–macrophage colony-stimulating factor, monocyte chemotactic protein-1, macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, and chemokine – C-C motif – ligand 2, 3, and CCL2, CCL3, CCL4). Furthermore, it also stimulated keratinocyte interleukin-8 production through the activation of mitogen-activated protein kinases (Radek et al., 2008; Shooshtarizadeh et al., 2010). Other peptides derived from chromogranins, termed vasostatins, have shown deleterious activity against Gram-positive bacteria, filamentous fungi, and yeasts (Dondossola et al., 2010). These studies have demonstrated that a neuroendocrine peptide may have antimicrobial activity against a wide variety of skin pathogens and may be up-regulated in the lesion. These data have demonstrated a direct link between the neuroendocrine and immune system (Shooshtarizadeh et al., 2010). Finally, the expression of catestatin in mice skin resulted in higher protection against infection and injury. Catestatin is a 21-amino acid residue, which is made endogenously from the proteolytic cleavage of chromogranin A (Radek et al., 2008).

Studies have shown that members of the neuropeptide bombesin family, in addition to direct activity against microorganisms, mediate a variety of biological activities in the gastrointestinal tract and central nervous system (CNS) of mammals, including smooth muscle contraction, hormone secretion, cell proliferation stimulation, and central-homeostatic mechanism regulation (Su et al., 2010). Another HDP family found in mammalian brains are hepcidins, which besides having direct antimicrobial activity, also reduce secretion of TNF-α, IL-1α, IL-1β, and IL-6 by regulating the COX-2 gene and PDE4D by using mechanisms dependent on pERK1/2 in macrophage cells (Su et al., 2010). In this view, due to LPS stimulation, a significant increase in hepcidin mRNA expression was observed in cortex and *substantia nigra*. These findings suggest that the CNS can use many peptides as anti-infective and immunomodulatory agents by delivering them quickly and accurately to innervated sites, indicating a new dimension for an immunomodulatory role for neuropeptides in inflammatory and immune responses (El Karim et al., 2008).

HOST-DEFENSE PEPTIDES: FUNDAMENTAL MOLECULES IN RESPONSE TO HUMAN MICROBIAL INFECTIONS

HDPs are evolutionarily ancient molecules that reinforce the human innate immune system against microbial invasion (Nijnik et al., 2009). A constitutive HDP expression has been observed in phagocytic cells and in body tissues (**Figure 3**). This occurrence

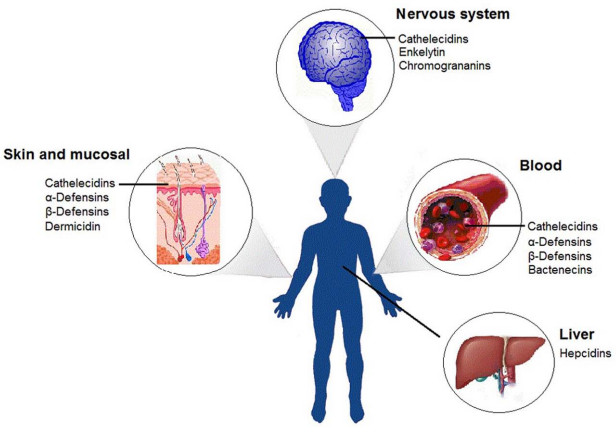

FIGURE 3 | Distribution of host-defense peptides described in human body. The host-defense peptides have a wide distribution in human body, being found mainly in skin, mucous membranes, blood, nervous system, and liver.

happens particularly at human interfaces between the environment and the host, such as skin and mucosal membranes (Hirsch et al., 2008). HDP synthesis and release are regulated by microbial stimuli, cytokines, neuroendocrine signals, and epigenetic regulation (Ganz, 2003).

Along these lines, intestinal mucosal pathologies, such as Crohn's disease and intestinal inflammation, result from the reduction of α-defensin expression in the small intestine (Inaba et al., 2010). The protective role of α-defensins against pathogenic bacteria in intestinal infections has been certified in α-defensin-deficient mice, since mice became more susceptible to *Salmonella typhimurium* infections (Inaba et al., 2010). Moreover, hBD1 and hBD2 are important HDPs in gastric-intestinal mucosa. The expression of both peptides increased in the gastric antrum mucosa, which is commonly induced by gastritis caused via *Helicobacter pylori* (Bajaj-Elliott et al., 2002).

Regarding HDPs produced in human skin, patients with infectious cellulitis presented an increased expression of LL-37 and β-defensin in comparison with those normal skins (Stryjewski et al., 2007). This datum suggests that HDPs may provide additional protection against microbial dissemination, confining the focus of infectious cellulitis to a single location. However, patients with atopic dermatitis presented an absence or reduced expression of LL-37, hBD2, hBD3, and dermicidin (Bernard and Gallo, 2011). These findings suggest that patient susceptibility to atopic dermatitis in skin infections could be higher due to a significant increase in either bacterial colonization, fungal, or viral infections (Bernard and Gallo, 2011). Recent studies have detected high levels of human β-defensin, hBD2, hBD3, and psoriasin in cutaneous infections after skin surgery (Kesting et al., 2010). Expression of hBD2-3 is reduced in burn wounds, while the expression hBD1 is unchanged (Milner and Ortega, 1999). However, the skin's lower layers continue to express hBD1-3 after wound recovery, maintaining a barrier against infection and preventing burn sepsis (Poindexter et al., 2006).

Sepsis is a major cause of mortality and high hospital costs and, despite the efforts that have been made by public health authorities in recent decades, the incidence of this syndrome continues to increase year after year due in particular to the increasing emergence of microorganisms resistant to the available antibiotics (Barnato et al., 2008). Several HDPs have been reported to modulate the host response to bacterial sepsis. LL-37 acted as a potent inflammation molecule mediator, protecting mice and rats from the lethal effects of endotoxemia and intra-abdominal sepsis. This protection occurs due to a reduction in the levels of TNF-α and LPS in plasma, which inhibit the activation of macrophages by LPS, lipoteichoic acid (LTA), and non-mannose-capped lipoarabinomannan, as well as regulating the expression of coding genes and receptors (CXCR-4, CCR2, and IL-8RB) of chemokines in macrophages (Torossian et al., 2007). The main mechanism of TNF-α expression inhibition by LL-37 in mice is believed to occur by the blocking of LBP-mediated transport of LPS in $CD14^+$ cells (Nagaoka et al., 2001). HDPs buforin II, indolicidin, and cecropin B have shown effectiveness in the treatment of rats with septic/endotoxic shock caused by multi-resistant *Escherichia coli* and LPS, significantly reducing the plasma levels of endotoxin and TNF-α when compared to animals treated with piperacillin (Vallespi et al., 2003). Limulus anti-LPS factor (LALF), a peptide isolated from the horseshoe crab, could increase the survival of mice after administration of a lethal *P. aeruginosa* dose. This activity may be correlated with a decreased TNF-α mRNA synthesis and elevation of systemic IL-2, IL-12, and IL-13 into animal spleen and livers (Vallespi et al., 2003). Mice with endotoxic shock derived from an LPS and treated with S-thanatin, an insect peptide, showed reduced levels of TNF-α accompanied by a significant increase (80%) of animal survival (Wu et al., 2011a). In another study, animals infected with multi-resistant *P. aeruginosa* and further treated with a single dose of tachyplesin III isolated from horseshoe crabs has shown reduced TNF-α levels in contrast to a clear survival increase (Cirioni et al., 2007). Treatment of amphibian source magainins (I, II, and III) in septic/endotoxic shock significantly reduced the concentrations of endotoxin and TNF-α in plasma rats, thus increasing host survival (Cirioni et al., 2002). These data suggest that peptides that could act against septic shock could be found at different sources and their activities are clearly related to TNF-α inhibition and endotoxin neutralization, which seem to be important mechanisms that increase the survival of infected animals.

NOVEL AND CLASSICAL STRATEGIES FOR HDPs ISOLATION

Currently, several limitations have driven the area in the development and production of HDP molecules away from natural peptides to the shorter and more stable synthetic forms. Libraries, synthetic strategies, and peptidomimetic technologies are being refined in order to overcome current limitations (Yeung et al., 2011).

Traditionally, since the beginning in 1928, with the discovery of lantibiotic Nisin produced by a *Lactococcus lactis*, until nearly the end of the twentieth century, the discovery and identification of peptide antimicrobial molecules from natural sources with pharmaceutical anti-infectious purposes have been carried out by conventional biochemical procedures. Both goals frequently started from an expected and confirmed biological activity: generally a direct inhibition of microbial growth of bacteria, virus, fungi, and parasites (using a medical concept of microbes). This primary evaluation of crude extracts was the starting point for combined steps of clarification, salt or acid precipitation, ultrafiltration, chromatographic purification, and the eventual available structural characterization. This approach, on the other hand, required an important volume of specimen, whole bodies or parts, to assure a significant level of antimicrobial activity. These procedures were achieved representing a real ecological challenge for the different life kingdoms if, finally, a large-scale pharmaceutical industry could be implemented for sustaining permanent production based on such an animal source (Bulet, 2008). Such natural sources were, in principle, associated with invertebrates, since HDPs are their major humoral tool to defeat a vast microbial challenge and must be effective in the absence of acquired immunity (Otero-Gonzalez et al., 2010). Today, multiple AMPs have been explored also for their immunomodulatory properties even when they are, *in vivo*, less potent for directly killing microbes than their conventional antibiotic counterparts (Lai and Gallo, 2009).

New trends in research are currently categorizing the discovery of new HDPs through the use of biological peptide libraries, thus providing a larger and more diverse quantity of molecules for evaluation. Such libraries based on phage (Pini et al., 2005), bacterial (Betscheider and Zangen, 2005), or ribosome display (Xie et al., 2006) are nevertheless time-consuming procedures and difficult to handle. In fact, a novel method for the identification of AMPs using a phage library and bacterial magnetic particles has been reported (Betscheider and Zangen, 2005; Pini et al., 2005). Using this library, six antibacterial peptides specifically active against *B. subtilis* have been identified. It was the first attempt that showed the functionality of a magnetic selection of HDPs targeting the bacterial inner membrane. Furthermore, these methodologies produce fusion instead of individual peptides and, additionally, only amino acids encoded in the genome can be used with a reasonable support of biological diversity. A clear advantage of these approaches is that peptides are biologically synthesized, avoiding the use of expensive chemicals and facilities (Mcphee et al., 2005). Furthermore, the advances in the genomic area including next generation sequencing and improvements in databank and bioinformatics tools have permitted the identification of many different compounds including HDPs. In a well-designed genetic approach reported in flatfish, the identification of active, novel antimicrobial molecules has been determined by screening both the genomic information and the mRNA transcripts from a number of different sequences encoding AMPs. Predictions of active peptide sequences from the genetic information were the starting point for the production of chemically synthesized peptides that have been tested for their cognate activities. A very active peptide was found to show inhibitory activity against a test panel of pathogens including antibiotic-resistant bacteria and fungi (Patrzykat et al., 2003).

As an attempt to expand the efficiency of discovering novel and effective HDPs, including unnatural HDPs, chemical peptide libraries are open to amino acids not encoded in the genome, D-amino acids, and even non-canonical amino acids. Screening large numbers of peptides using a synthesis of peptide arrays on cellulose membranes (SPOT technology) provides an extraordinary

tool for characterizing large quantities of peptides for a particular biological activity (Hilpert, 2010). This improved method using hydroxymethyl phenoxyacetic acid (HMPA) for 19 amino acids and 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) for proline, since acidic labile linkers in SPOT synthesis have been developed. Using this approach, reduced side-chain reactions, normally occurring during conventional alkaline peptide cleavage of cellulose membranes, was achieved. This method is suitable for synthesizing many thousands of different peptides subsequently used for direct multiple-biological assays (Ay et al., 2008).

Since the search for novel classes includes the purification process, the properties of target peptides such as hydrophobicity, cationicity, and small size have been broadly used in their purification. As follows, reversed-phase chromatography, ion exchange, and gel filtration are commonly included in the purification procedures for these molecules (Schroder, 2010). Although direct interaction of HDPs with different target molecules such as LPS (Gustafsson et al., 2010), lipid II (Schneider et al., 2010), and sphingolipid receptors are widely known (de Medeiros et al., 2010), affinity chromatography has not been widely used for their isolation. The use of this type of chromatography may improve the isolation of AMPs with desired affinities for biomolecules. For example, HDPs with the ability to bind LPS are very important in the treatment of sepsis, as previously described (Giuliani et al., 2010). However, the majority of molecules capable of inhibiting LPS have been synthesized on the basis of the Limulus antilipopolysaccharide factor (Andra et al., 2004). There is little doubt that the rational use of the LPS-affinity chromatography could enhance the number of natural HDPs with anti-LPS activity. In this case, affinity chromatography also acts as a screening procedure. In fact, it has isolated lipopolysaccharide-binding proteins from porcine milk and from the invertebrate hemolymph *Tachypleus tridentatus* using a similar chromatographic principle (Shahriar et al., 2006). Furthermore, HDPs are able to be induced, being another HDP property that could be exploited in their purification (Lavine et al., 2005). In fact, the microbial challenge test of different vertebrates and invertebrates permitted the isolation of augmented HDPs (Isobe et al., 2009). Thus, it could be possible to induce HDPs with a potent anti-proliferative activity against a particular group of pathogens (Lemaitre et al., 1997). The induction of these molecules may reveal basal HDPs and facilitate their purification by augmenting the concentration levels for further purification. Traditionally, once a peptide sequence had been identified and/or partially purified from a natural source, a structural approach had been applied with the aim to improve its antimicrobial activity and/or decrease its toxic or antigenic implications for experimental tests in animals or initial human trials. In this sense, amino acid substitutions based on the sequence data or on the three-dimensional structure such as the replacing of arginine residues with α-amino-3-guanidino-propionic acid of the natural peptides may help to reduce serum AMPs degradation. The modified peptide stability was increased by nearly 80% without affecting the antimicrobial activity (Knappe et al., 2010). In a completely different but valid approach (based on the membrane-binding activity of most AMPs), cell membrane affinity chromatography has been reported to screen antibacterial peptide molecules from plant extracts. In this case, cell membrane affinity chromatography was useful for primary screening of cell membrane receptors according to its chromatographic retention. This constitutes one of the first reports on a high-throughput screening (HTS) of AMPs by such a principle (Xiao et al., 2011).

High-throughput screening is a well-established method in the bio-industry and is now used for basic and applied scientific research (**Figure 4**). It involves the screening of large biological and chemical libraries for activity against biological targets by automation, miniaturized assays, and large-scale data processing (Mayr and Bojanic, 2009). While HTS was initially applied to relatively non-complex simple assays concerning single molecular objectives or using affinity interactions relationships, HTS has also been applied to more integrated complex biological multicomponent systems, such as whole-cell-based tests. The intense need for novel and efficient antimicrobial molecules highlights the existence of multi-drug-resistant microorganisms, where multiple-target or cell-based assays are frequently desired, and thus is obliging researchers to reflect on multi-target, high-throughput technologies (Blondelle and Lohner, 2010). Clearly, an improved understanding of non-lytic modes of HDP interaction with target microbes will lead to the design and discovery of more effective antimicrobial molecules (Marcos and Gandía, 2009). In the pharmaceutical industry, HTS means challenging millions of molecules in massive automated applications. On the other hand, in a current academic laboratory, it may imply screening only a small quantity of peptides, mainly by conventional artisanal techniques. HDP activity may be best described by the concept of "interfacial activity" (Wimley, 2010). Structure–function relationships are infrequently utilized. The next challenge is not the generation of an extensive repertoire of molecules, but HTS employed to efficiently identify the active members from a massive source. In general, HTS could be divided into two categories: biological and non-biological procedures (Rathinakumar and Wimley, 2010), which will be discussed next.

IN VITRO AND *IN SILICO* BIOLOGICAL ASSAYS FOR THE FUNCTIONAL EVALUATION OF HDPs

Broth dilution and agar diffusion methods, in which the activity of HDPs are directly estimated for the inhibition of microbial growth in a nutritive broth, could be in principle amended for HTS (Wiegand et al., 2008). Automated and massive variations of such procedures have been established in order to scale the number and diversity of HDPs to be verified. The use of luminescent bacteria for fast screening and characterization of short cationic AMPs synthetically immobilized in cellulose using peptide array technology has been developed (Hilpert et al., 2005). In this unusual approach, the authors used bacteria expressing the luciferase gene cassette (luxCDABE). Any synthetic peptide decreasing the energy level within the microbial cell will cause a quantifiable decrease in luminescence. The effectiveness of a tested molecule, at different concentrations, is reflected by the decreasing rate in luminescence. The assay is rapid and high-throughput and has shown significant correlation when compared with conventional suspension inhibitory culture assays performed with the same peptides synthesized by standard solid-phase peptide synthesis. Using this approach, 277 variants of the antimicrobial peptide

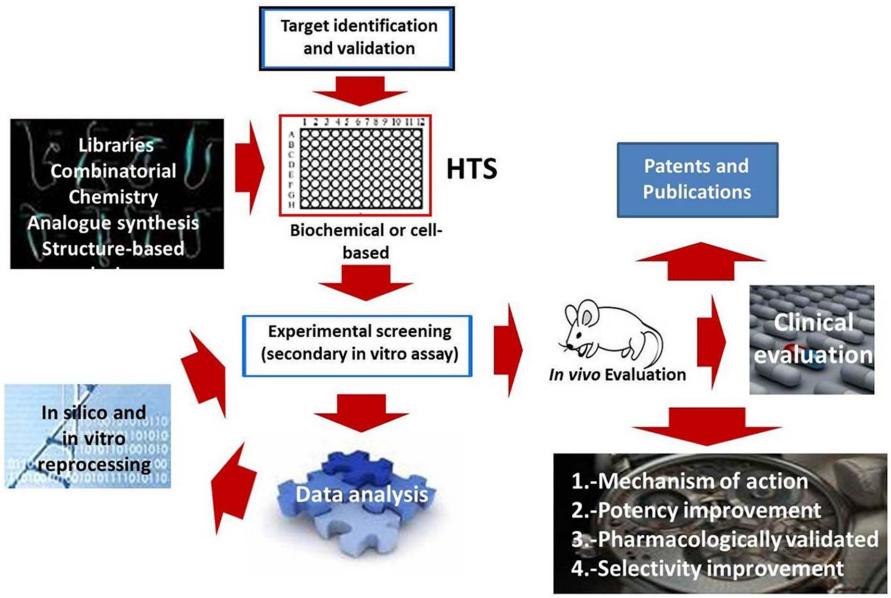

FIGURE 4 | A typical "end point" high-throughput screening flow for searching novel host-defense peptides.

Bac2A (a 12-mer linear variant of the HDP bactenecin) were studied. The antimicrobial activity of Bac2A derivatives ranged from superior to inactive compared to Bac2A (Hilpert et al., 2006). This technology allowed for a decreased amount of time required to convert leaded antimicrobial molecules into drugs.

In another interesting approach, a novel assay that had exploited fluorescence resonance energy transfer (FRET) was set up for antimicrobial peptide screening. It took advantage of changes in pH that occur in FRET efficiency due to the instability of increased yellow fluorescent protein against the stability of increased cyan fluorescent protein in a reduced-pH environment. Evaluation of antimicrobial activity was achieved through a difference of FRET efficiency between fusion molecules released from disrupted bacteria. It represented a clear example applicable to HTS of candidate peptide libraries (Kim and Cha, 2006). In this line of thought, a high-throughput method for screening cDNA libraries has been developed to detect possible AMPs. It was based on a fast dye inclusion assay for determining a bacterial viability loss. Colonies were grown on a membrane on a suitable medium until full colony development. The membrane containing the array of colonies is transferred onto an inductive medium containing a vital dye. Upon expression of any active peptides, the cell membrane becomes susceptible and permits dye infusion to induce visual identification of positive peptides (Loit et al., 2008). In this way, a random oligonucleotide library containing a potential pool of 100,000 peptides was screened. The authors found three novel antibacterial peptides. One of them has shown membrane disruption and bacterial aggregation activity (Loit et al., 2010). Unfortunately, these HTSs are not available yet for other HDP activities like immunomodulation or wounding repair. The development of techniques in these areas will boost the insertion of HDPs into the drug industry.

An early attempt for automating the screening of membrane-related AMPs was reported in which a colorimetric sensor by which particles composed of phospholipids and polymerized polydiacetylene lipids were shown to exhibit clearly visible color changes upon interactions with antimicrobial membrane peptides. The color changes in the system are due to a structural perturbation of the lipids after their interactions with AMPs. The assay was also valuable for detecting functionally related peptide analogs (Kolusheva et al., 2000). Alamethicin, gramicidin, and valinomycin have been investigated in such planar film systems (Volinskya et al., 2006). Otherwise, the *in silico*-based screening systems include computerized methods as well as simulation methods that mimic the interaction between peptides and membranes (Raventos et al., 2005). A mathematical model has been developed to predict, before synthesis, a peptide with an activity against *P. aeruginosa* (Jenssen et al., 2008). Using original descriptors for assessing the contact energy between spatially closed amino acids, as well as a set of inductive and conventional quantitative structure–activity relationship (QSAR) descriptors, it has been possible to model the antibacterial activity of peptides before the synthesis step. Cross-correlation and optimization of the selected descriptor values permitted these authors to build two models, using very limited number of peptides. Even when such models were significantly different in size, no significant difference was demonstrated in their predictive power, meaning that it was possible to use this tool to obtain useful and potent predictive models, even when using small sets of peptides with different structures. The development of biological assays in HTS has increased the number of known antimicrobial structures allowing the development of newer inductive QSAR descriptors, which in combination with more complex mathematical algorithms, improve the *in silico* identification of novel HDPs (Hilpert et al., 2008). Also, QSAR

descriptors have been developed for predicting antimicrobial peptide toxicity against host cells (Langham et al., 2008). Additionally, recent advances in molecular dynamics simulations of AMPs and membrane mimics offer new data to help us to understand the mechanism of action of these molecules (Langham and Kaznessis, 2010).

In a recent chemical approach, novel cationic AMPs were proposed with a simple strategy using acid-amide substitution to add a net positive charge to natural non-antibacterial amino acid sequences showing structures that were different from the reported cationic AMPs. Alterations produced in membranes by these modified peptides were evaluated and conformational changes were estimated from far-ultraviolet circular dichroism spectra. These novel cationic AMPs showed sequences completely distant from any other AMPs described before, suggesting that such modified natural sequences could be an interesting source of novel frames for cationic AMPs (Ueno et al., 2011).

LARGE-SCALE HDP PRODUCTION: OVERCOMING A MAIN PROBLEM

Structural and functional features, as well as several other significant physiological characteristics of these molecules as described in this review, are reasons why HDPs have received much attention from the scientific community for the development of new pharmaceuticals. However, there are real barriers in developing and producing natural HDPs. The challenge begins with the understanding of the fundamental principles of action in HDPs, as well as their structure–function relationship and pharmaceutical potential (Wimley, 2010). The lack of this understanding has been one of the major reasons why the development of these peptides into new drugs has slowed. This is due to the fact that only a few heterologous systems are capable of yielding a sufficient amount (in the range of ∼50–690 mg L^{-1}) of soluble and purified peptides in order to enlighten such extensive biological and structural questions (Xu et al., 2007; Li, 2009). Moreover, in order to accomplish pharmaceutical and biotechnological goals, it is necessary to produce these peptides in large-scale. This sort of production has been the biggest challenge in studies regarding screening, synthesis, and characterization of these molecules.

Despite two decades of continuous efforts, the limitation of small-scale production has impaired the successful use of HDPs in health care. For instance, only four peptides to date have reached global sales of over US$ 1 billion as therapeutic drugs. These have been prescribed for treatments for multiple sclerosis as Glatiramer acetate (also known as Cop-1 or Copaxone; $3.8 billion), for prostate cancer and breast cancer as leuprolide acetate (Lupron; $2.12 billion) and goserelin acetate (Zoladex; $1.14 billion), and for treatment of acromegaly and carcinoid syndrome as octreotide acetate (Sandostatin; $1.12 billion; Peptide Therapeutics Foundation, 2010 Report Summary). Moreover, in the last 5 years, only one HDP, the human bactericidal/permeability-increasing protein $rBPI_{23}$ (Neuprex – indicated for treatment of meningococcal sepsis) has been approved for marketing. Furthermore, only three HDPs have progressed to phase III of clinical-efficacy trials (Pexiganan, Iseganan, and Omiganan) and none of these have to date received FDA approval for clinical use (Hancock and Sahl, 2006; Yeung et al., 2011).

To change this scenario, current approaches need to be refined and new technologies are required to be employed in the development and production of HDPs. In order to recover and produce peptides, different methods can be employed such as the direct isolation from natural sources, chemical synthesis, or recombinant expression. The first strategy, despite extensive and efficient utilization at academic levels, it is extremely time-consuming and only low amounts of peptides are recovered from the host organism. This technique may even present environmental issues, especially for peptides isolated from species that occur in low numbers in nature (Li, 2011).

Chemical synthesis has been traditionally used to synthesize short and simple molecules and has allowed for the production of both natural and synthetic peptides (Zhou et al., 2010). However, it is tremendously costly and practically unfeasible for the synthesis of sequences larger than 15 residues of amino acids, running in the range of US$ 100–600/g (Hancock and Sahl, 2006). Moreover, the cost may increase substantially due to difficulties that arise during synthesis of certain sequences such as those containing one or more disulfide bonds, or showing multiple post-translational modifications (Tay et al., 2011).

In view of the limits and inefficiencies of both methods described above, the most widely used method is the heterologous expression system. In the last few decades, various systems have been developed to reach a cost-effective and large-scale production of several proteins (Huber et al., 2005) and HDPs such as human defensins LL-37 and IDR-1 (Bommarius et al., 2010).

In most cases, the effectiveness of heterologous expression for the production of HDPs involves the fusion of carrier proteins containing an enzymatic or chemical cleavage site that allows the target peptide to be released. Some of these proteins act as stabilizing molecules due to their ability to neutralize the positive charge of the peptide, thus resulting in a non-toxic, efficient, and soluble expression of the target peptide (Rao et al., 2004). According to the *Recombinantly produced* AMPs *Database* (http://faculty.ist.unomaha.edu/; Li and Chen, 2008), the carrier protein most frequently used is the thioredoxin, representing more than 20% of all reported fusion proteins, followed by the use of GST (glutathione transferase – ∼12%), PurF (amidophosphoribosyltransferase), and intein-mediated protein (∼8%). The SUMO protein (small ubiquitin-related modifier) has been a promising carrier protein and has been shown to increase expression levels as well as the solubility of peptides (Malakhov et al., 2004; Bommarius et al., 2010).

The heterologous expression system may also be composed of one or more epitope tags (Rubio et al., 2005). These are short and hydrophilic peptide sequences recognized by specific antibodies, thus offering efficient detection. The most used epitope tags are HA (YPYDVPDYA), FLAG (DYKDDDDK), cMyc (EQKLISEEDL), and 6xHis (HHHHHH). Besides being used for subcellular location, their small size provides efficient affinity purification and is more likely to interfere less with the peptide of interest regarding its folding and function, when compared to larger carrier proteins (Earley et al., 2006).

Among the available commercial options of heterologous expression vectors, the most commonly used are those which belong to the pET and pQE series, developed by Novagen and

Qiagen, respectively. These vectors have been largely used for expression of HDP derived from several organisms such as defensins from humans (e.g., α-defensin 6), bovine (β-defensin 12) and defensin-like from fungus (plectasin; Wu et al., 2011b). In addition, new mutant strains of *E. coli* BL21 (DE3) have been developed to overcome the lethality caused by the expression of AMPs (Li et al., 2010).

Regarding the prokaryotic systems, *E. coli* is the most popular organism used to express recombinant proteins. Due to its features such as rapid growth rate, low cost, large availability for commercial expression vectors, and extensive knowledge about its genetics and physiology, *E. coli* has been applied to innumerable studies of synthesis and expression of AMPs and HDPs from different organisms (Canales et al., 2011) and has also been used in large-scale production of these peptides (Bommarius et al., 2010).

The limitation of the use of prokaryotic systems is to perform post-translational modifications required for stability and biological activity of certain peptides. To overcome this limitation, the use of eukaryotic systems is the appropriate approach. The yeast species *Pichia pastoris* and *Saccharomyces cerevisiae* represent the most common eukaryotic host system used for expression of heterologous proteins (Holz et al., 2002). The yeast *P. pastoris* has been used for expression of HDPs derived from humans and plants, although none have reported large-scale production (Yang et al., 2007). Naturally, the disadvantage in using these organisms is their innate sensitivity against antifungal peptides (Thevissen et al., 2007).

Plants have been a promising alternative for eukaryotic systems and numerous strategies have been developed to optimize these organisms to attend commercial production (Desai et al., 2010). The most common plant hosts are *Nicotiana tabacum* and *Arabidopsis thaliana*, which have been reported in expression of AMPs derived from plants such as LjAMP1, SmAMP1, and SmAMP2, which have activities against 15 plant pathogens, including those of economic concern. Furthermore, considering AMPs in plants act like HDPs, such as defensins, the expression of these molecules, either in plants or in bacteria, has been successfully achieved. However, there is no report in the literature regarding expression of HDPs from animal sources expressed in plants. Diverse studies have demonstrated viable possibilities of using plants for production of biotechnological products, contributing to the development of new heterologous expression systems to reach large-scale production (Pogue et al., 2010). Nevertheless, it should be emphasized that there needs to be advances in technology before these organisms can be chosen over bacteria as an expression system for recombinant HDPs.

The heterologous expression technologies overviewed here are of paramount concern regarding the production of natural or synthetic peptides in large-scale for therapeutic research and development. The development of new technology and the widespread acceptance of protein therapy as listed above will very likely increase the percentage of HDPs and AMPs as therapeutic candidates for pharmaceutical and biotechnological industries.

CONCLUDING REMARKS

As focused in this report, promiscuous AMPs and HDPs are evolutionarily primordial defensive molecules widely distributed in different kingdoms suggesting that they have played an essential role in the development of multicellular organisms. This central function comes from the fact that these promiscuous peptides present the ability to bind to different targets. In addition to the direct effects against microorganisms, HDPs also show the ability to act in the host organism, thus monopolizing the control of infections at higher levels. This ability involves basic functions of acquired immunity such as cytokine signal transduction, TLR interaction, and primeval immune memory, thus acting as effective immune modulators in mammals. Unfortunately, even though research on HDPs has lead to a better understanding of their functionality, the number of outbreaks is continuously increasing while few alternatives are currently available to combat these infections.

In order to control this increasing problem, novel strategies for treating infectious illnesses will be necessary, requiring additional manipulation of immune responses in order to intensely diminish the infectious agents and also avoid tissue wounds due to inflammation. The interchange ability between immunogenicity, danger signaling, and immune modulation of HDPs could act in synergy with the immunity normally offered by humans. In this view, normal patients subjected to resistant bacteria would clearly benefit. Moreover, patients with a compromised immune system subjected to severe opportunistic infections could also have the opportunity to improve their quality of life, reducing multiple infectious symptoms, e.g., fever, skin abscesses, chest pain, and shortness of breath.

Nevertheless, many problems must be solved for efficient HDP selection and production. Regarding the selection issue, novel compounds must be screened from different and unusual sources exploring different biomes and environments. The production problems must be solved by using novel technologies of chemical synthesis, and also novel strategies of heterologous production. An additional problem is the low stability of some peptides. This can be improved by several methods including the production of mutants and also the design of novel particles that could protect the peptide against host–hostile environments, providing an efficient delivery system. One must ask whether peptides are capable of correctly modulating the host immune system. If not, the collateral effects will be worse than the proper infection. This does not seem to be the case, at least when considering the many trials using animal models. Nevertheless, several clinical trials using human beings must be performed in order to provide security for all patients. The use of the AMPs or HDPs is inevitable and extremely desirable, but more studies must be performed in order to really understand the mechanisms of action of such molecules. Indeed, researchers must use all the available technology possible including proteomics, genomics, transcriptomics, nanobiotechnology, and many others in order to reduce the detrimental effects on the population. The infectious storm is upon the human species and the best that we can do is be prepared for it by constructing and producing the best compounds possible.

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Mechanism of honey bacteriostatic action against MRSA and VRE involves hydroxyl radicals generated from honey's hydrogen peroxide

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It has been recently reported that honey hydrogen peroxide in conjunction with unknown honey components produced cytotoxic effects resulting in bacterial growth inhibition and DNA degradation. The objective of this study was twofold: (a) to investigate whether the coupling chemistry involving hydrogen peroxide is responsible for a generation of hydroxyl radicals and (b) whether •OH generation affects growth of multi-drug resistant clinical isolates. The susceptibility of five different strains of methicillin-resistant *Staphylococcus aureus* (MRSA) and four strains of vancomycin-resistant *Enterococcus faecium* (VRE) isolates from infected wounds to several honeys was evaluated using broth microdilution assay. Isolates were identified to genus and species and their susceptibility to antibiotics was confirmed using an automated system (Vitek®, Biomérieux®). The presence of the *mec*(A) gene, *nuc* gene and *van*(A) and (B) genes were confirmed by polymerase chain reaction. Results showed that no clinical isolate was resistant to selected active honeys. The median difference in honeys MICs against these strains ranged between 12.5 and 6.25% v/v and was not different from the MIC against standard *Escherichia coli* and *Bacillus subtilis*. Generation of •OH during bacteria incubation with honeys was analyzed using 3′-(*p*-aminophenyl) fluorescein (APF) as the •OH trap. The •OH participation in growth inhibition was monitored directly by including APF in broth microdilution assay. The growth of MRSA and VRE was inhibited by •OH generation in a dose-dependent manner. Exposure of MRSA and VRE to honeys supplemented with Cu(II) augmented production of •OH by 30-fold and increased honey bacteriostatic potency from MIC_{90} 6.25 to MIC_{90} < 0.78% v/v. Pretreatment of honeys with catalase prior to their supplementation with Cu ions fully restored bacterial growth indicating that hydroxyl radicals were produced from H_2O_2 via the Fenton-type reaction. In conclusion, we have demonstrated for the first time that bacteriostatic effect of honeys on MRSA and VRE was dose-dependently related to generation of •OH from honey H_2O_2.

Keywords: hydroxyl radicals, H_2O_2, 3′-(*p*-aminophenyl) fluorescein, growth inhibition, MRSA, VRE, mechanism of honey antibacterial activity

INTRODUCTION

Honey has well established function as an effective antibacterial agent with a broad spectrum of activity against Gram-positive and Gram-negative bacteria (for review, Lusby et al., 2002; Irish et al., 2011). Despite a progress in identification of compounds that are involved in growth inhibitory and bactericidal actions of honey, the mechanism underlying these activities remained unknown. A functional relationship between hydrogen peroxide produced in honey and antibacterial activity strongly pointed to H_2O_2 as the main contributor to this activity (White et al., 1963; Bang et al., 2003; Brudzynski, 2006). However, recent new findings question this view. Firstly, molecular H_2O_2 is relatively weak oxidant and it requires high concentrations to exert its cytotoxic effect (Rutala et al., 2008; Finnegan et al., 2010). Our data indicate that H_2O_2 content in honeys (0.4–2.6 mM), is much below its biocidal levels. Yet, even at these low H_2O_2 concentrations, honeys effectively inhibited bacterial growth and caused DNA strand breaks (Brudzynski et al., 2011). Secondly, oxidative stress of endogenous H_2O_2 was clearly augmented by the action of unknown honey components (Brudzynski et al., 2011). Thirdly, in our accompanying paper, we provided the first evidence that honeys of high bacteriostatic activity (MIC_{90} 12.5–6.25% v/v) possessed significantly higher levels of phenolic compounds of higher radical scavenging activities than honey of the average bacteriostatic activity (MIC_{90} 25% v/v), thus suggesting the involvement of phenolics and H_2O_2 in bacterial growth inhibition (Brudzynski et al., 2012).

In our prior research, we have screened over 200 samples of Canadian honeys of different botanical origin for the antibacterial activity using broth microdilution assay (Brudzynski and Kim, 2011). Fifty percent of screened honeys showed MICs exceeding

that of sugar solution. Among the group of "active" honeys, MICs of honeys originating from buckwheat differed consistently by four to 16 doubling concentrations from the MIC of sugar solution. Canadian buckwheat honeys characteristically contained higher amounts of hydrogen peroxide than other honeys (Brudzynski, 2006) and possessed higher than average content of polyphenols of high antioxidant activity as measured by the oxygen radical absorbent capacity (ORAC) method (Brudzynski and Miotto, 2011; Brudzynski et al., 2012). These results have converged to recognize that the coupling chemistry between H_2O_2 and polyphenols, rather than hydrogen peroxide alone, may exert oxidative effect causing bacterial growth arrest and DNA degradation (Brudzynski et al., 2011, 2012). High ORAC values of polyphenols are usually indicative of their efficient peroxyl radical scavenging abilities (Cao et al., 1997; Price et al., 2006). However, the same polyphenols could become powerful pro-oxidants when oxidized in the presence of oxygen from air. Such situation occurs during honey harvesting from the comb and during storage. Oxidized polyphenols further generate hydrogen peroxide and in the presence of transition metals such as Cu(I) or Fe(II) they drive the generation of hydroxyl radicals from H_2O_2 via the Fenton reaction (Puppo, 1992; Hanasaki et al., 1994; Cao et al., 1997; Sakihama et al., 2002).

Together, these facts brought about a new hypothesis that the oxidizing action of honey on bacterial cells may result from the generation of hydroxyl radicals from H_2O_2. Such coupling reaction has never been shown to be associated with honey function in spite of the fact that honey possesses all necessary substrates for the Fenton reaction; H_2O_2, polyphenols as well as transition metal ions (Bogdanov et al., 2007).

In contrast to H_2O_2, $^{\bullet}OH$ radicals are powerful oxidants that can oxidize molecules in all cellular compartments in a non-specific manner. $^{\bullet}OH$ cytotoxic effects have been shown for both prokaryotic and eukaryotic cells; bacteria, yeast, and human cells (Halliwell et al., 1985; Imlay and Linn, 1988; Perrone et al., 2008). For example, $^{\bullet}OH$ production from H_2O_2 by neutrophil granules is a first line of defense against bacteria during acute inflammation. Similarly, the H_2O_2-induced injury in *Escherichia coli* appears to be mediated by $^{\bullet}OH$ via Fenton reaction (Imlay and Linn, 1988; Imlay et al., 1988; Gutteridge et al., 1998). In the Fenton reaction *in vivo*, H_2O_2 is reduced to $^{\bullet}OH$ radicals in the presence of ferrous (Fe II) or cuprous (Cu II) ions, according to the equation $Me^n + H_2O_2 = Me^{n+1} + {}^{\bullet}OH + OH^-$. Importantly however, the efficiency of this reaction increases dramatically in the presence of polyphenols (Cao et al., 1997; Sakihama et al., 2002).

Therefore, it appeared likely to us that hydroxyl radicals generated from hydrogen peroxide in the Fenton reaction rather than H_2O_2 are main cytotoxic agents that underlie honey antibacterial activity. However, there was no direct experimental evidence that (a) honey can generate hydroxyl radicals from H_2O_2 and (b) that H_2O_2-derived $^{\bullet}OH$s were responsible for bacterial growth and survival. In this study, we took advantage of 3′-(*p*-aminophenyl) fluorescein (APF) as $^{\bullet}OH$ trap to investigate generation of hydroxyl radicals during exposure of bacterial cultures to honey.

Since H_2O_2 is a common component in honeys, a byproduct of glucose oxidation by honeybee glucose oxidase, we also explored a possibility that generation of hydroxyl radicals from honey hydrogen peroxide represents a general mechanism by which honey affects bacterial growth. In support of such notion are the following facts: (a) honeys from different botanical and geographical origins have been shown to possess antibacterial activity and (b) at least in some honeys, this activity has been shown to be directed against both antibiotic-sensitive as well as multi-resistant bacteria. This putative mechanism against antibiotic-resistant bacteria may function in honeys derived from *Leptospermum* spp., (manuka), *Fagopyrum esculentum* (buckwheat), *Koompassia excelsa* (tualang), and honeydew honeys (Willix et al., 1992; Cooper et al., 2000, 2002a,b; French et al., 2005; Brudzynski and Lannigan, 2008; Blair et al., 2009; Tan et al., 2009; Majtan et al., 2010).

To provide a better insight into the involvement of hydroxyl radicals on bacterial growth, we have chosen several clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE) and monitored simultaneously their growth (by absorbance) and $^{\bullet}OH$ generation (by fluorescence) upon their incubation with active honeys. If the $^{\bullet}OH$ generation proves to be implicated in the growth inhibition of standard bacteria (*E. coli* and *Bacillus subtilis*) as well as MRSA and VRE, then perhaps it could be concluded that honey action resembles that of antibiotics. In the latter case, the oxidative damage evoked by hydroxyl radicals has been suggested as a common mechanism for antibiotic-mediated cell death (Gutteridge et al., 1998; Kohanski et al., 2010).

MATERIALS AND METHODS

HONEYS

Honeys were donated by beekeepers and included both commercial (pasteurized) and apiary (raw) samples. The list of honeys, their plant origin main physico-chemical characteristics is given in **Table 1**.

BACTERIAL STRAINS

Standard strains of *B. subtilis* (ATCC 6633) and *E. coli* (ATCC 14948) purchased from Thermo Fisher Scientific Remel Products (Lenexa, KS 66215, USA) were grown in Mueller–Hinton Broth (MHB; Difco Laboratories) overnight in a shaking water bath at 37°C. Overnight cultures were diluted with broth to the equivalent of the 0.5 McFarland Standard.

Clinical isolates were obtained from the Clinical Microbiology Laboratory of the London Health Science Centre, London, ON, Canada, three strains of vancomycin-resistant *Enterococcus faecium* and four strains of MRSA. On receipt these were subcultured from swabs onto Mueller–Hinton II agar (Difco Laboratories). Originally the organisms were isolated and processed at the Department of Clinical Microbiology of the London Health Science Centres. An automated system (Vitek®, Biomérieux®) was used to identify bacterial isolates to genus and species and their susceptibility to antibiotics (**Table 2** and **3**).

IDENTIFICATION OF CLINICAL ISOLATES AND CONFIRMATION OF ANTIBIOTIC RESISTANCE

Isolates were identified to genus and species and their susceptibility to antibiotics was confirmed using an automated system (Vitek®, Biomérieux®). The presence of the *mec (A) gene*, *nuc gene*, and *van (A) and (B)* genes were determined by polymerase chain reaction.

Table 1 | List of honeys.

Honey	Botanical source	Color ($A_{560-A720}$)	Hydrogen peroxide (mM/L)*	Water activity Aw
Spl. 11	Sweet clover (mix) *Melilotus officinalis*	0.131	2.49 ± 0.03	0.550
Spl. 15	Blueberry *Vaccinium corymbosum*	0.267	1.75 ± 0.02	0.605
Spl. 23	Buckwheat *Fagopyrum esculentum*	0.975	2.12 ± 0.022	0.690
Spl. 58	Buckwheat	0.989	2.68 ± 0.04	0.576
Spl. 76	Buckwheat	0.906	2.56 ± 0.06	0.591
Spl. 77	Buckwheat	1.266	2.70 ± 0.06	0.597
Spl. 81	Buckwheat	0.331	2.32 ± 0.08	0.580
Spl. 103	Buckwheat	0.203	2.40 ± 0.08	0.601
Manuka	*Leptospermum scoparium*	0.539	1.04 ± 0.17	0.617
H203	Buckwheat	NA	0.248 ± 0.02	NA
H204	Buckwheat	NA	0.744 ± 0.01	NA
H205	Buckwheat	NA	1.168 ± 0.05	NA
H206	Buckwheat	NA	1.112 ± 0.02	NA

∗Hydrogen peroxide concentration was measured at honey dilution of 8× (25% v/v) and represents an average of three experimental trials, where each honey was tested in triplicate

Table 2 | Susceptibility of clinical isolates to antibiotics.

Bacterium	Antibiotics																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
MRSA 3	+		R	R	R	R	R	S		R	S	R	S	R	S	S		S	S	S
MRSA 4	+		R	R	S	R	R	S		R	S	R	S	R	S	S		S	S	S
MRSA 5	+		R	R	R	R	R	R		R	S	I	S	R	S	S		S	S	S
VRE 0	−	R	R	R		R	R		S	R	S		R		S		S	R		R
VRE 2	−	R	R	R		R	R		R	R	S		I		S		R	R		R

1, Beta-lactamase; 2, ampicilli; 3, benzylpenicillin; 4, ciprofloxacin; 5, clindamycin; 6, erythromycin; 7, gatifloxacin; 8, gentamicin; 9, gentamicin high level (synergy); 10, levofloxacin; 11, linezolid; 12, moxifloxacin; 13, nitrofurantoin; 14, oxacillin; 15, quinupristin/dalfopristin; 16, rifampicin; 17, streptomycin high level (synergy); 18, tetracycline; 19, trimethoprim/sulfamethoxazole; 20, vancomycin.

Table 3 | Identification of clinical isolates and confirmation of antibiotic resistance.

Plate	Identification	*mec (A)*	*nuc*	*van (A)*	*van (B)*	PB2 test
VRE 0	Vancomycin-resistant *Enterococcus faecium*	NA	NA	+	−	NA
VRE 2	Vancomycin-resistant *Enterococcus faecium*	NA	NA	+	−	NA
VRE 3	Vancomycin-resistant *Enterococcus faecium*	NA	NA	+	−	NA
MRSA 3	Methicillin-resistant *Staphylococcus aureus*	+	+	NA	NA	+
MRSA 4	Methicillin-resistant *Staphylococcus aureus*	+	+	NA	NA	+
MRSA 5	Methicillin-resistant *Staphylococcus aureus*	+	+	NA	NA	+
MRSA 6	Methicillin-resistant *Staphylococcus aureus*	+	+	NA	NA	

This work was conducted by the Clinical Microbiology Laboratory, London Health Sciences Centre, London, ON, Canada.

Reproducibility for each strain was determined at minimum two experiments and maximum three conducted in triplicate. MIC was determined by using a protocol as described in Methods according to the National Committee for Clinical Laboratory Standards (NCCLS, 1996).

The overnight cultures of each strain were diluted in MHB to obtain 10^8 cfu/ml using a 0.5 McFarland standard.

POLYMERASE CHAIN REACTION

The procedure for identification and confirmation for both MRSA and VRE were as follows: Nasal and perineal swabs were inoculated onto chromogenic media designed to identify possible MRSA and VRE isolates (Colorex VRE, Alere, Inc., London, ON, Canada; MRSASelect™, Bio-Rad Redmond, WA, USA). Colonies suspected of being MRSA or VRE were confirmed using PCR. For MRSA confirmation the organism was assayed for the presence of the *mec (A)* gene and nuclease. Suspected VRE were assayed for

the presence of *van (A)* and *van (B)* genes. Sequences used were:

MecA1: TGGCTATCGTGTCACAATCG (20 bases)
MecA2: CTGGAACTTGTTGAGCAGAG (20 bases)
Nuc1: GCGATTGATGGTGATACGGTT (21 bases)
Nuc2: AGCCAAGCCTTGACGAACTAAAGC (24 bases)
VanA1: GGGAAAACGACAATTGC (17 bases)
VanA2: GTACAATGCGGCCGTTA (17 bases)
VanB1: ATGGGAAGCCGATAGTC (17 bases)
VanB2: GATTTCGTTCCTCGACC (17 bases)

PCR was run according to a standard protocol that is used routinely in clinical microbiology laboratories.

ANTIBACTERIAL ASSAY

The antibacterial activity of honeys was performed using a broth microdilution assay in sterile, 96-well microplates (Costar, Thermo Fisher Scientific, Canada) in compliance with requirements of NCCLS (1996). Wells in rows B to G contained 110 μl of inoculated broth (10^6 cfu/ml final concentrations for each of microorganisms). Wells in row A were filled with 200 μl of 50% solution of honey in sterile water and were inoculated with 20 μl of 10^7 cfu/ml of bacterial culture. Serial twofold dilutions of honey were prepared by mixing and transferring 110 μl of honey with 110 μl of inoculated broth from row A to row H of a microplate. Row G contained only inoculum and served as a positive control and row H contained sterile MHB and served as a blank. In a single experiment, each honey was tested in triplicate.

After overnight incubation of plates at 37°C in a shaking water bath, bacterial growth was measured at A_{595} nm using the Synergy HT multidetection microplate reader (Synergy HT, Bio-Tek Instruments, Winooski, VT, USA).

The contribution of color of honeys to the absorption was corrected by subtracting the absorbance of wells before (zero time) and after overnight incubation.

Statistical analysis and dose–response curves were obtained using K4 software provided by Synergy HT, Bio-Tek Instruments, Winooski, VT, USA.

Conversion of honey dilutions to honey concentrations (% v/v):

Dilutions	**2×**	**4×**	**8×**	**16×**	**32×**	**64×**
Concentrations	50%	25%	12.5%	6.25%	3.125%	1.56%

DETERMINATION OF MICs

The absorbance readings obtained from the dose–response curve were used to construct growth inhibition profiles (GIPs). The minimal inhibitory concentrations (MIC_{90}) were determined from the GIPs curves and represented the lowest concentration of the honeys that inhibited the bacterial growth by 90% as measured by the absorbance at A_{595} nm.

QC/QA controls: estimation of the MICs was conducted only then when control bacterial strains in microdilution assay (row G) show sufficient growth. For each bacterial strain, 3–6 MICs were established in independent experiments. The obtained median MICs were the same or differed by± one doubling concentration.

HYDROXY-RADICALS MEASUREMENTS

3′-(*p*-Aminophenyl) fluorescein (APF; Invitrogen, Canada) was used for the detection of hydroxyl radicals produced by honeys. A broth microdilution assay in 96-well microplate format containing inoculum and twofold serially diluted honeys was adapted to assess both hydroxyl radicals generated and bacterial growth. To each well the APF solution was added to a final concentration of 10 μM (in 50 mM potassium phosphate buffer, pH 7.4). The experimental wells (containing bacterial inoculum and honey dilutions) as well as the assay controls (bacterial inoculum) were supplemented with 10 μM APF while the negative control consisted of experimental wells without APF.

Plates were incubated in a shaking water bath at 37°C for 18 h. The plates were analyzed for both, bacterial growth using absorbance at A_{595} nm and hydroxyl radical generation using fluorescence excitation and emission wavelengths at 490 and 520 nm, respectively.

To measure hydroxyl radical generation in the presence of Cu ions, honeys were incubated with 400 μM of $CuCl_2$ dissolved in 50 mM potassium phosphate buffer, pH 7.4.

HYDROGEN PEROXIDE ASSAY

The hydrogen peroxide concentrations of the honeys were determined using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit in 96-well microplate format according to the manufacturer manual (Molecular Probes, Invitrogen, Burlington, ON, Canada) and as described previously (Brudzynski et al., 2011). The Synergy HT multidetection microplate reader was used to measure the fluorescence formed during the reaction of honey's hydrogen peroxide with the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxasine). The measurements were conducted at an emission wavelength of 590 nm, and an excitation wavelength of 530 nm. The standard curve constructed from the known concentration of H_2O_2 was used to calculate the hydrogen peroxide concentrations of the honeys. Each of the honey samples, and the standard curve, were tested in triplicate.

CATALASE-TREATMENT OF HONEYS

Honey were treated with catalase (13 800 U/mg solid; Sigma-Aldrich, Canada) at ratio of 1000 units per 1 ml of 50% honey solution in sterile water for 2 h at room temperature.

RESULTS

BACTERIOSTATIC EFFECT OF HONEY AGAINST ANTIBIOTIC-RESISTANT CLINICAL ISOLATES

From a large pool of over 200 honeys screened for their bacteriostatic activity against *E. coli* and *B. subtilis* (Brudzynski and Kim, 2011), we selected eleven honeys that showed MIC_{90} exceeding those of sugar solution (artificial honey). The list included six buckwheat honeys (H23, 58, 76, 77, 81, and 103), one clover honey (H11) and one blueberry honey (H15). The MIC_{90} of these honeys ranged from 6.25 to 12.5% (v/v), with exception of H15, with MIC_{90} of 25% (v/v). New Zealand Active Manuka honey (MIC_{90} 6.25% v/v) was used as a reference (**Figure 1**).

These honeys were analyzed for their ability to inhibit growth of two vancomycin-resistant *Enterococcus faecium*, VRE 0 and VRE 2 and three strains of MRSA, MRSA 3, 4, and 5. **Figure 1** shows

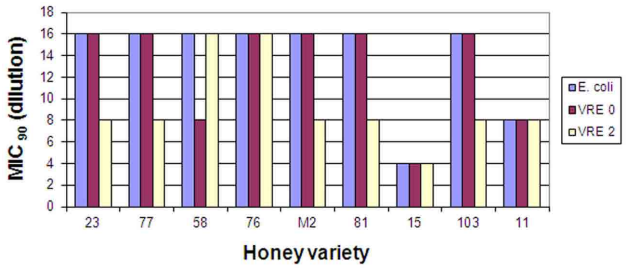

FIGURE 1 | Bacteriostatic activity of eleven honey varieties against two clinical isolates of vancomycin-resistant *Enterococcus faecium* strains, VRE 0 and VRE 2. Reproducibility for each strain was determined at minimum in three separate experiments conducted in triplicate.

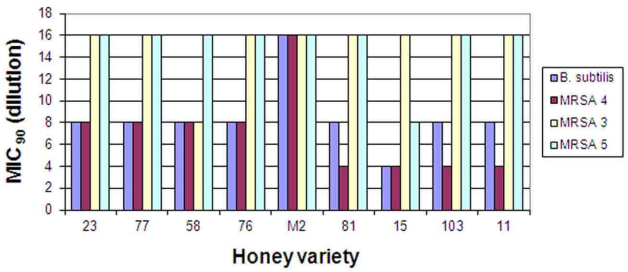

FIGURE 2 | Bacteriostatic activity of eleven honey varieties against two clinical isolates of methicillin-resistant *Staphylococcus aureus* strains, MRSA 3, 4, and 5. Reproducibility for each strain was determined at minimum in three separate experiments conducted in triplicate.

that both VRE strains were susceptible to honeys. The median differences in honeys bacteriostatic activity varied only by twofold from the low MIC to the high MIC for each strains (MIC_{90} 6.25 and 12.5% (v/v), respectively), with exception of honey H15. Both VRE strains displayed the same multi-drug resistant pattern (**Table 2**). There was no difference in MIC values of honeys against multi-drug resistant VRE strains and *E. coli* (**Figure 1**).

On the other hand, the susceptibility of the three strains of MRSA to honeys differed two- to four-fold ranging from MIC_{90} 6.25–25% (v/v). MRSA 4 was the most resistant strain while MRSA 3 and 5 were more susceptible to different honeys. MRSA 4 was more resistant to honey action than *B. subtilis* (**Figure 2**).

The antibiograms performed on MRSA and VRE strains showed similar multi-resistance profiles to several classes of antibiotics (**Table 2**).

CONFIRMATION OF THE PRESENCE OF RESISTANCE GENES IN CLINICAL ISOLATES

These pathogens isolated from wounds were identified to the genus and species using the automated system Vitek II, Biomerieux. The presence or absence of antibiotic resistance genes [*van(A)*, *van(B)*, *mec(A)*, and *nuc*] was confirmed by the polymerase chain reaction. The presence of penicillin-binding protein (PBP2′) in the *Staphylococcus aureus* strains were confirmed using an Oxoid agglutination kit.

Despite the multi-resistance pattern to antibiotics (**Table 2**), supported by a positive identification of the antibiotic-resistant genes (**Table 3**), all strains of MRSA and VRE were sensitive to honey bacteriostatic action (**Figures 1** and **2**). This observation together with the lack of significant differences in susceptibility between the antibiotic-resistant and standard bacteria suggested that bacteriostatic action of honey may proceeds via a common mechanism.

DETECTION OF HYDROXYL RADICAL FORMATION FOLLOWING INCUBATION OF MRSA AND VRE WITH HONEYS

In our most recent study, we have shown that hydrogen peroxide is implicated in bacterial growth inhibition but its inhibitory efficiency was modulated by unknown honey components (Brudzynski et al., 2011). We hypothesized that the formation of $^{\bullet}OH$ from H_2O_2 rather than H_2O_2 itself may be directly implicated in bacterial growth inhibition.

To investigate the generation of $^{\bullet}OH$ from honey's hydrogen peroxide and its effect on bacterial growth, we exposed MRSA and VRE culture to serially diluted honeys in the presence of 3′-(*p*-aminophenyl) fluorescein (APF) as $^{\bullet}OH$ trap (Setsukinai et al., 2003). APF is a non-fluorescent product but in the presence of $^{\bullet}OH$ it became oxidized to a fluorescent form. It has been shown that, the $^{\bullet}OH$ trapping by APF resulted in a dose-dependent increase in the fluorescence (Setsukinai et al., 2003).

Using broth microdilution assay supplemented with APF, we analyzed in the same experiment the growth of MRSA and VRE by monitoring the increase in turbidity/absorbance at A_{595} nm and the generation of hydroxyl radicals by monitoring the increase in fluorescence.

Two new bacterial strains, MRSA 6 and VRE 3 were exposed separately to five freshly obtained buckwheat honeys: H203, 204, 205, and 206. The incubation of each honey with MRSA 6 and VRE 3 cultures resulted with steady accumulation of hydroxyl radicals up to the 16× honey dilution, as assessed by increase in fluorescence (**Figures 3A,B**). The peak of $^{\bullet}OH$ generation corresponded to the MIC of honey. Further honey dilutions resulted in a decrease of $^{\bullet}OH$ levels and concomitant loss of growth inhibition. Thus, there was a causal relationship between $^{\bullet}OH$ generation and bacterial growth inhibition.

No major differences were observed in the growth inhibition patterns between the six honeys; neither in the final MIC values against MRSA 6 and VRE 3 nor in the levels of $^{\bullet}OH$ generation during incubations. Therefore, **Figures 3A,B** represent an average from the all honeys used against VRE (**Figure 3A**) and against MRSA (**Figure 3B**).

The relationship between honey dilutions and hydroxyl radical production was strongly reminiscent of that between honey dilutions and hydrogen peroxide production (Brudzynski et al., 2011). Previous studies have established that honey dilutions facilitate the production of endogenous H_2O_2 by glucose oxidase (White et al., 1963; Bang et al., 2003; Brudzynski et al., 2011). In most of our test honeys, a steady increase in H_2O_2 content with honey dilution was observed, reaching the peak of production between 4× and 16× dilution and declining thereafter (Brudzynski et al., 2011).

When the results on $^{\bullet}OH$ generation during incubation of honeys with MRSA and VRE were combined and presented

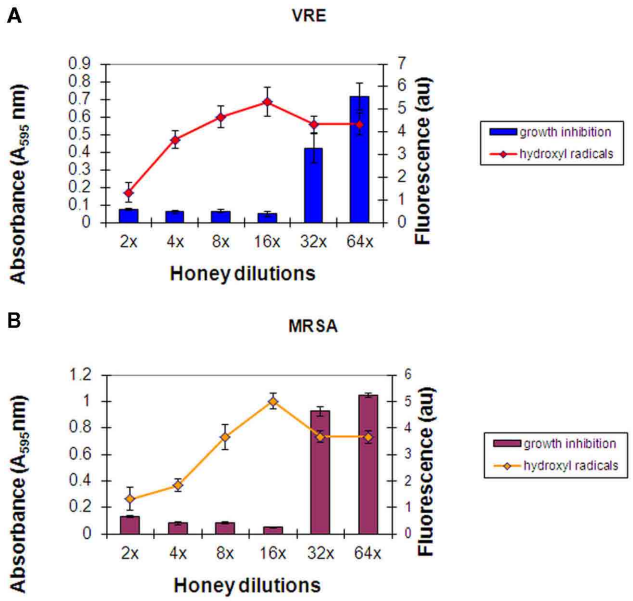

FIGURE 3 | (A,B) Hydroxyl radical generation during incubation of honey with MRSA 6 and VRE 3 cultures. Columns represented mean values ± SEM ($n = 18$) of absorbance (turbidity) of VRE **(A)** and MRSA **(B)** cultures (106 cfu/well) at each honey dilutions. A line represents mean ± SEM ($n = 18$) of hydroxyl radical produced at each honey dilution. Honey represents combined effect of H203, 204, 205, and 206 measured in triplicate.

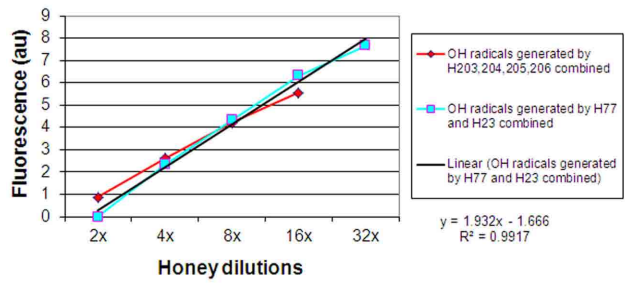

FIGURE 4 | Linear relationship between honey dilutions and hydroxyl radical generation from honey's hydrogen peroxide.

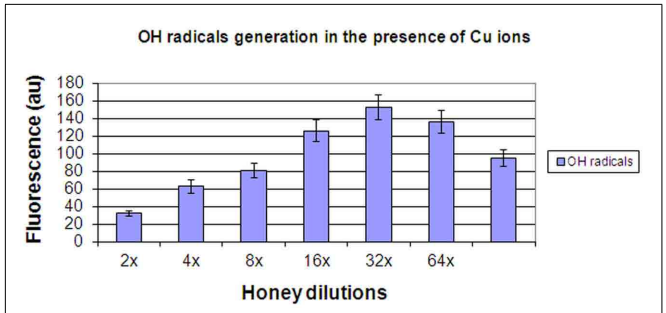

FIGURE 5 | The generation of hydroxyl radicals by honeys H77 and H76 (combined) supplemented with 400 μM Cu(II).

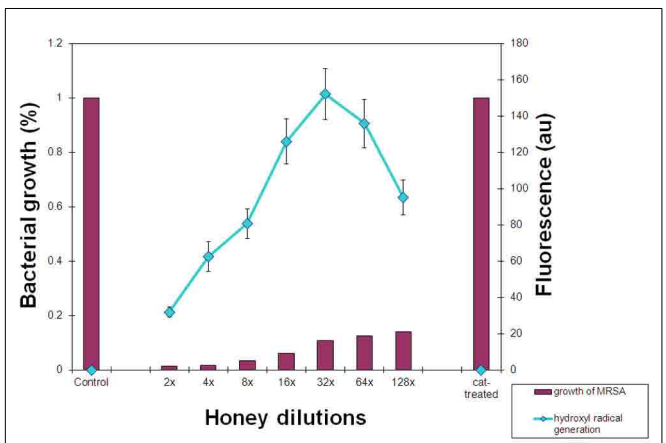

FIGURE 6 | Relationship between hydroxyl radical generation and inhibition of MRSA cultures by honeys H77 and H76 supplemented with Cu ions. Columns represent growth of MRSA expressed as a percentage of control (growth of MRSA in the absence of honey, 100%) exposed to the serially diluted honey. A line represents fluorescence of hydroxyl radicals trapped by APF during incubation of honeys with MRSA. Cat-treated sample represents growth of MRSA in the presence of honey pre-treated with catalase and then supplemented with Cu ions.

graphically, it appeared that there was a linear relation between the increase in •OH concentrations and honey dilution. **Figure 4** shows the linearity up to 16-fold dilution in case of H203–206 (median H_2O_2 content of 0.8 mM) and 32-fold dilution for honeys H77 and H76 (median H_2O_2 content of 2.63 mM, **Table 1**). This finding suggested that honey dilutions, the increased production of H_2O_2, and increased generation of •OH are interdependent phenomena.

EFFECT OF CU IONS AND CATALASE ON THE HYDROXYL RADICAL PRODUCTION AND HONEY BACTERIOSTATIC ACTIVITY

Hydroxyl radicals are generated as a result of the coupling chemistry between hydrogen peroxide and metal ions Fe(II) or Cu(II) via Fenton reaction. To test whether the Fenton reaction is responsible for •OH production from endogenous H_2O_2, we supplemented honeys with Cu ions (400 μM) and incubated with MRSA cells in broth microdilution assay.

The results confirmed that upon addition of Cu ions to honeys the generation of •OH radicals from H_2O_2 was substantially enhanced (**Figure 5**). Approximately 30-fold higher level of •OH was observed in the presence of Cu ions compared to control honeys. Similarly to results presented in **Figure 4**, a curve of •OH radical generated under these conditions was essentially linear up to 32-fold dilution. These data indicate that Cu ions facilitated decomposition of H_2O_2 to generate •OH, as expected from the classical Fenton reaction.

The increased levels of H_2O_2 decomposition and hydroxyl radical generation in the presence of Cu ions dramatically augmented honey bacteriostatic potency. When honeys H77 and H76 supplemented with Cu ions were incubated with MRSA 6 cells, the bacterial growth was inhibited beyond 128-fold honey dilution, increasing honey MIC < 0.78% v/v (**Figure 6**). The direct

connection between the increase in •OH generation and the increase in growth inhibition of MRSA by honey supplemented with Cu is consistent with the conclusion that hydroxyl radicals are primarily responsible for this cytotoxic effect.

To demonstrate that honey H_2O_2 is a substrate in the generation of hydroxyl radicals in these experiments, we first pre-treated honeys H77 and H76 with catalase to remove H_2O_2 and then supplemented them with Cu ions. Addition of catalase completely abolished inhibitory action of honey and bacterial growth was fully restored. In the same time, the •OH generation was reduced to 0 in catalase-treated honeys (**Figure 6**).

Together, these results indicate that •OH radicals were generated from the honey endogenous H_2O_2 via the Fenton reaction. The •OH radical exerted dose-dependent inhibitory effect on MRSA and VRE growth.

DISCUSSION

In this study, the 3′-(*p*-Aminophenyl) fluorescein (APF) •OH trap technique was used to provide direct evidence that hydroxyl radical generation from honey hydrogen peroxide caused inhibition of growth of multi-drug resistant clinical isolates of MRSA and VRE, as well as growth of standard bacteria, *E. coli* and *B. subtilis.* Application of APF allowed us to measure simultaneously (in the same experiment) the generation of hydroxyl radicals and their effects on growth of MRSA and VRE cells.

Firstly, we have established that the antibiotic-resistant clinical isolates, VRE 0 and VRE 2, carrying vancomycin-resistance genes *van(A)* and MRSA 3, 4, and 5 carrying *mec(A)* and PBP2′ genes, all were sensitive to honey actions. The variability in honeys median MICs against MRSA and VRE were comparable to those against *E. coli* and *B. subtilis* and ranged from 6.25 to 12.5% v/v. Similar results have been previously obtained for honeys of *Leptospermum* origin, (manuka; Willix et al., 1992; Cooper et al., 2002a,b; French et al., 2005; Blair et al., 2009), *K. excelsa* (tualang; Tan et al., 2009), and *F. esculentum* (buckwheat; Brudzynski and Lannigan, 2008).

The presence of several antibiotic resistance genes therefore, did not interfere with the honey action against these microorganisms, providing an important clue that the underlying mechanism may concern a common biochemical pathway affecting all bacteria. The observed slight variability in susceptibility of different MRSA to honey may be a reflection of their ability to counteract the oxidative stress.

Secondly, exposure of MRSA and VRE to tested honeys resulted in a steady accumulation of hydroxyl radicals, as evidenced by increased fluorescence of APF, reaching the peak between 16- and 32-fold honey dilution. The dilution of honey at which the maximal •OH generation was observed corresponded to honey MIC. After reaching this culmination point, both hydroxyl radical generation and growth inhibition showed a decline, indicating a functional interplay between these two events.

The relationship between generation of •OH and the extent of growth inhibition strongly resembled the relationship between the production of H_2O_2 upon honey dilution and inhibition of bacterial growth (Brudzynski et al., 2011). This observation implied that honey H_2O_2 might be a main source of •OH radicals. A generally accepted mechanism of •OH generation is the Fenton reaction in which H_2O_2 is reduced to •OH in the presence of transition metal ions (Puppo, 1992; Hanasaki et al., 1994; Cao et al., 1997; Urbanski and Beręsewicz, 2000). Honey is naturally enriched in transition metals such as Fe(II) or Cu(II) (Bogdanov et al., 2007) and produces its own H_2O_2, thus fulfilling requirements for the Fenton –type reaction to occur. Consistently with this premise, supplementation of honey with Cu(II) caused a remarkable increase in •OH production. This increase was completely abolished by pretreatment of honey with catalase prior to addition of Cu ions, clearly indicating that honey H_2O_2 was a source from which of •OH radicals were generated. A coupling chemistry via the Fenton reaction is then the most plausible mechanism operating in honey since, similarly to results observed here, removal of H_2O_2 or chelation of metal ions prevented hydroxyl radical generation in Fenton reaction (Puppo, 1992; Ali and Konishi, 1998; Urbanski and Beręsewicz, 2000).

Furthermore, a tight functional relationship was observed between levels of hydroxyl radicals produced and honey bacteriostatic potency. We observed that MICs of honeys increased from 6.25% v/v to less than 0.78%v/v (below 128-fold dilution) after Cu-supplementation. Again, this augmented bacteriostatic activity was catalase-sensitive: removal of H_2O_2, which in turn abolished •OH generation, resulted in the full restoration of bacterial growth.

These results indicate that the oxidative stress caused by honey action on bacterial cells resulted from hydroxyl radical generated from honey's hydrogen peroxide rather than from the action of molecular H_2O_2 itself. The formed hydroxyl radicals inhibited the growth of MRSA and VRE in a dose-dependent manner. Thus, we established here a functional link between the generation of hydroxyl radicals from honey H_2O_2 and bacterial growth inhibition.

There is a tremendous need for novel antibacterial agents to treat infections caused by antibiotic-resistant bacteria. Honey, with its long history of usage as an antibacterial agent in traditional and folk medicine (for review, Lusby et al., 2002), has recently brought renewed attention of researchers working in the area of drug discovery and development. Accumulated evidence from basic research and clinical trials allowed manuka honey (derived from *Leptospermum* species) to be recognized as a therapeutic agent. Its antibacterial effectiveness in wound healing has been documented in many case studies and randomized controlled trials (for review, Molan, 2006). As the result, at least two types of formulations based on manuka honey, Medihoney™and Active Honey Absorbent Dressing, API-MED™have been approved to be used in clinical treatments of infected wounds.

One of the principle limitations of many antibacterial agents derived from natural products, including honey, is lack of knowledge about a molecular mechanism that lead to bacterial cell death. In this context, our finding that hydroxyl radicals generated by honeys (other than manuka honey) underlie its antibacterial activity may be critical for designing effective antibacterial therapy for the following reasons: (a) generation of hydroxyl radical is a common property of honeys of European and North American origin, (b) the hydroxyl radical-based mechanism of honey action did not discriminate between antibiotic-sensitive and antibiotic-resistant bacteria, and (c) hydroxyl radical levels generated by honey could serve as a diagnostic tool to predicted antibacterial efficacy of honeys in clinical applications.

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Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components

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Essential oils are aromatic and volatile liquids extracted from plants. The chemicals in essential oils are secondary metabolites, which play an important role in plant defense as they often possess antimicrobial properties. The interest in essential oils and their application in food preservation has been amplified in recent years by an increasingly negative consumer perception of synthetic preservatives. Furthermore, food-borne diseases are a growing public health problem worldwide, calling for more effective preservation strategies. The antibacterial properties of essential oils and their constituents have been documented extensively. Pioneering work has also elucidated the mode of action of a few essential oil constituents, but detailed knowledge about most of the compounds' mode of action is still lacking. This knowledge is particularly important to predict their effect on different microorganisms, how they interact with food matrix components, and how they work in combination with other antimicrobial compounds. The main obstacle for using essential oil constituents as food preservatives is that they are most often not potent enough as single components, and they cause negative organoleptic effects when added in sufficient amounts to provide an antimicrobial effect. Exploiting synergies between several compounds has been suggested as a solution to this problem. However, little is known about which interactions lead to synergistic, additive, or antagonistic effects. Such knowledge could contribute to design of new and more potent antimicrobial blends, and to understand the interplay between the constituents of crude essential oils. The purpose of this review is to provide an overview of current knowledge about the antibacterial properties and antibacterial mode of action of essential oils and their constituents, and to identify research avenues that can facilitate implementation of essential oils as natural preservatives in foods.

Keywords: antimicrobial, mode of action, synergy, terpenes, terpenoids, phenylpropenes, carvacrol, thymol

INTRODUCTION

Essential oils are aromatic and volatile liquids extracted from plant material, such as flowers, roots, bark, leaves, seeds, peel, fruits, wood, and whole plant (Deans and Ritchie, 1987; Hammer et al., 1999; Sánchez et al., 2010). Essential oils have been used for centuries in medicine, perfumery, cosmetic, and have been added to foods as part of spices or herbs. Their initial application was in medicine, but in the nineteenth century their use as aroma and flavor ingredients increased and became their major employment. Almost 3000 different essential oils are known, and 300 are used commercially in the flavor and fragrances market (Burt, 2004).

Essential oils are considered to be secondary metabolites and important for plant defense as they often possess antimicrobial properties (Fraenkel, 1959; Tajkarimi et al., 2010). The antibacterial properties of secondary metabolites were first evaluated using essential oil vapors by De la Croix in 1881 (Burt, 2004). Since then, essential oils or their components have been shown to not only possess broad-range antibacterial properties (Deans and Ritchie, 1987; Oussalah et al., 2007), but also antiparasitic (George et al., 2009), insecticidal (Essam, 2001; Kim et al., 2003), antiviral (Schnitzler et al., 2011), antifungal (Fitzgerald et al., 2003; Kalemba and Kunicka, 2003; Silva et al., 2011; Tserennadmid et al., 2011), and antioxidant (Brenes and Roura, 2010) properties. Furthermore, they also function as growth enhancers for animals (Brenes and Roura, 2010; Ahmadifar et al., 2011).

Although the food industry primarily uses essential oils as flavorings, they represent an interesting source of natural antimicrobials for food preservation. However, application of essential oils as food preservatives requires detailed knowledge about their properties, i.e., the minimum inhibitory concentration (MIC), the range of target organisms, the mode of action, and the effect of food matrix components on their antimicrobial properties. The purpose of this review is to provide an overview of current knowledge about the antimicrobial mode of action of essential oil constituents, and to identify research avenues that can facilitate implementation of essential oil constituents as natural food preservatives in foods.

ESSENTIAL OIL CONSTITUENT CLASSES: THEIR ANTIMICROBIAL ACTIVITY AND MODE OF ACTION

Plants produce a variety of compounds with antimicrobial activity. Some are always present while others are produced in response

to microbial invasion or physical injury (Roller, 2003). Identifying the most active antimicrobial compounds of essential oils is cumbersome because essential oils are complex mixtures of up to 45 different constituents (Delaquis et al., 2002; Djenane et al., 2011; Espina et al., 2011), and the composition of a particular essential oil may vary depending on the season of harvest, and the methods used to extract the oil (Nannapaneni et al., 2009; Pereira and Meireles, 2010; Sánchez et al., 2010; Demuner et al., 2011; Djenane et al., 2011; Paibon et al., 2011). Essential oil constituents are a diverse family of low molecular weight organic compounds with large differences in antimicrobial activity. The active compounds can be divided into four groups according to their chemical structure: terpenes, terpenoids, phenylpropenes, and "others." This section will provide an overview of what is currently known about the antimicrobial properties and the mode of action of selected essential oil constituents. Although studies have been performed on the mode of action of some essential oils (**Table 1**), analyzing the mode of action behind each constituent in the oils can reveal details of its antimicrobial properties that might be concealed when studied in a mixture with many other compounds. We will thus focus this review on the individual constituents of essential oils.

Most studies concerning the antimicrobial mode of action of essential oil constituents have been performed on bacteria, while less is known about their action on yeast and molds. Gram-negative bacteria are generally less susceptible than Gram-positive bacteria (Trombetta et al., 2005). The outer membrane of Gram-negative bacteria contain hydrophilic lipopolysaccharides (LPS), which create a barrier toward macromolecules and hydrophobic compounds, providing Gram-negative bacteria with higher tolerance toward hydrophobic antimicrobial compounds like those found in essential oils (Nikaido, 1994, 2003). Most essential oil constituents have several targets (**Table 2**). It is therefore difficult to predict how susceptible a microorganism is and why the susceptibility varies from strain to strain. Predictions about the mode of action of crude essential oils require thorough investigations of their constituents' target site, their mode of action, and their interactions with the surrounding environment. In this context, the following is known about the mode of action of some selected essential oil constituents.

TERPENES

Terpenes are hydrocarbons produced from combination of several isoprene units (C_5H_8). Terpenes are synthesized in the cytoplasm of plant cells, and the synthesis proceeds via the mevalonic acid pathway starting from acetyl-CoA. Terpenes have a hydrocarbon backbone which can be rearranged into cyclic structures by cyclases, thus forming monocyclic or bicyclic structures (Caballero et al., 2003). The main terpenes are monoterpenes ($C_{10}H_{16}$) and sesquiterpene ($C_{15}H_{24}$), but longer chains such as diterpenes ($C_{20}H_{32}$), triterpenes ($C_{30}H_{40}$), etc., also exist. Examples of terpenes include *p*-cymene, limonene, terpinene, sabinene, and pinene (**Figure 1**).

Terpenes do not represent a group of constituents with high inherent antimicrobial activity. For example, *p*-cymene, one of the major constituents in thyme, had no antimicrobial activity against several Gram-negative pathogens even at 85700 μg/mL concentration (Bagamboula et al., 2004). In a large scale experiment, limonene, α-pinene, β-pinene, δ-3-carene, (+)-sabinene, and α-terpinene showed no or low antimicrobial activity against 25 different genera of bacteria that pose problems in animals, plants, and food products (Dorman and Deans, 2000). Koutsoudaki et al. (2005) compared the effect of α-pinene, β-pinene, *p*-cymene, β-myrcene, β-caryophyllene, limonene, and γ-terpinene against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*, and their antimicrobial activity were low or absent. *p*-Cymene and γ-terpinene were ineffective as fungicides against *Saccharomyces cerevisiae* (Rao et al., 2010). These *in vitro* tests indicate that terpenes are inefficient as antimicrobials when applied as single compounds.

p-Cymene

The carvacrol precursor *p*-cymene is a monoterpene that has a benzene ring without any functional groups on its side chains. *p*-Cymene is not an efficient antimicrobial compound when used alone (Juven et al., 1994; Mann et al., 2000; Aligiannis et al., 2001; Bagamboula et al., 2004), but it potentiate the activity of compounds like carvacrol (Ultee et al., 2002; Rattanachaikunsopon and Phumkhachorn, 2010) and polymyxin B nonapeptide (Mann et al., 2000).

Several studies indicate that *p*-cymene is likely to act as a substitutional impurity in the membrane, which partly perturbs the membrane of microorganisms. *p*-Cymene has a high affinity for membranes and causes membrane expansion and affect the membrane potential of intact cells (Ultee et al., 2002). Investigations on cell and vesicle systems confirm that *p*-cymene has no effect on the membrane permeability, but cause a decrease in the enthalpy and melting temperature of membranes (Cristani et al., 2007), supporting the hypothesis that *p*-cymene acts as a substitutional impurity in the membrane.

Even though the action of *p*-cymene on the cell membrane is well established, its effect on protein synthesis and cell motility has also been investigated. *p*-Cymene had a negligible effect on the protein synthesis of *E. coli* cells (Burt et al., 2007), while its effect on the membrane potential resulted in decreased cell motility, as a proton motive force is needed for flagellar movement (Gabel and Berg, 2003; Burt et al., 2007).

TERPENOIDS

Terpenoids are terpenes that undergo biochemical modifications via enzymes that add oxygen molecules and move or remove methyl groups (Caballero et al., 2003). Terpenoids can be subdivided into alcohols, esters, aldehydes, ketones, ethers, phenols, and epoxides. Examples of terpenoids are: thymol, carvacrol, linalool, linalyl acetate, citronellal, piperitone, menthol, and geraniol (**Figure 1**).

The antimicrobial activity of most terpenoids is linked to their functional groups, and it has been shown that the hydroxyl group of phenolic terpenoids and the presence of delocalized electrons are important for antimicrobial activity. For example, the antimicrobial activity of the carvacrol derivatives carvacrol methyl ether and *p*-cymene were much lower than carvacrol (Dorman and Deans, 2000; Ultee et al., 2002; Ben Arfa et al., 2006). Exchanging the hydroxyl group of carvacrol with methyl ether affects its hydrophobicity, antimicrobial activity, and changes how

Table 1 | Overview of crude essential oils and their identified target sites and modes of action.

Common name of plant from which essential oil is derived	Species of plant from which essential oil is derived	Major constituents of essential oil	Model organisms and measured MIC	Mechanism	Reference
African basil	*Ocimum gratissimum*	Thymol (53.2%), γ-terpinene (25.7%), eugenol (12.7%), *p*-cymene (7.3%)	*E. coli* (6 μg/mL) *Klebsiella* sp. (6 μg/mL) *L. innocua* *L. monocytogenes* *Proteus mirabilis* (12 μg/mL) *Pseudomonas aeruginosa* (≥24 μg/mL) *Salmonella enteritidis* (3 μg/mL) *Shigella flexneri* (3 μg/mL) *Staphylococcus aureus* (0.75 μg/mL)	Permeabilized membrane	Nakamura et al. (1999), Cimanga et al. (2002), Nguefack et al. (2004b)
Bishop's weed	*Trachyspermum ammi* L.	Thymol (49.6%), β-cymene (16.3%), eugenol (3%), β-pinene (2.5%)	Three Gram-positive strains (12.5–175 μg/mL) Six Gram-negative strains (12.5–462.5 μg/mL)	Potassium and ATP leakage; cell lysis	Paul (2011)
Cinnamon	*Cinnamomum verum*	E-cinnamaldehyde (73.35%), β-caryophyllene (4.09%), linalool (3.55%), cinnamyl acetate (2.96%), eugenol (2.68%)	*Campylobacter jejuni* (0.05% v/v) *Enterobacter aerogenes* *E. coli* (0.05% v/v) *Listeria monocytogenes* (0.03% v/v) *P. aeruginosa* (0.125% v/v) *S. enteritidis* (0.05% v/v) *S. aureus* (0.04–0.125% v/v)	Inhibited histidine decarboxylase *P. aeruginosa*: depolarized and permeabilized membranes; leakage and coagulation of cytoplasmic content; inhibited respiration activity *S. aureus*: entered a viable but non-cultivable state, and lost membrane integrity	Wendakoon and Morihiko (1995), Smith-Palmer et al. (1998), Bouhdid et al. (2010)
	Cinnamomum cassia	Cinnamaldehyde (75.3%), coumarin (10.6%), cinnamic alcohol (3%)	*B. cereus* (339 μg/mL) *E. coli* (2640 μg/mL) *L. monocytogenes* (2640 μg/mL) *Salmonella infantis* (2640 μg/mL) *S. aureus* (1320 μg/mL)	Released cellular content; reduced intracellular pH; affected membrane integrity	Alzoreky and Nakahara (2003), Oussalah et al. (2006)
Coriander	*Coriandrum sativum*	Linalool (25.9–64.4%), (E)-2-decenal (0–20.2%), decanol (0.14–8.4%), (E)-2-decen-1-ol (0–7.9%)	*Candida* species (0.05–0.4% v/v) *L. monocytogenes* (0.018–0.074% v/v)	Damage cytoplasmic membrane; released cellular content	De et al. (1999), Gill et al. (2002), Silva et al. (2011)
Cloves	*Syzygium aromaticum*	Eugenol (64%), eugenyl acetate (16.3%), caryophyllene (14.5%)	*C. jejuni* (0.05% v/v) *E. aerogenes* *E. coli* (0.04% v/v) *L. monocytogenes* (0.03% v/v) *S. enteritidis* (0.04% v/v) *S. aureus* (0.04% v/v)	Inhibited histidine decarboxylase (*E. aerogenes*)	Wendakoon and Morihiko (1995), Smith-Palmer et al. (1998)

(Continued)

Table 1 | Continued

Common name of plant from which essential oil is derived	Species of plant from which essential oil is derived	Major constituents of essential oil	Model organisms and measured MIC	Mechanism	Reference
Cumin	*Cuminum cyminum* L. (seed)	Cumin aldehyde (29%), α-terpinen-7-al (20.7%), γ-terpinene (12.9%), *p*-cymene (8.6%)	*B. cereus* (0.05 μL/mL) *B. subtilis* (0.05 μL/mL or 1000 μg/mL)	Mild changes in cytoplasm; cell envelope intact	De et al. (1999), Pajohi (2011)
Garlic	*Allium sativum*	Allicin (70%)	*Candida albicans* (470–940 μg/mL) *E. coli* (15–15000 μg/mL or 3.95% v/v) *L. monocytogenes* (8.8% v/v) *Salmonella typhi* (7% v/v) *S. aureus* (12–15000 μg/mL or 5% v/v)	Induced leakage from *E. coli* cells	Hughes and Lawson (1991), Kumar and Berwal (1998), Ankri and Mirelman (1999), Perry et al. (2009)
Kaffir lime	*Citrus hystrix*	Citronellol (10.7%), limonene (7.3%), linalool (5.8%)	*Aspergillus flavus* (560 μg/mL) *Aspergillus parasiticus* (1130 μg/mL)	Reduced aflatoxin production	Rammanee and Hongpattarakere (2011)
Lemon grass	*Cymbopogon citratus*	Geranial (45.7%), myrcene (3.9%), 6-methylhept-5-en-2-one (2.7%)	*L. innocua* *L. monocytogenes* *S. aureus*	Permeabilized membrane	Baratta et al. (1998), Nguefack et al. (2004b)
Lime	*Citrus aurantifolia*	Limonene (69.1%), *p*-cymene (12.8%)	*A. flavus* (560 μg/mL) *A. parasiticus* (1130 μg/mL)	Reduced aflatoxin production; extra- and intracellular damages to cells	Rammanee and Hongpattarakere (2011)
Mustard oil			*E. coli* (0.2% v/v) *S. typhi* (0.2% v/v)	Affected membrane integrity; released cell content; decreased intracellular ATP and pH, while external ATP increased	Turgis et al. (2009)
Menthol	*Mentha longifolia*	Menthol (32.5%), menthone (20.7%), pulegone (17.8%), 1,8-cineole (5.6%), terpineol-4 (4.9%)	*Salmonella typhimurium* (1560 μg/mL) *E. coli* (780 μg/mL) *Micrococcus luteus* (190 μg/mL) *S. aureus* (780 μg/mL)	Damaged cell wall	Hafedh et al. (2010)
Oregano	*Origanum compactum*	Carvacrol (36.5%), thymol (29.7%), *p*-cymene (24.3%), γ-terpinene (1.1%)	*P. aeruginosa* (1% v/v) *S. aureus* (0.031% v/v)	Dissipated potassium gradient; depolarized membranes; permeabilized membranes; inhibited cell respiration; affected cell structure: coagulated cytoplasmic material; liberation of membrane vesicles; mesosome-like structures	Bouhdid et al. (2009), Babili et al. (2011)
	Origanum vulgare	Carvacrol (68.1%), *p*-cymene (15.9%), α-pinene (2.6%), myrcene (2%)	*E. coli* (0.625 μL/mL) *P. aeruginosa* (1648 μg/mL) *S. aureus* (0.6 μL/mL or 575 μg/mL) Six bacteria (20–40 μL/mL) Three *Candida* spp. (10–20 μL/mL)	Suppressed enterotoxin production; released cellular content; cell morphological changes; permeabilized membranes; leaked potassium and phosphate; dissipated pH gradients	Lambert et al. (2001), Burt and Reinders (2003), De Souza et al. (2008, 2010)

Rosemary	*Rosmarinus officinalis*	Carnosic acid, carnosol, rosmadial, genkwanin, rosmarinic acid	Model membranes *C. albicans* (10 μg/mL) *Saccharomyces cerevisiae* (5 μg/mL) *B. subtilis* (10 μg/mL) *E. coli* (0.1% v/v or 40 μg/mL) *S. aureus* (0.1% v/v or 20 μg/mL)	Membrane-rigidifying effects; affected lipid polymorphism	Panizzi et al. (1993), Smith-Palmer et al. (1998), Pérez-Fons et al. (2006)
Savory	*Satureja montana*	Thymol (29%), *p*-cymene (12%), linalool (11%), carvacrol (10.7%)	*C. albicans* (5 μg/mL) *S. cerevisiae* (5 μg/mL) *E. coli* (0.05% v/v or 40 μg/mL) *L. monocytogenes* (0.05% v/v) *S. aureus* (0.013% v/v or 5 μg/mL) *S.* Typhimurium (0.05% v/v) *Clostridium perfringens* (1.56% v/v)	Increased extracellular ATP; reduced intracellular pH; affected membrane integrity; structural damages; and cell lysis	Panizzi et al. (1993), Oussalah et al. (2006, 2007), De Oliveira et al. (2011)
Spanish oregano	*Coridothymus capitatus*		*E. coli* (0.025% v/v) *L. monocytogenes* (0.025% v/v) *S. aureus* (0.013% v/v) *S.* Typhimurium (0.025% v/v)	Increased extracellular ATP; released cellular content; reduced intracellular pH; affected membrane integrity	Oussalah et al. (2006, 2007)
Thyme	*Thymus eriocalyx*	Thymol (63.8%), α-phellandrene (13.3%), cis-sabinene hydroxide (8.1%)	*L. monocytogenes* (125 μg/mL)	Damaged cell envelope	Rasooli et al. (2006)
	Thymus vulgaris	Thymol (31.4%), *p*-cymene (17%), carvacrol (12.4%), γ-terpinene (11.1%)	*C. albicans* (1 μg/mL) *S. cerevisiae* (2 μg/mL) *B. subtilis* (2 μg/mL) *C. jejuni* (0.04% v/v) *Erwinia amylovora* (1600 μg/mL) *Erwinia carotovora* (1600 μg/mL) *E. coli* (0.05% v/v or 2 μg/mL) *L. innocua* *L. monocytogenes* (0.02% v/v) *S. aureus* (0.02% v/v or 5 μg/mL) *S. enteritidis* (0.04% v/v)	Permeabilized membrane; caused changes in outer membrane protein profile of *Erwinia* strains	Panizzi et al. (1993), Smith-Palmer et al. (1998), Nguefack et al. (2004b), Horváth et al. (2009)
	Thymus x-porlock	α-Phellandrene (38.7%), thymol (31.7%), *cis*-sabinene hydroxide (9.6%)	*L. monocytogenes* (125 μg/mL)	Damaged cell envelope; clumping of intracellular material	Rasooli et al. (2006)
	Gnaphalium affine	Eugenol (18.2%), linalool (10.6%), *trans*-caryophyllene (8.9%), α-terpineol (6%), *p*-cymene (5.8%)	Six bacteria (0.39–1.56 μg/mL) Five fungi (0.2 μg/mL)	Disrupted cell walls and membranes	Zeng et al. (2011)
	Sphallerocarpus gracilis	α-Asarone (33.1%), γ-terpinene (25,6%), *p*-cymene (17.4%)	Eight Gram-positive bacteria (160–640 μg/mL) Four Gram-negative bacteria (80–320 μg/mL) One fungus (no MIC)	Altered cell morphology	Gao et al. (2011)

Table 2 | Overview of essential oil components and their identified target sites and modes of action.

Compound (plant origin)	Chemical classification	MIC	Mechanism	Reference
Carvacrol (oregano and thyme)	Monoterpenoid phenol	*S. cerevisiae* (79.8–112.5 μg/mL) *Candida* strains (mean MIC 75–100 μg/mL) *B. cereus* (900 μg/mL) *Enterococcus faecalis* (225 μg/mL) *E. amylovora* (800 μg/mL) *E. carotovora* (1600 μg/mL) *E. coli* (225–2500 μg/mL) *L. monocytogenes* (450–1500 μg/mL) *S. aureus* (450–1250 μg/mL) *Staphylococcus epidermidis* (450 μg/mL) *S. typhimurium* (150–250 μg/mL) *P. fluorescens* (1.84 μg/mL) *Vibrio vulnificus* (250 μg/mL) *Yersinia enterocolitica* (225 μg/mL)	Yeast: Depolarized and permeabilized membranes Transient Ca^{2+} surge Might act on specific signaling pathways rather than non-specific membrane damages Upregulated genes involved in drug efflux, alternative metabolism, stress response, and autophagy Down-regulated genes involved in RNA metabolism and ribosome biogenesis Impaired ergosterol biosynthesis Bacteria: Permeabilized cell membranes, and vesicles Affected fatty acid and phospholipid head-group composition Decreased melting temperature and transition enthalpy thus decreased membrane fluidity Dissipated pH gradient and membrane potential Depleted intracellular ATP, with no leakage of ATP (Gill and Holley, 2006a; claim release of ATP) ATPase inhibition Incubated at sub-lethal concentration cells increased GroEL and decreased flagellin protein Caused changes in outer membrane protein profile Inhibited toxin production by *B. cereus* Damaged cell morphology	Kim et al. (1995), Helander et al. (1998), Cosentino et al. (1999), Ultee et al. (1999, 2000, 2002), Lambert et al. (2001), Ultee and Smid (2001), Di Pasqua et al. (2006, 2007), Gill and Holley (2006a,b), Burt et al. (2007), Cristani et al. (2007), Xu et al. (2008), Horváth et al. (2009), Rao et al. (2010), Ahmad et al. (2011), La Storia et al. (2011)
Eugenol (clove)	Phenylpropene phenol	*Aspergillus niger* (3000 μg/mL) *C. albicans* (3000 μg/mL) *S. cerevisiae* (490–3000 μg/mL) *B. thermosphacta* (2.12 μg/mL) *Bacillus licheniformis* (250 μg/mL) *E. coli* (800–3000 μg/mL) *L. monocytogenes* (800 to above 1000 μg/mL) *L. sakei* (985 μg/mL for kill) *M. luteus* (250 μg/mL) *P. aeruginosa* (1500–3000 μg/mL) *P. fluorescens* (2.12 μg/mL) *S. aureus* (2.12–750 μg/mL) *S. enterica* serovar Thypimurium (3.18–500 μg/mL) *V. vulnificus* (500 μg/mL)	Yeast: Cells lysis and damaged cell surface Small Ca^{2+} burst Bacteria: Inhibited: ATPase, histidine decarboxylase, and extracellular enzyme production (at sub-lethal concentrations) Permeabilize membranes Leaked ATP and potassium ions	Zemek et al. (1979, 1987), Thoroski (1989), Kim et al. (1995), Wendakoon and Morihiko (1995), Chang et al. (2001), Walsh et al. (2003), Bennis et al. (2004), Gill and Holley (2004, 2006a,b), Yamazaki et al. (2004), Rao et al. (2010)

Thymol (thyme)	Monoterpenoid phenol	*S. cerevisiae* (112.5–270 μg/mL) *Candida* strains (mean MIC 100–150 μg/mL) *B. cereus* (450 μg/mL) *B. thermosphacta* (0.58 μg/mL) *E. amylovora* (1600 μg/mL) *E. carotovora* (1600 μg/mL) *E. coli* (225–5000 mg/mL) *E. faecalis* (225 μg/mL) *L. monocytogenes* (450 μg/mL) *P. fluorescens* (2.88 μg/mL) *S. aureus* (225–310 μg/mL) *S. enterica* serovar Thypimurium (0.96 μg/mL) *S. epidermidis* (225 μg/mL) *S. typhimurium* (56.25–150 μg/mL) *Y. enterocolitica* (225 μg/mL)	Yeast: Disrupted cell membrane Impaired ergosterol biosynthesis Lysed cells Damaged cell surface Ca^{2+} bursts Similar up- and down-regulation of genes as carvacrol, except of repression of genes involved in vitamin B1 biosynthesis and sulfur metabolism Bacteria: Interacted with phospholipid membranes causing fluidifying effect Affected lipid composition Permeabilized cell membranes and vesicles Decreased melting temperature and transition enthalpy of membranes Leaked H^+ and K^+ ions and ATP Depolarized cells Affected cell morphology Impaired citrate metabolic pathway Inhibited enzymes involved in ATP synthesis Upregulated GroEL, DnaK, and outer membrane proteins Caused changes in outer membrane protein profile Altered gene response	Helander et al. (1998), Cosentino et al. (1999), Lambert et al. (2001), Walsh et al. (2003), Bennis et al. (2004), Trombetta et al. (2005), Di Pasqua et al. (2006, 2007, 2010), Cristani et al. (2007), Shapira and Mimran (2007), Xu et al. (2008), Horváth et al. (2009), Rao et al. (2010), Ahmad et al. (2011)
p-Cymene (oregano and thyme)	Monoterpene	*E. coli* (2500 μg/mL) *S. aureus* (1250 μg/mL)	Decreased membrane melting temperature and transition enthalpy Decreased membrane potential Reduced cell motility Incorporate and expand membranes Might perturb membrane of microorganisms	Ultee et al. (2002), Burt et al. (2007), Cristani et al. (2007)
γ-Terpinene (oregano and thyme)	Monoterpene	*E. coli* (5000 μg/mL) *S. aureus* (2500–34000 μg/mL)	Decreased membrane melting temperature and transition enthalpy Might perturb the membrane of microorganisms	Carson and Riley (1995), Cristani et al. (2007)
Cinnamaldehyde (cinnamon)	Phenylpropene aldehyde	*B. cereus* (0.3 μg/mL) *B. thermosphacta* (0.84 μg/mL) *E. aerogenes* *E. faecalis* (250 μg/mL) *E. coli* (397–1322 μg/mL) *L. monocytogenes* (3965 μg/mL) *P. aeruginosa* (750–1500 μg/mL) *P. fluorescens* (3.15 μg/mL) *S. aureus* (2.1–750 μg/mL) *S. typhimurium* (397 μg/mL)	Yeast: Inhibited cell wall synthesizing enzymes, and cytokinesis Bacteria: Concentration dependent ATPase inhibition Lost cell motility Studies argued both for and against membrane disintegration properties Inhibited: histidine decarboxylase and cytokinesis No significant protein leakage No effect on cell morphology (an effect was observed by Di Pasqua et al. (2007)	Zemek et al. (1987), Wendakoon and Morihiko (1995), Helander et al. (1998), Bang et al. (2000), Chang et al. (2001), Kwon et al. (2003), Gill and Holley (2004, 2006a,b), Yamazaki et al. (2004), Di Pasqua et al. (2007), Domadia et al. (2007), Hemaiswarya et al. (2011)

(Continued)

Table 2 | Continued

Compound (plant origin)	Chemical classification	MIC	Mechanism	Reference
Linalool (basil and citrus oils)	Monoterpenoid alcohol	*C. albicans* (2145 μg/mL) *B. cereus* (1073 μg/mL) *C. jejuni* (515 μg/mL) *E. coli* (515–2145 μg/mL) *L. monocytogenes* (1000–2145 μg/mL) *S. aureus* (1073–2145 μg/mL) *S. typhimurium* (1000 μg/mL) *V. vulnificus* (1000 μg/mL)	Permeabilized membranes	Carson and Riley (1995), Kim et al. (1995), Bagamboula et al. (2004), Fisher and Phillips (2006, 2008), Ait-Ouazzou et al. (2011)
Carvone (caraway and dill)	Monoterpenoid ketone	*E. coli* (1500 μg/mL) *S. typhimurium* (1500 μg/mL)	No effect on outer membrane and ATP pool	Helander et al. (1998), Ceylan and Fung (2004)
Linalyl acetate (bergamot)	Monoterpenoid acetate ester	*E. coli* (5000 μg/mL) *S. aureus* (1250 μg/mL)	Perturbed membrane permeability Released cellular content Might interact with intracellular components	Trombetta et al. (2005), Schipilliti et al. (2011)
Vanillin (vanilla)	Phenylpropene phenolic aldehyde	*S. cerevisiae* (3195 μg/mL) *Z. bailii* (3043 μgmL) *Z. rouxii* (1978 μg/mL) *E. coli* (2282 μg/mL) *Lactobacillus plantarum* (11411 μg/mL) *Listeria innocua* (5325 μg/mL) 18 yeasts and molds (456–1460 μg/mL)	Inhibited cell respiration Permeabilized cell membranes Dissipated potassium and pH gradients Stimulated ATP production in some cells and had no affect on remaining cells	Fitzgerald et al. (2003, 2004, 2005)
Limonene (orange, lemon, and mandarin)		*B. thermosphacta* (1.68 μg/mL) *E. coli* (8.4 μg/mL) *P. fluorescens* (8.4 μg/mL) *S. aureus* (1.68 μg/mL) *S. enterica* serovar Thypimurium (8.4 μg/mL)	Changed membrane fatty acid composition Damaged cell morphology	Di Pasqua et al. (2006, 2007), Espina et al. (2011)
Menthol (peppermint)	Monoterpenoid phenol	*C. albicans* (2500 μg/mL) *B. cereus* (1250 μg/mL) *Enterobacter aerogenes* (1250 μg/mL) *E. coli* (1250–2500 μg/mL) *Klebsiella pneumonia* (2500 μg/mL) *L. monocytogenes* (1250 μg/mL) *Proteus vulgaris* (1250 μg/mL) *P. aeruginosa* (2500 μg/mL) *S. typhimurium* (625 μg/mL) *S. aureus* (620 μg/mL) *S. epidermidis* (625 μg/mL) *Yersinia enterocolitica* (2500 μg/mL)	Perturbed membrane permeability Released cellular content Might interact with intracellular components	Işcan et al. (2002), Trombetta et al. (2005), Bassolé et al. (2010)

Allyl isothiocyanate (mustard oil)	Organosulfur	*Aeromonas hydrophilia* (200 μg/mL) *E. coli* (51–200 μg/mL) *Lactobacillus brevis* (1000 μg/mL) *Leuconostoc mesenteroides* (500 μg/mL) *Pediococcus pentosaceous* (1000 μg/mL) *P. fluorescens* (200 μg/mL) *S. aureus* (200 μg/mL) *B. subtilis* (200 μg/mL)	AITC readily cross membranes Caused leakage of metabolites No cell lysis React with SH- and amino-group containing compounds Inhibited: oxygen uptake, acetate kinase, thioredoxin reductase, and cytochrome *c* oxidase activity (yeast) Function as a non-specific inhibitor of intracellular enzymes and alter proteins by oxidative cleavage of disulfide bonds and attack of free amino groups in lysine and arginine	Kojima (1971), Kawakishi and Namiki (1982), Kawakishi and Kaneko (1985, 1987), Delaquis (1995), Shofran et al. (1998), Lin et al. (2000), Ahn (2001), Luciano et al. (2008), Luciano and Holley (2009)
Allicin (garlic)	Organosulfur	*C. albicans* (7 μg/mL) *E. coli* (28 μg/mL) *S. aureus* (28 μg/mL)	Readily permeates phospholipid membranes Inhibited: thiol-containing enzymes, RNA, DNA, and protein synthesis, acetyl-CoA synthetase (yeast), and the bacterial acetyl-CoA-forming system Might react with non-SH amino acids	Feldberg et al. (1988), Focke et al. (1990), Hughes and Lawson (1991), Rabinkov et al. (1998), Miron et al. (2000), Kyung (2011)
Citral (citrus fruits)	Monoterpenoid aldehyde	*Aspergillus fumigates* (714 μg/mL) *C. albicans* (447 μg/mL) *E. coli* (447–500 μg/mL) *L. monocytogenes* (500 μg/mL) *Microsporum gypseum* (447 μg/mL) *S. typhimurium* (500 μg/mL) *S. aureus* (447 μg/mL) *Trichophyton mentagrophytes* (714 μg/mL) *V. vulnificus* (100 μg/mL)	The sigma factor RpoS increase resistance of *E. coli* cells against citral, damaged cell membranes	Onawunmi (1989), Kim et al. (1995), Nazer et al. (2005), Fisher and Phillips (2008), Somolinos et al. (2010)
T-cadinol (myrrh)	Sesquiterpenoid alcohol	*S. aureus* (24 μg/mL) *E. coli* (no inhibition) *Trichophyton mentagrophytes* (2.3 μg/mL) *Candida parapsilosis* (no inhibition)	Lysed cells	Claeson et al. (1992)

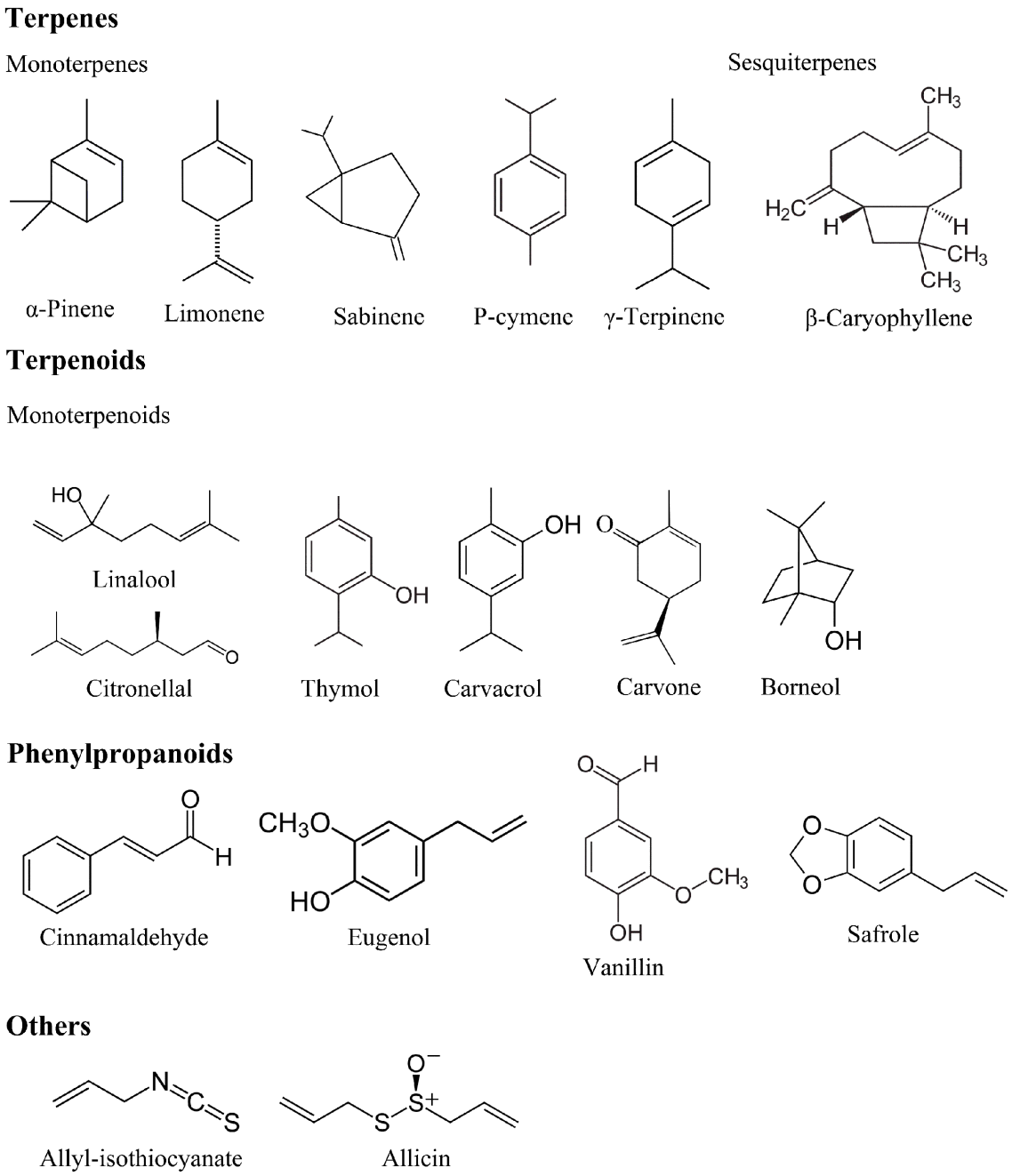

FIGURE 1 | Chemical structures of selected essential oil constituents.

the molecule interacts with the membrane (Veldhuizen et al., 2006). Carvacrol's antimicrobial activity is comparable to that of 2-amino-*p*-cymene, which indicates that the hydroxyl group is important, but not essential for carvacrol's activity (Veldhuizen et al., 2006). The antimicrobial activity of essential oils can often be correlated to its content of phenolic constituents (Aligiannis et al., 2001; Kalemba and Kunicka, 2003; Rhayour et al., 2003).

The terpenoids are a large group of antimicrobial compounds that are active against a broad spectrum of microorganisms, with the most active monoterpenoids identified so far being carvacrol and thymol. Dorman and Deans (2000) investigated the effect of many terpenoids against 25 different bacterial strains, and showed that all terpenoid compounds, except borneol and carvacrol methyl ester, exhibited a broad antimicrobial activity. The antimicrobial activity of carvacrol, thymol, linalool, and menthol were evaluated against *Listeria monocytogenes*, *Enterobacter aerogenes*, *E. coli*, and *Pseudomonas aeruginosa*. The most active compound was carvacrol followed by thymol with their highest MIC being 300 and 800 μg/mL, respectively (Bassolé et al., 2010). These results confirm the high antimicrobial activity of

a broad collection of terpenoids, and because their chemical structures are closely related to that of terpenes, the increased activity compared to terpenes can be attributed to the functional moieties.

Thymol

The mode of action of thymol, a phenolic monoterpenoid and one of the major constituents of thyme oil, has received much attention from researchers. Thymol is structurally very similar to carvacrol, having the hydroxyl group at a different position on the phenolic ring (**Figure 1**). The antimicrobial action of phenolic compounds, such as thymol and carvacrol, are expected to cause structural and functional damages to the cytoplasmic membrane (Sikkema et al., 1995). The primary mode of antibacterial action of thymol is not fully known, but is believed to involve outer- and inner membrane disruption, and interaction with membrane proteins and intracellular targets.

Studies have shown that thymol interacts with cell membranes. The interaction affects membrane permeability, and this has been documented by loss of membrane potential, cellular uptake of ethidium bromide, and leakage of potassium ions, ATP, and carboxyfluorescein (Helander et al., 1998; Lambert et al., 2001; Walsh et al., 2003; Xu et al., 2008). Although the protective properties of lipopolysaccharide (LPS) against thymol had been confirmed using random transposon-insertion mutants, treatment of *E. coli* cells with thymol caused release of LPS and disruption of the outer membrane (Helander et al., 1998; Shapira and Mimran, 2007). The outer membrane disruption could not be prevented by addition of magnesium, suggesting that thymol did not disrupt the membrane by chelating cations (Helander et al., 1998). Thymol integrates at the polar head-group region of a lipid bilayer causing alterations to the cell membrane, which at low concentrations induce adaptational changes in the membrane lipid profile in order to compensate for thymol's fluidifying effects and to maintain the membrane function and structure (Turina et al., 2006; Di Pasqua et al., 2007).

In addition to interacting with membrane phospholipids, evidence has accumulated that documents thymol's interaction with membrane proteins and intracellular targets, which hinder cell recovery after temporary exposure. The ability of thymol to interact with proteins was examined using the protein bovine serum albumin (BSA) and the organic compound deferoxamine, which is also rich in amine groups but otherwise known for its Fe^{3+}-chelating properties. These compounds react similarly to that of amine groups in bacterial membrane proteins (Juven et al., 1994). Based on the antimicrobial activity of thymol in the absence and presence of the thymol-inhibiting deferoxamine or BSA, Juven et al. (1994) hypothesized that thymol forms a complex with membrane-bound or periplasmic proteins by means of hydrogen bonds and hydrophobic interactions. Interaction with membrane proteins was further supported by Di Pasqua et al. (2010) who exposed *Salmonella enterica* to sub-lethal concentrations of thymol, and observed accumulation of misfolded outer membrane proteins and upregulation of genes involved in synthesis of outer membrane proteins. Contrarily, down-regulation of outer membrane proteins was shown in *Erwinia* spp. (Horváth et al., 2009). Upon exposure to thymol, *S. enterica* upregulated production of the chaperon proteins Heat Shock Protein 60 (GroEL), and Heat Shock Protein 70 (DnaK), which are key proteins in the protection against thermal stress and misfolding of proteins (Di Pasqua et al., 2010; Hartl et al., 2011). Thymol also impaired the citrate metabolic pathway and affected many enzymes directly or indirectly involved in the synthesis of ATP (Di Pasqua et al., 2010). Thymol's intracellular action indicates that it affects important energy-generating processes, which lower a cells' ability to recover after exposure to thymol.

The mode of action of thymol against yeast and fungi has been sparsely investigated, but studies point to interactions with the cell envelope and intracellular targets. Thymol disrupted vesicles and cell membranes, and impaired ergosterol biosynthesis in *Candida* strains, which consequently affected cell membrane integrity because ergosterol regulates membrane fluidity and asymmetry similarly to cholesterol in animal cells (Ghannoum and Rice, 1999; Cristani et al., 2007; Ahmad et al., 2011). Interestingly, thymol induced cell lysis and only altered the cell structure of proliferating *S. cerevisiae* cells, indicating the effect of thymol depends on cell proliferation (Bennis et al., 2004). Contrary to this, Rao et al. (2010) proposed that thymol activates specific signaling pathways in yeast, rather than causing non-specific lesion of membranes. This proposal was based on the observation that thymol caused cytosolic Ca^{2+} bursts and transcription responses similar to Ca^{2+} stress and nutrient starvation (Rao et al., 2010).

Carvacrol

Carvacrol is a phenolic monoterpenoid and a major constituent of oregano. Together with its closely related isomer thymol, it is one of the most extensively studied essential oil constituents. The antimicrobial effect of carvacrol is expected to be similar to that of thymol, causing structural and functional damages to the cell membrane (Sikkema et al., 1995). The primary mode of action of carvacrol is its ability to position in the membrane where it increase permeability, however, other more specific actions may be important and will be discussed.

Carvacrol has been demonstrated to affect the outer membrane of Gram-negative bacteria (La Storia et al., 2011). Disintegration of the outer membrane caused release of LPS from Gram-negative bacteria (Helander et al., 1998). Although carvacrol affects the outer membrane, its site of action is thought to be the cytoplasmic membrane, resulting in passive transport of ions across the membrane. Carvacrol has a hydroxyl group that has been proposed to function as a transmembrane carrier of monovalent cations across the membrane, carrying H^+ into the cell cytoplasm and transporting K^+ back out (Ultee et al., 2002; Ben Arfa et al., 2006). However, Veldhuizen et al. (2006) found the hydroxyl group of carvacrol not to be essential for antimicrobial activity, and proposed that although the transmembrane proton carrier mechanism plays a role in the antimicrobial activity, the relatively high activity of a non-hydroxyl compound ruled it out as the main mode of action of carvacrol.

The evidences for the membrane as carvacrol's site of action are many, and the results suggest that the mode of action of carvacrol is to increase fluidity and permeability of membranes. It has been proposed that cells exposed to carvacrol change the fatty acid composition of the membrane as an adaptation mechanism

to maintain optimal membrane structure and function because of carvacrol's effect on fluidity (Ultee et al., 2000; Di Pasqua et al., 2006, 2007). It is well established that increased membrane fluidity enhances the permeability of membranes (Nikaido, 1994). Membrane permeabilization by carvacrol has been confirmed by monitoring the efflux of H^+, K^+, carboxyfluorescein, and ATP, and the influx of nucleic acid stains (Helander et al., 1998; Ultee et al., 1999; Lambert et al., 2001; Cristani et al., 2007; Xu et al., 2008).

Besides the interaction with membranes, carvacrol has been proposed to interact with membrane proteins and periplasmic enzymes (Juven et al., 1994), but the evidence for this is limited. The only example used isolated bacterial membranes with ATPase activity as the indicator for direct molecular binding of carvacrol in an assay with excess amounts of ATP added (Gill and Holley, 2006b). Carvacrol has also been proposed to have intracellular targets, but the studies documenting this are few and do not identify the possible intracellular targets. Inhibitory concentrations of carvacrol caused over-expression of outer membrane proteins in *Erwinia amylovora* cells (Horváth et al., 2009), indicating that carvacrol possibly affect outer membrane protein folding or insertion. In another study, *E. coli* cells grown in the presence of sub-lethal concentration of carvacrol produced significant amounts of GroEL, indicating that protein folding was affected. Furthermore, it inhibited the synthesis of flagellin, which caused new cells to be without flagella (Burt et al., 2007). Cells that had flagella exhibited decreased motility at increasing carvacrol concentration, indicating that carvacrol disrupts the membrane potential and thereby the proton motive force needed to drive flagellar movement (Gabel and Berg, 2003; Burt et al., 2007).

The mechanism of antifungal activity of carvacrol resembles that of thymol, showing disruption of Ca^{2+} and H^+ homeostasis, up- and down-regulation of gene transcription similar to Ca^{2+}-stress and nutrient starvation (Rao et al., 2010), disruption of membrane integrity and impairment of ergosterol biosynthesis in *Candida* strains (Ahmad et al., 2011).

PHENYLPROPENES

Phenylpropenes constitute a subfamily among the various groups of organic compounds called phenylpropanoids that are synthesized from the amino acid precursor phenylalanine in plants. Phenylpropanoids have their name from the six-carbon aromatic phenol group and the three-carbon propene tail of cinnamic acid, produced in the first step of phenylpropanoid biosynthesis. The phenylpropenes constitute a relatively small part of essential oils, and those that have been most thoroughly studied are eugenol, isoeugenol, vanillin, safrole, and cinnamaldehyde (**Figure 1**).

Comparison of molecules that are chemically similar to eugenol and isoeugenol indicated that the free hydroxyl groups are important for their activity against bacteria, but not yeast (Laekeman et al., 1990). Some of isoeugenol's activity might be attributed to the double bond in the α, β positions of the side chain, and a methyl group in the γ position (Jung and Fahey, 1983). Furthermore, the antimicrobial activity of phenylpropenes depends on the kind and number of substituents on the aromatic ring, selected microbial strains, and the experimental test parameters such as choice of growth medium, temperature, etc. (Pauli and Kubeczka, 2010).

The antibacterial activity of eugenol was evaluated against 25 different bacterial strains of which only one strain was not inhibited (Dorman and Deans, 2000). Isoeugenol and eugenol showed pronounced inhibition activity against yeasts and 6 out of 10 Gram-positive and Gram-negative bacteria at 1000 μg/mL (Laekeman et al., 1990). The antimicrobial properties of isoeugenol appear more potent than eugenol, as lower MIC values are found against a variety of bacteria, yeast, and molds (Zemek et al., 1979, 1987). Interestingly, isoeugenol and eugenol have higher antimicrobial activity against Gram-negative bacteria, yeasts, and molds than Gram-positive bacteria (Mygind, unpublished). This is unusual for essential oil constituents because they normally are more effective against Gram-positive bacteria.

Cinnamaldehyde appears less potent than eugenol. In a study of *L. monocytogenes* and *Lactobacillus sakei*, 3965 and 66080 μg/mL of cinnamaldehyde, but only 821 and 985 μg/mL of eugenol were required to obtain a bactericidal effect (Gill and Holley, 2004). However, when tested against *E. coli* and *Salmonella typhimurium*, the antimicrobial activity of cinnamaldehyde equals that of the potent monoterpenoids thymol and carvacrol (Helander et al., 1998). Another phenylpropene, vanillin, inhibits a range of yeasts, molds, and bacteria (Fitzgerald et al., 2003, 2004, 2005; Rupasinghe et al., 2006). It should be noted that some yeasts were able to convert sub-lethal concentrations of vanillin into non-inhibitory compounds (Fitzgerald et al., 2003).

Eugenol

Eugenol is a major constituent in clove essential oil, and its antimicrobial activity is linked to its ability to permeabilize the cell membrane and interact with proteins. Eugenol's action on membranes occurs mainly by a non-specific permeabilization. The non-specific permeabilization of the cytoplasmic membrane by eugenol has been demonstrated in various studies as increased transport of potassium and ATP out of the cells (Walsh et al., 2003; Gill and Holley, 2006a; Hemaiswarya and Doble, 2009). Eugenol induced minor changes in the fatty acid profile of *Pseudomonas fluorescens*, *E. coli*, *Brochotrix thermosphacta*, *S. enterica*, and *S. aureus*, and cell damages to *E. coli* and *B. thermosphacta* cells (Di Pasqua et al., 2006, 2007).

The hydroxyl group of eugenol is thought to bind to and affect the properties of proteins, thereby contributing to eugenol's inhibitory effect at sub-lethal concentrations. Consistent with this, eugenol has proven to inhibit the activity of the following enzymes: ATPase, histidine decarboxylase, amylase, and protease (Thoroski, 1989; Wendakoon and Morihiko, 1995; Gill and Holley, 2006b). Inhibition of the ATPase may be important for cell killing at high eugenol concentrations because energy generation needed for cell recovery is impaired (Gill and Holley, 2006b).

The antifungal mode of action of eugenol needs further investigation, but it is known to depend on cell proliferation. Eugenol treatment altered cell membrane and cell wall structures of proliferating *S. cerevisiae* cells resulting in the release of cellular content (Bennis et al., 2004).

Cinnamaldehyde

Aldehyde groups are reactive and have the ability to cross-link covalently with DNA and proteins through amine groups, thereby

interfering with their normal function (Feron et al., 1991). However, the mode of action of cinnamaldehyde, a phenylpropene aldehyde, is inconclusive. At least three things are believed to occur: At low concentrations, cinnamaldehyde inhibits different enzymes involved in cytokinesis or less important cell functions. At higher but sub-lethal concentrations, it acts as an ATPase inhibitor, and at lethal concentrations it perturbs cell membrane. Cinnamaldehyde was suggested to inhibit cytokinesis as a mode of action on *B. cereus* because cells could not separate although septa were present after division (Kwon et al., 2003). It has been established that cinnamaldehyde binds to the FtsZ protein, inhibiting its GTP dependent polymerization and thereby preventing cell division (Domadia et al., 2007; Hemaiswarya et al., 2011). The FtsZ protein is an attractive target for antimicrobial therapies as it is evolutionary distant from eukaryotic tubulin, and the predicted interaction of H2 and H3 of cinnamaldehyde with G295 and V208 of FtsZ, respectively, is conserved among FtsZ proteins from several species (Domadia et al., 2007; Hemaiswarya et al., 2011). Other enzymes, e.g., the histidine decarboxylase, is also inhibited by cinnamaldehyde (Wendakoon and Morihiko, 1995).

At sub-lethal concentrations, cinnamaldehyde gains access to the periplasm and inhibits the activity of transmembrane ATPase. Sub-lethal concentrations of cinnamaldehyde did not affect the integrity of the outer membrane of *E. coli*, but it inhibited growth and bioluminescence of *Photobacterium leiognathi*, indicating that cinnamaldehyde does gain access to the periplasm and possibly also the cytoplasm (Helander et al., 1998). The ability of cinnamaldehyde to access the periplasm was confirmed by demonstrating a decrease in ATPase activity of isolated cell membranes at increasing concentrations of cinnamaldehyde (13.6–1362 μg/mL; Gill and Holley, 2006a,b). ATPase inhibition was, however, suggested not to be the primary cause of cell death because the concentration required to inhibit the ATPase also resulted in membrane disruption of *E. coli* cells (681–1362 μg/mL; Gill and Holley, 2006a).

Many studies have demonstrated that cinnamaldehyde interacts with the cell membrane, but it is not yet clear how it perturbs membranes. It is not a general mode of action of cinnamaldehyde to disrupt membranes as illustrated by Di Pasqua et al. (2007). Cinnamaldehyde altered the membrane lipid profile with large increases in saturated fatty acids, yielding a more rigid membrane probably compensating for a fluidifying effect of cinnamaldehyde, and cell structure of *E. coli*, *S. enterica*, *P. fluorescens*, and *B. thermosphacta*, while only *S. aureus* demonstrated disintegration of the cell envelope (Di Pasqua et al., 2006, 2007). *Cinnamomum verum* essential oil (73% cinnamaldehyde) caused membrane depolarization, loss of membrane integrity, reduced respiratory activity, and coagulation of cytoplasmic material of *P. aeruginosa*, while exposure of *S. aureus* cells caused them to enter a viable but non-cultivable state (Bouhdid et al., 2010).

Among fungi, the primary mode of action of cinnamaldehyde has also been proposed to be inhibition of cell division. This was proposed because cinnamaldehyde inhibited the cell wall synthesizing enzymes in *S. cerevisiae* by functioning as a non-competitive inhibitor of β-(1,3)-glucan synthase and a mixed inhibitor of chitin synthase isozymes (Bang et al., 2000).

Vanillin

The mode of action of the phenylpropene phenolic aldehyde vanillin is not well understood, but it has been proposed to function as a membrane active compound that might have intracellular targets.

The proposed membrane and protein interactions of vanillin are based on one study. Vanillin inhibited respiration of *E. coli* and *Listeria innocua* cells, and disrupt the potassium and pH homeostasis of *Lactobacillus plantarum* cells (Fitzgerald et al., 2004). Propidium iodide staining demonstrated that treatment with vanillin disrupted membrane integrity of only a sub-population of cells and it was proposed that although vanillin primarily is a membrane active compound, it may also have intracellular target sites (Fitzgerald et al., 2005).

Not much is known about vanillin's mechanism of antifungal activity, but it has been suggested that the aldehyde moiety of vanillin plays an important role in its antifungal activity. The rationale for this is that *S. cerevisiae* convert vanillin into vanillic acid and vanillyl alcohol, which possess no antimicrobial activity, confirming the key-role of the aldehyde moiety (Feron et al., 1991; Fitzgerald et al., 2005).

OTHER ESSENTIAL OIL CONSTITUENTS

Essential oils contain a number of different degradation products originating from unsaturated fatty acids, lactones, terpenes, glycosides, and sulfur- and nitrogen-containing compounds (Caballero et al., 2003). Two examples of sulfur- and nitrogen-containing compounds with known antimicrobial activity are allicin and allyl isothiocyanate (AITC).

Allicin (diallyl thiosulfinate) is found in garlic and plays an important role in plant defense (Ankri and Mirelman, 1999). Inside the garlic cloves, the amino acid cysteine is converted to alliin (*S*-allyl-L-cystein-*S*-oxide), a known sulfoxide with no antimicrobial activity (Block, 1992; Ankri and Mirelman, 1999). Conversion of alliin to the antimicrobial allicin requires the enzyme alliinase. Studies suggest that alliin and alliinase are located in two different compartments (Ankri and Mirelman, 1999), and when garlic cloves are crushed, alliinase comes into contact with alliin and produces allicin (Ankri and Mirelman, 1999). Allicin has a pungent smell of garlic and exhibits antibacterial, antifungal, antiparasitic, and antiviral properties (Kyung, 2011). Allicin is equally effective against Gram-negative and Gram-positive bacteria (Cavallito and Bailey, 1944). Allicin is bactericidal with LD_{50} values against different bacteria ranging between 3 and more than 100 μg/mL, while a bacteriostatic effect was observed at much lower concentrations reflected in MIC values for fungal pathogens were in the range 0.15–1.5 μg/mL (Ankri and Mirelman, 1999). Although allicin shows great potential as a food preservative when evaluated *in vitro*, conflicting results have been obtained in food matrixes (Kyung, 2011).

Isothiocyanates, also known as mustard oils, are common essential oil constituents from plants belonging to the mustard family (Brassicaceae), such as mustard, broccoli, horseradish, and turnips (Nielsen and Rios, 2000). Isothiocyanates arise in plants as a result of enzymatic cleavage of released glucosinolates from intracellular compartments by membrane-bound myrosinase upon damage to the plant (Delaquis, 1995). Myrosinase

promotes hydrolysis and intramolecular rearrangement of intermediates, resulting in the three main groups of substances: nitriles, thiocyanates, and isothiocyanates (Zhang and Talalay, 1994). The latter group includes the non-phenolic volatile AITC which can constitute close to 90% of the oil composition (Ward et al., 1998). Allyl isothiocyanate in vapor and liquid forms has demonstrated high bactericidal activity against various food spoilage microorganisms and food pathogens, including *E. coli* O157:H7 (Luciano and Holley, 2009), *S. typhimurium*, *L. monocytogenes*, and other aerobic Gram-negative spoilage bacteria (Delaquis, 1997), and a broad spectrum of fungi (Delaquis, 1997; Nielsen and Rios, 2000).

Allicin

The antimicrobial activity of allicin has been known since it was first isolated and studied by Cavallito and Bailey (1944), since then the mode of action of allicin have been elucidated in great detail. Allicin is shown to target intracellular enzymes by interacting with their free SH groups.

Allicin is readily transported across the cell membrane into the cytoplasm where it can exert its antimicrobial action. Garlic extract, with allicin as major component, induced no significant changes in *E. coli* and *S. aureus* cell morphology, supporting the hypothesis that allicin acts intracellularly (Perry et al., 2009). In another study, Miron et al. (2000) showed that allicin freely permeated phospholipid vesicles and reacted with encapsulated SH-containing molecules.

The antimicrobial activity of allicin is ascribed to its reactive chemical group that binds to and inhibits a broad-range of intracellular targets. Allicin's -S(O)-S- group reacts with SH groups of enzymes (Rabinkov et al., 1998). Allicin irreversibly inhibited the thiol-protease papain, $NADP^+$-dependent alcohol dehydrogenase from *Thermoanaerobium brockii*, and a NAD^+-dependent alcohol dehydrogenase from horse liver (Rabinkov et al., 1998). Interestingly, all three enzymes could be reactivated with thiol-containing components like dithiothreitol, 2-mercaptoethanol, and glutathione (Rabinkov et al., 1998), demonstrating that the interaction leading to inhibition is reversible. Focke et al. (1990) also demonstrated reversible inhibition by specific binding of allicin to the enzymes involved in acetyl-CoA synthesis in bacteria, plants, yeasts, and mammals. Allicin only partially and transiently inhibited the DNA replication and protein synthesis in *S. typhimurium*, while RNA synthesis was reduced by more than 90% for at least 30 min, suggesting RNA synthesis as the primary target of allicin (Feldberg et al., 1988). Collectively, these studies indicate that allicin is a non-specific inhibitor of many enzymes. Allicin could potentially be used in combination with other antimicrobials because it has inhibiting effects on RNA synthesis and thereby reduce or hinder cell protection mechanisms induced by other antimicrobials.

Allyl isothiocyanate

The mode of action behind AITC's antimicrobial activity is not yet fully understood, but since it might penetrate membranes and no single site of action has been described, it is generally regarded as a non-specific inhibitor of periplasmic or intracellular targets.

It is not yet clear if AITC rapidly crosses membranes and enters the cytoplasm of prokaryotic and eukaryotic cells, or if it has an effect on cell membranes. Ahn (2001) visualized the AITC-treated cells by transmission electron microscopy (TEM) and showed that AITC altered the internal cell structures without causing ATP leakage or cell wall damages to *L. monocytogenes*. However, it did reduce the internal levels of ATP, indicating that cellular energy-generating processes were affected. In contrast, another study showed that AITC caused cell membrane damages to *E. coli* and *Salmonella* Montevideo leading to leakage of cellular metabolites, but not cell lysis (Lin et al., 2000).

The mode of action of AITC is due to its chemical group. The central carbon atom of isothiocyanate ($R-N=C=S$) is highly electrophile and reacts readily, and under mild conditions with oxygen-, sulfur-, or nitrogen-centered nucleophiles resulting in carbamates, thiocarbamates, or thiourea derivatives, respectively (Zhang and Talalay, 1994; Verma, 2003). Inside a cell, AITC can react with glutathione, sulfites, amino acids, oligopeptides, proteins, and water (Kawakishi and Namiki, 1982; Kawakishi and Kaneko, 1985, 1987; Cejpek et al., 2000). AITC cleaves the cysteine disulfide bond in proteins through an oxidative process (Kawakishi and Namiki, 1982), but also attacks free amino groups and arginine residues (Kawakishi and Kaneko, 1987). The antimicrobial mode of action of AITC is thus related to its general inhibition of enzymes and alteration of proteins by oxidative cleavage of disulfide bonds (Delaquis, 1995; Luciano and Holley, 2009).

The action of AITC on yeast is not well understood and warrants more investigation. Allyl isothiocyanate stalls oxygen uptake of yeasts, and uncouples the oxidative phosphorylation through the inhibition of cytochrome c oxidase in the electron transport chain (Kojima, 1971). Due to its very general mode of action in prokaryotes, similar effects are likely observed in eukaryotes also.

EXPERIMENTAL APPROACHES TO INVESTIGATE THE ANTIMICROBIAL MODE OF ACTION

The diversity of essential oil constituents is enormous and presents a wide range of compounds. Some have low or no efficiency against microorganisms while others are potent antimicrobials. The majority of antimicrobial compounds found in essential oils are terpenoids and phenylpropenes with the most active being phenols, although some aldehydes and non-phenolic substances also present promising antimicrobial activity. The target site and mode of action of most essential oil components is still not well understood, especially in yeast. Commercial applications of essential oils would benefit from deeper insight into the mode of action behind individual compounds, as this could facilitate the exploitation of, e.g., synergistic combinations with more powerful antimicrobial properties.

Many different techniques have been applied to elucidate the mode of action of essential oils and their constituents. Here we will present and discuss the most common experimental approaches. After establishing the killing or inhibition activity of a compound, an array of experiments can be performed to identify how a compound interacts with the cell to cause the observed effects. In this context, it is important to distinguish between experiments that identify the target site from those that elucidate the mode of action. The site of action refers to the part of the cell which interacts with the compound, e.g., the cell membrane, cell wall, or intracellular proteins, enzymes, nucleic acids, or metabolites. The mode

of action, however, yields more elaborate knowledge about the molecular mechanisms or intermolecular interactions behind the inhibition or killing effects. An overview of methods addressing the site or mode of action of antimicrobial compounds is provided in **Table 3**.

LOCATING THE SITE OF ACTION

High-resolution microscopy, such as electron microscopy or atomic force microscopy (AFM), can reveal the most extreme consequences of exposure to an antimicrobial compound, i.e., deformation of cells occurring from lysis or from damages to the cell wall. An advantage of TEM is that ultra-thin cross sections can reveal ultrastructural changes in the interior of the cell. Scanning electron microscopy (SEM) and AFM only image the cell surface. AFM has one important advantage over electron microscopy, in that it allows measurements in liquid under physiological conditions, avoiding difficult sample preparation and the artifacts associated herewith (Alessandrini and Facci, 2005). A limitation of both AFM and electron microscopy is, however, that specific cellular structures must be identified according to morphology unless some form of labeling can be applied. While antibodies conjugated to metal nanoparticles have been used with TEM in a few studies (Romero et al., 2010), no labeling techniques have been reported for SEM and AFM. It is, however, possible to combine AFM with optical microscopy and thus take advantage of the numerous options for fluorescent labeling of biomolecules.

An important site of action is the cell membrane, and indeed, many essential oil constituents have been proposed to act on the bacterial membrane. Interaction of antimicrobial compounds with the membrane can affect the transport of nutrients and ions, the membrane potential, and the overall permeability of the cell. These effects are investigated by measuring the efflux of intracellular ions like K^+ and H^+ (Ultee et al., 1999; Lambert et al., 2001). Efflux of small ions is not necessarily indicative of complete loss of membrane function, and can be observed in viable cells where growth is inhibited because the cell uses energy for repair or survival rather than cell proliferation (Bouhdid et al., 2010). Effects on the cell membrane that lead to cell death is more accurately predicted by detecting the efflux of larger molecules like ATP or carboxyfluorescein diacetate (cFDA) after esterase reaction (Xu et al., 2008), or by influx of large polar organic DNA-binding stains like ethidium bromide (Lambert et al., 2001) and propidium iodide (Bouhdid et al., 2010). It should be pointed out that it is always good practice to validate the observed effects by combining several techniques. Monitoring the release of calcein encapsulated in membrane vesicles can for example be used as a complimentary technique to confirm the membrane as the site of action (Miron et al., 2000).

If no effects are observed on cell structure and membrane functionality, it is assumed that the site of action is intracellular. The target can be proteins and enzymes in general, or it can be essential cellular processes involved in biosynthesis or energy generation. An intracellular site of action can for example be determined by incorporation of radioactively labeled substrates used in particular biosynthesis pathways (Schneider et al., 2010). Lack of or decreased incorporation is then taken as an indication of the process being affected by the antimicrobial compound. For example, radiolabeled nucleotides or amino acids can be used to detect if DNA replication or protein synthesis takes place, respectively (Schneider et al., 2010).

Some compounds have multiple sites of action, and in that case it can be difficult to pinpoint which one is ultimately responsible for cell death. For example, a compound that affects membrane permeability will also affect the membrane potential and thereby energy generation by respiration. It is thus difficult to distinguish direct effects on energy-generating processes from the indirect effect a permeable membrane has on these processes. At sublethal concentrations, changes to the transcriptome and proteome during exposure can reveal how the cell responds to the compound, and upregulation of genes involved in certain metabolic or biosynthesis pathways can be indicative of which cell structures or processes that are affected (Burt et al., 2007; Rao et al., 2010).

ELUCIDATING THE MODE OF ACTION

The probably most comprehensive approach to investigate the mode of action of a particular compound is to perform random transposon mutagenesis in order to search for mutations that compensate for the antimicrobial effect of a particular compound. In this way, it is possible to identify the mode of action of compounds that interact very specifically with, e.g., a single enzyme or with particular proteins or lipids in the membrane (Shapira and Mimran, 2007; Van Hoang et al., 2011). The approach is, however, not suited for investigating antimicrobial compounds that act simultaneously on several components in the cell, as a single mutation is unlikely to facilitate compensation for the antimicrobial effect on the cell as a whole.

Antimicrobial compounds that act on the membrane can cause depolarization or increased permeability through various mechanisms. For example, some antimicrobial peptides form pores (Cotter et al., 2005; Fantner et al., 2010) while other compounds, such as certain essential oil constituents, have a fluidifying effect on the membrane (Trombetta et al., 2005; Cristani et al., 2007). Membrane properties like lipid packing can be investigated in membrane vesicles by LAURDAN staining combined with spectrofluorometry (Nielsen and Otzen, 2010), and membrane fluidity can be investigated directly in bacteria by differential scanning calorimetry (Trombetta et al., 2005) or fluorescence anisotropy measurements of DPH using a spectrofluorometer (Liao et al., 2010). AFM imaging has also in recent years allowed high-resolution visualization of native membranes on a solid support. Structural changes resulting from integration of an antimicrobial compound into the membrane can thus be visualized directly (Brasseur et al., 2008), and the effect on membrane rigidity can be quantified by AFM force spectroscopy (Sullan et al., 2010). Functionalizing the AFM tip with the antimicrobial compound of interest furthermore allows investigation of interaction forces between the compound and its target. This approach was for example used to map binding events of vancomycin on the surface of bacteria and confirmed that binding occurred at the site of cell wall synthesis in dividing cells (Gilbert et al., 2007).

ESSENTIAL OILS IN FOOD PRESERVATION

Food-borne diseases are a growing public health problem worldwide. It is estimated that each year in the United States, 31 species of

Table 3 | Overview of experimental approaches used to identify target sites and modes of action of antimicrobial compounds.

Target site or mode of action	Experiment	Reference
Changes in cell morphology	Scanning electron microscopy (SEM) visualizes the cell structure with sub-micron resolution.	Burt and Reinders (2003), Kwon et al. (2003), Bennis et al. (2004), Di Pasqua et al. (2007), Turgis et al. (2009), De Souza et al. (2010), Gao et al. (2011), Lv et al. (2011), Paul (2011)
	Transmission electron microscopy (TEM) can visualize changes in cell morphology, damages to cell wall and cell membrane, and coagulation of intracellular content.	Claeson et al. (1992), Gustafson et al. (1998), Ahn (2001), Carson et al. (2002), Rasooli et al. (2006), Bouhdid et al. (2009, 2010), Fisher and Phillips (2009), De Oliveira et al. (2011), Pajohi (2011), Rammanee and Hongpattarakere (2011), Zeng et al. (2011)
	Atomic force microscopy (AFM) visualizes cells at nanometer resolution in liquid under physiological conditions, and can provide information about changes in cell topography and elasticity.	Perry et al. (2009), Hafedh et al. (2010), La Storia et al. (2011)
Disruption of cytoplasmic membrane	Cell lysis or release of cellular content can be detected spectrophotometrically at 260 nm.	Carson et al. (2002), Bennis et al. (2004), Turgis et al. (2009), De Souza et al. (2010), Lv et al. (2011), Paul (2011)
	Measurement of potassium or phosphate leakage from the cells using ion-selective electrodes, atomic absorption spectroscopy, or flame photometry.	Lambert and Hammond (1973), Ultee et al. (1999), Cox et al. (2000), Lambert et al. (2001), Walsh et al. (2003), Fitzgerald et al. (2004), Shapira and Mimran (2007), Bouhdid et al. (2009, 2010)
	Measurement of ATP leakage from the cells using an assay based on luciferase activity quantified by bioluminescence.	Helander et al. (1998), Ultee et al. (1999, 2002), Ahn (2001), Fitzgerald et al. (2004), Gill and Holley (2004, 2006b), Fisher and Phillips (2009), Turgis et al. (2009), Sánchez et al. (2010), Paul (2011)
	Uptake of fluorescent DNA-binding stains, such as propidium iodide (PI), SYTO9, ethidium bromide (EB), and carboxyfluorescein diacetate (cFDA), using fluorescence microscopy or flow cytometry.	Cox et al. (2000), Lambert et al. (2001), Fitzgerald et al. (2004), Nguefack et al. (2004a), Gill and Holley (2006a), Paparella et al. (2008), Bouhdid et al. (2009, 2010), Somolinos et al. (2010), Ahmad et al. (2011), Ait-Ouazzou et al. (2011)
	Leakage of the self-quenching dyes calcein or carboxyfluorescein encapsulated in phospholipid vesicles is as an increase in fluorescence intensity as the intravesicular concentration decreases.	Cox et al. (2000), Miron et al. (2000), Trombetta et al. (2005), Cristani et al. (2007)
	Changes in concentration gradients of ions across a cell membrane can be detected by fluorometry using bis-oxonol or $DiSC_3(5)$, or by flow cytometry using bis-oxonol, $DiOC_2(3)$, or BOX.	Ultee et al. (1999, 2002), Veldhuizen et al. (2006), Xu et al. (2008), Bouhdid et al. (2009, 2010), Fisher and Phillips (2009), Sánchez et al. (2010), Silva et al. (2011)
Disruption of outer membrane in Gram-negative bacteria	Damages to the outer membrane is detected by monitoring the uptake of the hydrophobic fluorescent probe. 1-*N*-phenyl-l-napthylamine (NPN) into the membrane using fluorescence microscopy.	Helander et al. (1998), Fisher and Phillips (2009)
	Release of phospholipid and LPS from the outer membrane is detected by capillary gas chromatography and compared with an internal fatty acid standard. The release of proteins from the outer membrane is detected by a electrophoresis (SDS-PAGE) in which the protein profiles of cell-free supernatants of treated and untreated cells are compared.	Helander et al. (1998)
	Changes in the protein profile of the outer membrane can be measured by separating the proteins according to mass and labeling for detection by laser induced fluorescence.	Horváth et al. (2009)
Changes in membrane properties	Membrane expansion can be detected by relief of fluorescence self-quenching by the liposome probe octadecyl rhodamine β chloride.	Ultee et al. (2002)
	Effects on membrane melting temperature, fluidity, and phase separation can be detected using differential scanning calorimetry, Fourier-transform infrared spectrometer, nuclear magnetic resonance, or small-angle X-ray diffraction.	Ultee et al. (2000), Trombetta et al. (2005), Pérez-Fons et al. (2006), Cristani et al. (2007)

(Continued)

Table 3 | Continued

Target site or mode of action	Experiment	Reference
	Changes in yeast cell's ergosterol biosynthesis can be evaluated by comparing the intracellular content of ergosterols of cells grown in the absence or presence of antimicrobials. The content of ergosterols in an intracellular extract can be calculated using data obtained from a spectrophotometrically scan of the extract between 240 and 300 nm.	Ahmad et al. (2011)
Disruption of membrane potential	Changes in concentration gradients of ions across a cell membrane can be detected either with a spectrofluorometer using bis-oxonol or $DiSC_3(5)$, or by flow cytometry using bis-oxonol, $DiOC_2(3)$, or BOX.	Ultee et al. (1999, 2002), Veldhuizen et al. (2006), Xu et al. (2008), Bouhdid et al. (2009, 2010), Fisher and Phillips (2009), Sánchez et al. (2010), Silva et al. (2011)
Disruption of intracellular pH homeostasis	5-(and 6-)carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) is readily taken up by bacteria and hydrolyzed by esterases to 5-(and 6-)carboxyfluorescein succinimidyl ester (cFSE). The intracellular pH can then be determined from the ratio of the fluorescent signal of cFSE at the pH-sensitive 490 nm and the pH-insensitive 440 nm.	Breeuwer et al. (1996), Ultee et al. (1999, 2002), Fitzgerald et al. (2004), Fisher and Phillips (2009), Turgis et al. (2009), Sánchez et al. (2010)
	Intracellular pH is measured with pH-sensitive fluorescent probes pHluorin (cytoplasmic) and BCECF AM (vacuoles).	Rao et al. (2010)
Disruption of intracellular Ca^{2+} homeostasis	Intracellular Ca^{2+} concentration is measured after transformation with a plasmid containing the gene for aequorin. Aequorin emits light upon binding Ca^{2+}, and Ca^{2+} is thus quantified by measuring luminescence.	Rao et al. (2010)
Disruption of cellular respiration	Disruption of the cellular respiration can be detected by oxygen consumption measured with oxygen electrodes or by reduction of the stain 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which is reduced by the electron transport chain to the insoluble and fluorescent formazan.	Cox et al. (2000), Fitzgerald et al. (2004), Bouhdid et al. (2009, 2010)
Complex reaction mechanism	Reaction with thiol groups in a variety of targets can be monitored by complex formation with cysteine or glutathione as free SH-group compounds. Complex formation is then detected with high performance liquid chromatography (HPLC), liquid chromatography coupled with mass spectroscopy (LC–MS), or NMR.	Rabinkov et al. (1998), Miron et al. (2000), Luciano et al. (2008), Luciano and Holley (2009)
	Competitive binding of thiol groups can also be tested by adding thiol-containing compounds, e.g., the protein bovine serum albumin (BSA) or the organic compound desferal to the growth medium and test if the antimicrobial effect can be relieved.	Juven et al. (1994)
Inhibition of particular enzymes	Inhibition of the cell wall synthesizing enzymes β-(1,3)-glucan synthase and chitin synthase have been monitored using the radioactive substrates UDP[^{14}C]-Glu and UDP[U-^{14}C]-GlcNAc by a liquid scintillation counter.	Bang et al. (2000)
	Changes in enzyme activity of proteases, alcohol dehydrogenases, thioredoxin reductase, acetate kinase, decarboxylases, α-amylase, subtilisin, acetyl-CoA-forming enzyme systems, and ATPase has been investigated, using techniques such as spectrophotometer, luminometer, HPLC, pH monitoring, liquid scintillation counter, and zone of proteolysis.	Thoroski (1989), Focke et al. (1990), Wendakoon and Morihiko (1995), Rabinkov et al. (1998), Gill and Holley (2006b), Luciano and Holley (2009)
Inhibition of cell division	The effect on FtsZ assembly and hence on the cell division apparatus can be investigated using light scattering assay, GTP hydrolysis, TEM, isothermal titration calorimetry, saturation transfer difference NMR spectroscopy (STD NMR), and *in silico* molecular modeling.	Domadia et al. (2007), Hemaiswarya et al. (2011)
Changes in transcriptome	Random mutation can be used to identify the role of a particular genes in resistance or susceptibility mechanisms. Transcriptional up- and down-regulation can subsequently be detected for genes of interest using RT-qPCR, or for a large number of genes simultaneously using microarrays.	Somolinos et al. (2010), Shapira and Mimran (2007)

(Continued)

Table 3 | Continued

Target site or mode of action	Experiment	Reference
	Changes in RNA, DNA, and protein biosynthesis can be detected by continuous incorporation of radioactive labeled uridine, thymidine, and leucine, respectively.	Feldberg et al. (1988)
Changes in proteome	Expression of specific proteins can be determined by SDS-PAGE gel electrophoresis and western blotting followed by identification of peptide fragments by mass spectrometry.	Burt et al. (2007), Liu et al. (2010)
	Changes in a proteome profile can be detected by 2D-PAGE electrophoresis separation followed by selection and excision of up- or down-regulated protein-spots, which are then identified by mass spectrometry.	Di Pasqua et al. (2010), Liu et al. (2010)
Changes in toxin production	The effect on excreted toxin production can be measured using enzyme linked immunosorbent assay (ELISA) and spectrophotometric quantification.	Ultee and Smid (2001), De Souza et al. (2010)

pathogens cause 9.4 million cases of food-borne illnesses (Scallan et al., 2011). Successful control of food-borne pathogens requires the use of multiple preservation techniques in the manufacturing and storage of food products. A recent consumer trend toward preference for products with lower salt and sugar content presents an increased need for efficient food preservatives, as lowering the salt and sugar content would otherwise compromise the product's shelf-life (Zink, 1997). A wide range of preservatives are used to extend the shelf-life of a product by inhibiting microbial growth. However, an increasingly negative consumer perception of synthetic food additives has spurred an interest in finding natural alternatives to the traditional solutions (Zink, 1997). Although originally added to change or improve taste, the antimicrobial activity of essential oils makes them an attractive choice for substituting synthetic preservatives.

PERSPECTIVES AND LIMITATIONS IN APPLICATION OF ESSENTIAL OILS IN FOOD

A range of essential oil components have been accepted by the European Commission for their intended use as flavorings in food products. The registered flavorings are, e.g., linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral, and limonene, all of which are considered to present no risk to the health of the consumer. The United States Food and Drug Administration (FDA) also classifies these substances as generally recognized as safe (GRAS). The crude essential oils classified as GRAS by FDA include amongst others clove, oregano, thyme, nutmeg, basil, mustard, and cinnamon. There are regulatory limitations on the accepted daily intake of essential oils or essential oil components, so before they can be used in food products, a daily intake survey should be available for evaluation by FDA.

Despite the demonstrated potential of essential oils and their constituents *in vitro*, their use as preservatives in food has been limited because high concentrations are needed to achieve sufficient antimicrobial activity. In many food products, the hydrophobic essential oil constituents are impaired by interactions with food matrix components, such as fat (Cava-Roda et al., 2010; Rattanachaikunsopon and Phumkhachorn, 2010), starch (Gutierrez et al., 2008), and proteins (Cerrutti and Alzamora, 1996; Kyung, 2011). Furthermore, the antimicrobial potency of essential oil constituents also depends on pH (Juven et al., 1994), temperature (Rattanachaikunsopon and Phumkhachorn, 2010), and the level of microbial contamination (Somolinos et al., 2010). Extrapolation of results from *in vitro* tests to food products is thus difficult at best, and a lower performance of the antimicrobial compound must be expected. For example, Cilantro oil had significant antibacterial activity at 0.018% *in vitro*, but when applied to a ham model, even 6% cilantro oil had no antimicrobial activity (Gill et al., 2002). Before being added to food products, it is therefore useful to investigate how essential oils or their constituents interact with food components *in vitro*. Food matrix interactions with the essential oils or their constituents can be investigated by measuring the growth of microorganisms in culture medium containing a range of concentrations of fat, protein, or starch as well as the antimicrobial compound of interest. Such experiments have been performed using a so-called food model media (Gutierrez et al., 2009), and can be used to provide quick answers to which kind of food products the compound in question can be used in.

The intense aroma of essential oils, even low concentrations, can cause negative organoleptic effects exceeding the threshold acceptable to consumers (Lv et al., 2011). Having to increase the concentration of essential oils to compensate for their interactions with food matrix components is therefore highly unfortunate and limits their application to spicy foods where the acceptable sensory threshold is relatively high. Different strategies can be used to circumvent this problem. One option is to use essential oils in active packaging rather than as an ingredient in the product itself. Essential oils can be encapsulated in polymers of edible and biodegradable coatings or sachets that provide a slow release to the food surface or to the headspace of packages of, e.g., fruit, meat, and fish (Pelissari et al., 2009; Sánchez-González et al., 2011). Sachets that release volatile essential oils into the headspace environment are simply placed within an enclosed food package (Ahvenainen, 2003). The advantage of incorporating volatile components of essential oils in films or edible coatings is that the diffusion rate of the agents away from the food product can be reduced,

thereby maintaining the active compounds in the headspace or on the product surface for extended periods of time (Phillips and Laird, 2011; Sánchez-González et al., 2011). A way to minimize organoleptic effects of essential oils added to the matrix of a food product is to encapsulate essential oils into nanoemulsions. This approach increases the stability of volatile components, protecting them from interacting with the food matrix, and increases the antimicrobial activity due to increased passive cellular uptake (Donsí et al., 2011).

Lowering the concentration of essential oils without compromising their antimicrobial activity can also be obtained by applying them in combination with other antimicrobial compounds that provide a synergistic effect (Nguefack et al., 2012). Synergies are known to occur for essential oil combinations, and it is therefore a field with countless opportunities to find potent antimicrobial blends, which may be the key to implementing essential oils in food preservation without simultaneous organoleptic effects.

SYNERGIES BETWEEN ESSENTIAL OIL COMPONENTS

The interaction between antimicrobials in a combination can have three different outcomes, synergistic, additive, or antagonistic. Synergy occurs when a blend of two antimicrobial compounds has an antimicrobial activity that is greater than the sum of the individual components. An additive effect is obtained when the combination of antimicrobials has a combined effect equal to the sum of the individual compounds. Antagonism occurs when a blend of antimicrobial compounds has a combined effect less than when applied separately (Davidson and Parish, 1989; Burt, 2004).

The combined effect of a blend is analyzed by using measurements of the MIC to calculate the fractional inhibition concentration index (FIC_{Index}) according to the formulas defined by (Davidson and Parish, 1989): $FIC_A = MIC_{A+B}/MIC_A$, $FIC_B = MIC_{B+A}/MIC_B$, $FIC_{Index} = FIC_A + FIC_B$. The MIC_{A+B} value is the MIC of compound A in the presence of compound B, and vice versa for MIC_{B+A}. Calculating the FIC value for either substance A or B then requires determination of the MIC for the individual components. Theoretically, a FIC_{Index} near 1 indicates additive interactions, while below 1 implicates synergy, and above 1 antagonism (Davidson and Parish, 1989). However, this definition has been replaced by a more general one where the FIC_{Index} results are interpreted as synergistic if $FIC_{Index} < 0.5$, additive if $0.5 < FIC_{Index} < 4$, or antagonistic if $FIC_{Index} > 4$ (Odds, 2003).

The antimicrobial activity of a given essential oil may depend on only one or two of the major constituents that make up the oil. However, increasing amounts of evidence indicate that the inherent activity of essential oils may not rely exclusively on the ratio in which the main active constituents are present, but also interactions between these and minor constituents in the oils. Various synergistic antimicrobial activities have been reported for constituents or fractions of essential oils when tested in binary or ternary combinations (Delaquis et al., 2002; Pei et al., 2009; García-García et al., 2011; Nguefack et al., 2012). For example, García-García et al. (2011) found the most synergistic binary combination against *L. innocua* to be carvacrol and thymol, and the most active ternary combination to be carvacrol, thymol, and eugenol. Reports on greater antimicrobial activity of crude essential oils compared to blends of their major individual components suggests that trace components in the crude essential oils are critical to the activity and may have a synergistic effect (Marino et al., 2001; Delaquis et al., 2002; Burt, 2004; Koutsoudaki et al., 2005). In contrast to this, trace components may also cause antagonistic interactions, which were seen by comparing the antimicrobial effect of pure carvacrol to oregano oil where carvacrol is a major constituent. Pure carvacrol was 1500 times more effective than the crude essential oil (Rao et al., 2010). Among individual essential oil constituents, synergy has been observed for carvacrol and *p*-cymene on *B. cereus* (Ultee et al., 2002; Rattanachaikunsopon and Phumkhachorn, 2010). It appears that *p*-cymene swells bacterial cell membranes, probably enabling easier entrance of carvacrol into the cell membrane where it exerts its action (Ultee et al., 2002). Furthermore, Bassolé et al. (2010) showed that if linalool or menthol was combined with eugenol it showed the highest synergy, suggesting that a monoterpenoid phenol combined with a monoterpenoid alcohol is an effective combination.

Little is currently known about what governs synergy and antagonism among essential oil constituents. Four theoretical mechanisms of antimicrobial interactions produce synergy: (i) sequential inhibition several steps in a particular biochemical pathway, (ii) inhibition of enzymes that degrade of excrete antimicrobials, (iii) interaction of several antimicrobials with the cell wall, or (iv) interaction with the cell wall or membrane that leads to increased uptake of other antimicrobials (Davidson and Parish, 1989; Eliopoulos et al., 1996). Another possibility for synergistic effects could be that antimicrobials have different mode of actions, thereby attacking two different sites on or in the cell, which indirectly depend on each other. Even less is known about the cause antagonism, it is hypothesized to occur when: (i) combining bacteriostatic and bactericidal antimicrobials, (ii) antimicrobials have the same site of action, (iii) antimicrobials interact with each other (Davidson and Parish, 1989), Larson (1985) in Roller (2003). The hypothesized synergistic or antagonistic interactions are based on 15 year old results, and with the emergence of new techniques this field is likely to see some significant advances in our understanding of how antimicrobial compounds affect each other when acting in concert.

In practice, the knowledge needed to exploit synergistic combinations of essential oils in food products is (i) the site and mode of action of each essential oil constituent, and (ii) the mechanisms resulting in synergy or antagonism between several compounds, and (iii) how each compound interacts with food matrix components in a way that affects is antimicrobial properties. When the mechanistic details for synergistic interactions are better understood, it will be easier to exploit synergies using intelligent combinations of constituents to combat food spoilage microorganisms.

OUTLOOK

An attractive application of essential oils and their constituents is in food products to prolong the shelf-life of foods by limiting growth or survival of microorganisms. The organoleptic impact of essential oils and their components in food products currently limits their usage to spicy foods normally associated with herbs, spices, or seasonings. Synergistic interactions should therefore be

exploited to lower the organoleptic impact and thereby facilitate the use in a broader range of products.

The lack of detailed knowledge about the mode of action of the individual essential oil constituents is also the underlying cause for our superficial understanding about what governs synergy and antagonism. Future research should thus explore the mode of action of individual essential oil constituents further, while also initiating systematic investigations into the mechanisms of synergy among different constituents. Many studies have investigated the site of action, while few proceed to reveal the mode of action. Furthermore, most work to date has focused on prokaryotes, and little is known about how essential oils interact with yeast and fungi. Regardless of the microorganism, future research into the mode of action will need a standardization of investigation methods, complementary experiments that validate results, and implementation of new techniques. Taking a systems biology approach to investigating the mode of action of antimicrobial compounds will no doubt further this field. Transcriptomic and proteomic analyses can identify pathways targeted by an antimicrobial, whereas nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography, and computer modeling help identify key residues involved in the molecular interactions between target and the antimicrobial (Wang et al., 2006; Domadia et al., 2007; Liu et al., 2010; Schneider et al., 2010). Interactions with cell surface structures or cell membranes can be studied in detail by AFM force spectroscopy (Brasseur et al., 2008; Dufrêne, 2008). These techniques provide valuable information about an antimicrobial's specific intracellular targets, the structural nature of interaction, and what governs susceptibility, adaptation, and resistance mechanisms (Schneider et al., 2010).

Synergistic blends that have commercial interest must be evaluated under the relevant environmental conditions which reflect the food matrixes to which they should be applied, as interactions with food matrix ingredients could decrease their activity. Investigating the molecular interactions behind the inhibition of food matrix ingredients opens an entirely different research direction, which focuses on formulating essential oils in foods with the aim to minimize organoleptic effects without compromising the antimicrobial properties. Encapsulation and controlled/sustained release of potent synergistic combinations could potentially reduce the organoleptic impact and simultaneously increase the antimicrobial potency if the encapsulation material facilitates close interaction with the microorganisms. New strategies for nanoencapsulation may thus provide an interesting platform for this research avenue in the future.

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Conventional therapy and promising plant-derived compounds against trypanosomatid parasites

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Leishmaniasis and trypanosomiasis are two neglected and potentially lethal diseases that affect mostly the poor and marginal populations of developing countries around the world and consequently have an important impact on public health. Clinical manifestations such as cutaneous, mucocutaneous, and visceral disorders are the most frequent forms of leishmaniasis, a group of diseases caused by several *Leishmania* spp. American trypanosomiasis, or Chagas disease, is caused by *Trypanosoma cruzi*, a parasite that causes progressive damage to different organs, particularly the heart, esophagus, and lower intestine. African trypanosomiasis, or sleeping sickness, is caused by *Trypanosoma brucei* and is characterized by first presenting as an acute form that affects blood clotting and then becoming a chronic meningoencephalitis. The limited number, low efficacy, and side effects of conventional anti-leishmania and anti-trypanosomal drugs and the resistance developed by parasites are the major factors responsible for the growth in mortality rates. Recent research focused on plants has shown an ingenious way to obtain a solid and potentially rich source of drug candidates against various infectious diseases. Bioactive phytocompounds present in the crude extracts and essential oils of medicinal plants are components of an important strategy linked to the discovery of new medicines. These compounds have proven to be a good source of therapeutic agents for the treatment of leishmaniasis and trypanosomiasis. This work highlights some chemotherapeutic agents while emphasizing the importance of plants as a source of new and powerful drugs against these widespread diseases.

Keywords: leishmaniasis, Chagas disease, sleeping sickness, *Trypanosoma* spp., *Leishmania* spp., chemotherapy, medicinal plants, phytotherapy

INTRODUCTION

Leishmaniasis and trypanosomiasis are neglected tropical diseases caused by protozoans of the Trypanosomatid family, a diverse group of flagellated parasites that show similar cellular structures and undergo morphological alterations during their life cycles. The human diseases caused by trypanosomatids such as leishmaniasis, African trypanosomiasis (or sleeping sickness), and American trypanosomiasis (or Chagas disease) are transmitted by insects that affect 20 million people and cause 100,000 deaths per year, primarily in the tropical and subtropical areas of the world. In these regions, half of a billion people are at risk of infection (Stuart et al., 2008).

According to the World Health Organization (WHO), an ideal drug for the treatment of parasitic diseases should fulfill the following requirements: (i) parasitological cure in all the phases of the disease; (ii) effective in single or few doses; (iii) low cost for the patients; (iv) no collateral or teratogenic effects; (v) no need for hospitalization; and (vi) no induction of resistance. As described below, this ideal drug does not exist for the treatment of these parasitoses, and it will take a long time before such a drug is available. Additionally, because the vaccine approach has not produced satisfactory results in clinical trials, chemotherapy based on drugs that are not highly effective and cause side effects is the only treatment available for these maladies.

Considering the low number and efficacy of drugs available for the treatment of these diseases as well as their side effects and the resistance developed by parasites, the research in phytosciences, mainly regarding the properties of bioactive phytocompounds in the crude extracts and essential oils of medicinal plants, may lead to the discovery of new medicines with appropriate efficiency that are more accessible to the patients (Alviano and Alviano, 2009). Overall this review aims to provide a better understanding of the recent developments of the phytosciences to treat leishmaniasis

and trypanosomiasis neglected tropical diseases in terms of drug discovery and development in the world today.

LEISHMANIASIS, CHAGAS DISEASE, AND SLEEPING SICKNESS

Leishmaniasis is a generic term for diverse clinical manifestations including cutaneous, mucocutaneous, and visceral disorders caused by species of the genus *Leishmania*. All *Leishmania* species display similar morphologies and present two main developmental stages throughout their life cycles. The extracellular replicative forms, the promastigotes, are found in the gut of the insect vectors, the phlebotomines sandfly. The amastigote is the obligatory intracellular form observed in the mononuclear phagocytic system cells of vertebrate hosts. During blood feeding, the infected sandflies expel, together with saliva, a number of infective promastigotes found at the cardiac valve. Cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL) have a high impact in several countries of the world due to their morbidity and mortality rates. Many *Leishmania* species act as etiological agents of cutaneous and MCL; one of the most well known agents of the diffuse cutaneous form is *Leishmania amazonensis* and MLC forms is *L. brasiliensis*. On the other hand, *L. donovani* and *L. chagasi* are the only etiological agents of VL. The CL is characterized by lesions on the face, hands, and/or feet. MCL involves the nasal, oral, and pharyngeal mucosa and causes difficulty in eating as well as an increased risk of secondary infections that have a significant mortality rate (Murray et al., 2005; Piscopo and Mallia, 2007; David and Craft, 2009). The VL is characterized by fever, weakness, and fatigue are worsened by anemia, which is caused by a persistent inflammatory state, hypersplenism, and sometimes by bleeding. As the disease advances, splenomegaly and hepatomegaly can increase, causing abdominal distension and pain. These VL symptoms often persist for a long time before the patient either seeks medical care or dies from secondary infections, severe anemia, or organ failure (Chappuis et al., 2007; Piscopo and Mallia, 2007).

The life cycle of *Trypanosoma cruzi*, the etiological agent of Chagas disease, involves blood sucking by triatomine bugs that serve as insect vectors and a mammalian host where the parasite undergoes an obligate intracellular amastigote replicative form and an extracellular non-replicative trypomastigote form in the bloodstream. The epimastigote replicative form and a non-replicative infective metacyclic trypomastigote form are also observed in the insect vector. This disease is mainly transmitted by insect vectors feces, but transmission may also occur by blood transfusion, organ transplant, congenital and oral routes, and laboratory accidents. The prevalence and incidence of Chagas disease and the associated mortality are constantly changing as a consequence of vector control programs, rural-urban human migration, and changes in the socio-economic status of risk areas (Lewinsohn, 2003; Moncayo and Ortiz Yanine, 2006).

Despite the fact that most people infected with the parasite never appear to become symptomatic, Chagas disease presents in two stages: the acute stage, which appears shortly after the infection, and the chronic stage, which may last several years. In the acute phase, fever, lymphadenopathies, hepatomegaly, and splenomegaly may appear, as well as more severe problems, such as acute myocarditis and meningoencephalitis, which may be fatal in 2–8% of the cases in the absence of specific treatment (Moncayo and Ortiz Yanine, 2006; Develoux et al., 2009).

African trypanosomiasis or sleeping sickness occurs only in 36 countries in sub-Saharan Africa that contain vector insects of the genus *Glossina* (tsetse flies). Sleeping sickness is a serious public health problem in these countries because 55 million people are at risk of infection and there are an estimated 30,000 new cases annually. If left untreated, the disease is almost always fatal. Its etiologic agents are two subspecies of *Trypanosoma brucei*, *T. b. gambiense* in West Africa, and *T. b. rhodesiense* in East Africa, and the last is responsible for most severe form of the same disease. The trypanosomes of the *brucei* group primarily infect connective tissues. Hemolytic anemia occurs in the early stages of infection, followed by lymph node, spleen, and liver hypertrophy and cardiovascular and endocrine disorders. In the final phase of the infection, the parasites reach the nervous system and cause inflammatory reactions that lead to the meningoencephalitis associated with the clinical aspects of sleeping sickness (Gehrig and Efferth, 2008; Astelbauer and Walochnik, 2011).

CURRENT CHEMOTHERAPY AGAINST LEISHMANIASIS, CHAGAS DISEASE, AND SLEEPING SICKNESS AND IMPACT OF PARASITE RESISTANCE

Chemotherapy is the main tool used to control parasitic infections. The drugs available for these parasites control are often ineffective and sometimes life-threatening, adverse side effects, and some of these drugs require hospitalization (Croft et al., 2006; Ashutosh and Goyal, 2007; Piscopo and Mallia, 2007).

The drugs available for leishmaniasis infections are the pentavalent antimonial formulations of sodium stibogluconate (Pentostan) and *N*-methyl glucantime (Glucantime), which target the amastigotes of both CL and VL. Of the various proposed mechanisms of action for these two drugs, we can mention the production of ATP inhibition of the citric acid cycle and glycolysis by converting the pentavalent antimony the active trivalent antimony which is more active and more toxic, inhibition of oxidation of fatty acids in amastigotes and induction of apoptosis and inhibition of the enzyme DNA topoisomerase (Berman et al., 1987; Frézard et al., 2009). Variations in the clinical responses to these drugs have been a persistent problem in the treatment of leishmaniasis over the past 50 years due to intrinsic differences in species sensitivity and the development of resistance. When these drugs are ineffective or cannot be prescribed, treatment with amphotericin B, pentamidine, or paromomycin is indicated. However, as none of these drugs are free of adverse effects, the search for alternative therapeutic agents is essential. The combination of amphotericin B liposome is an alternative employed to reduce adverse effects, increasing the efficiency of the drug. As fluconazole and ketoconazole, azoles which initially were designed for the treatment of fungal infections, have been used for treating CL. Miltefosine has been recently approved as the first oral drug for VL. It yields cure rates of approximately 98% and is used to treat cases of resistance to antimoniates; however, it has been shown to induce parasite resistance *in vitro* (Croft et al., 2006; Santos et al., 2008; Goto and Lindoso, 2010; Tiuman et al., 2011).

Nifurtimox (Nif; Bayer 2502) and benznidazole (Bz; RO 7-1051) have been used for the treatment of Chagas disease since the end of 1960. The mechanism of action of Nif is based on a partial deficiency in the ability of *T. cruzi* to detoxify free radicals. Nif leads to the formation of nitro anion radicals that in turn produce highly toxic reduced oxygen metabolites. However, Nif is no longer commercially available. Instead of producing oxidative damage, the Bz mechanism of action might involve covalent modifications of parasite proteins, lipids, and DNA by nitro reduction intermediates (Coura and de Castro, 2002). The likelihood of curing Chagas disease with Nif and Bz varies according to the phase of the disease, the period of treatment, the dosage, and the age of the patient. Usually, satisfactory results are achieved when the patients are treated in the acute phase, in recent chronic infection, in congenital infection, and after laboratory accidents. The major limitation of these compounds is the low efficacy in the treatment of patients in the chronic phase of the disease (Coura and de Castro, 2002).

Treating infections with Nif may lead to collateral effects such as psychic alterations, anorexia, excitability, sleepiness, and digestive manifestations (nausea, vomiting, and diarrhea). Doses of Bz for Chagas disease treatment may cause hypersensitivity leading to dermatitis with cutaneous eruptions and generate neuropathologies such as paresthesia and polyneuritis of the peripheral nerves. However, the most serious reaction to Bz is the depression of bone marrow that leads to thrombocytopenic purpura and agranulocytosis. Due to these characteristic side effects, Nif and Bz should not be used by elderly or pregnant patients or by patients presenting any severe disease associated with Chagas disease, such as cardiac, respiratory, renal, or hepatic insufficiency, systemic infection, and neoplasia (Coura and de Castro, 2002).

The use of nanotechnology as liposomes, antibody conjugates, and nanoparticles as drug carriers can overcome anatomical barriers and take the drug directly to the site of action, reaching the target microorganisms, and reducing their side effects. Alternatively, amphotericin B can be used as a second line treatment in Chagas disease using nano-drug delivery systems (Yardely and Croft, 1999; Romero and Morilla, 2010). The E1224 azole compound, a prodrug transformed into ravuconazole *in vivo*, discovered and developed by Esai Pharmaceuticals had preclinical and clinical phase I studies completed. This promising compound for the treatment of Chagas disease, has been tested in phase II clinical studies in adult patients in Bolivia [Drugs for Neglected Diseases Initiative (DNDi), 2012]. Furthermore, the ravuconazol was evaluated extensively in animal models (Diniz et al., 2010).

The treatment for sleeping sickness depends on the stage of the disease. The drugs used in the first stage of the disease have a low toxicity and are easy to administer. Therefore, early identification results in an increased potential for curing the disease. Treatment success in the second stage of disease depends on a drug that can cross the blood-brain barrier to reach the parasite. Such drugs are toxic and complicated to administer. There are four drugs typically used for the treatment of sleeping sickness. Early infections are treated with pentamidine and suramin. Pentamidine is used for the treatment of the first stage of *T. b. gambiense* sleeping sickness. It has few undesirable effects and is generally well tolerated by patients. Suramin is used for the treatment of the first stage of sleeping sickness by *T. b. rhodesiense* but can cause allergic reactions and some undesirable effects in the urinary tract. In their final stages, both forms of infections are treated with melarsoprol, a drug derived from arsenic. This drug has many side effects, the most dramatic of which is reactive encephalopathy, which can be fatal, and kills up to 5% of patients. It is supposed that the mechanism of action is the interaction of the drug with a transporter specific for adenosine reducing the absorption of these nucleoside by the parasite and by the arsenic binding to glycerol 3-phosphate dehydrogenase, resulting in inhibition of glycolysis, and low levels of ATP (Denise and Barret, 2001). Another option is eflornithine, which is less toxic but only effective against *T. b. gambiense* infections. A combination treatment with nifurtimox and eflornithine was recently (2009) introduced, but is not effective for *T. b. rhodesiense* (Wilkinson and Kelly, 2009; Astelbauer and Walochnik, 2011; Jacobs et al., 2011).

One major drawback to the treatment of leishmaniasis, Chagas disease, and sleeping sickness is the emergence of resistance to current chemotherapeutics. Due to their high toxicity, drugs usually used for the treatment of these diseases have to be administered in low doses, allowing drug resistance to develop. Because there are few drugs available for the treatment of these parasitoses, resistance has a considerable impact on the control of these maladies.

The primary mechanism generally observed in parasite resistance is a decrease in the drug concentration within the parasite cell. The drug level may be lowered by a variety of mechanisms, including decreased uptake, increased efflux, and inhibition of drug activation and inactivation of active drug by the metabolism. In recent years, a large-scale increase in clinical resistance of *Leishmania* to pentavalent antimonials has been reported. The mechanisms of resistance of *Leishmania* spp. and *T. cruzi* against the chemotherapeutics developed in the field are not elucidated, and most of our knowledge stems from work on laboratory mutants (Nogueira et al., 2006; Ashutosh and Goyal, 2007).

The resistance mechanisms in *T. b. rhodesiense* and *T. b. gambiense*, recently reviewed by Gehrig and Efferth (2008), are frequently mediated by a reduced net drug uptake. Another drug resistance mechanism for melarsoprol is the overexpression of efflux pumps (Mäser et al., 2003).

A study using RNA interference in *T. brucei* showed the loss of function of a nitroreductase and an amino acid transporter necessary for activation of the prodrug nifurtimox and acquiring of eflornithine, respectively, showing the mechanisms of resistance to these two drugs (Baker et al., 2011).

PLANTS AS PROMISING SOURCES OF ANTI-LEISHMANIAL AND ANTI-TRYPANOSOMAL COMPOUNDS

Considering the lack of vaccines, the toxicity of the chemotherapies, the side effects of the treatment, and the resistance of the parasites to the drugs, there is an evident need to discover drugs that can be used as therapeutics for leishmaniasis, sleeping sickness, and Chagas disease. In recent years, many pharmacologically active and microbicidal compounds derived from plants have been discovered (Cowan, 1999; Alviano and Alviano, 2009; Izumi et al., 2011), indicating that phytoscience is important in the search of novel compounds with a potential to control these diseases.

Table 1 | Plants and identified antiprotozoal bioactive phytocompounds.

Scientific name	Major components	Effects	Parasites	Reference
Drymis brasiliensis	Polygodial	Cell proliferation (promastigotes), Cell viability (trypomastigotes), mitochondrial changes, nuclear changes, plasma membrane damages (promastigotes)	*L. amazonensis*, *L. braziliensis*, *L. chagasi*, *L. major*, *Trypanosoma cruzi*	Corrêa et al. (2011)
Baccharis retusa	Sakuranetin	Cell proliferation (promastigotes), Cell viability (trypomastigotes), activity in intracellular amastigotes	*L. amazonensis*, *L. braziliensis*, *L. chagasi*, *L. major*, *T. cruzi*	Grecco et al. (2012)
Ocimum gratissimum	Eugenol	Cell lysis e proliferation (promastigotes), mitochondrial changes, stimulation of NO production in macrophages	*L. amazonensis* *Trypanosoma brucei rhodesiense*	Ueda-Nakamura et al. (2006), Abiodun et al. (2012)
Croton cajucara	Linalool, acetyl aleuritolic acid	Mitochondrial changes (promastigotes), activity in intracellular amastigotes. Cell viability (trypomastigotes), activity in intracellular amastigotes, mitochondrial, and kinetoplast changes (epimastigotes)	*L. amazonensis, T. cruzi*	Rosa et al. (2003), Campos et al. (2010)
Piper claussenianum	Trans-nerolidol	Cell proliferation, activity in intracellular amastigotes, increased NO production in infection macrophages, decreased arginase activity of the parasite	*L. amazonensis*	Marques et al. (2010, 2011)
Lippia alba	Geranial, neral, geraniol, and trans-β-caryophyllene	Cell proliferation (epimastigotes) and intracellular amastigotes activity	*T. cruzi*	Escobar et al. (2010)
Lippia origanoides	Oxygenated monoterpenes	Cell proliferation (promastigotes)	*L. chagasi*	Escobar et al. (2010)
Moringa stenopetala	Benzyl-isothiocyanate	Cell proliferation (bloodstream forms)	*Trypanosoma brucei brucei*	Nibret and Wink (2010)
Hagen abyssinica	Ledol	Cell proliferation (bloodstream forms)	*T. b. brucei*	Nibret and Wink (2010)
Leonotis ocymifolia	Caryophyllene oxide	Cell proliferation (bloodstream forms)	*T. b. brucei*	Nibret and Wink (2010)
Cupania cinerea	Cupacinoside, taraxerol	Cell proliferation (bloodstream forms)	*T. b. rhodesiense*	Gachet et al. (2011)
Kola acuminata	Proanthocyanidin	Cell proliferation (bloodstream forms and procyclic forms), cell lysis and changes in plasma membrane	*T. brucei* (clone221a)	Kubata et al. (2005)
Salvia hydrangea	Salvadione, perovskone	Cell proliferation (bloodstream forms)	*T. b. rhodesiense*	Farimani et al. (2011)
Carlina acaulis	*Carlina* oxide (polyacetylene)	Cell proliferation (bloodstream forms)	*T. b. brucei*	Herrmann et al. (2011)
Syzygium aromaticum	Eugenol	Cell proliferation and viability (epimastigotes and bloodstreams trypomastigotes) and loss of nuclear content, and masses of condensed chromatin (trypomastigotes)	*T. cruzi*	Santoro et al. (2007)
Ocimum basilicum	Linalool	Cell proliferation and viability (epimastigotes and bloodstreams trypomastigotes), cytoplasmic extraction and nuclear alteration (epimastigotes)	*T. cruzi*	Santoro et al. (2007)
Achillea millefolium	Chamazulene	Cell proliferation and viability (epimastigotes and bloodstream trypomastigotes)	*T. cruzi*	Santoro et al. (2007)

(Continued)

Table 1 | Continued

Scientific name	Major components	Effects	Parasites	Reference
Zanthoxylum chiloperone	Chantin-6-one	Cell lysis (bloodstream trypomastigotes), anti amastigotes activity and *in vivo* activity in infected mice	*T. cruzi*	Ferreira et al. (2011)
Centaurea salmantica	Cynaropicrin	Cell proliferation (bloodstream trypomastigotes), *In vivo* activity (reduction of parasitemia)	*T. b. rhodesiense, T. b. gambiense*	Zimmermman et al. (2012)
Lippia sidoides	Thymol	Cell proliferation, accumulation of lipid droplets, wrinkled, or ruptured membranes and the loss of cytoplasm (promastigotes)	*L. amazonensis*	Medeiros et al. (2011)
Cymbopogon citratus	Citral	Cell proliferation, ultrastructural alterations like mitochondrial and kinetoplast swelling and disruption of nuclear membrane, loss of mitochondrial membrane potential and other alterations	*L. infantum, L. major, L. tropica*	Machado et al. (2012)
Ambrosia scabra	Psilostachyin C	Cell proliferation (epimastigotes and promastigotes), ultrastructural changes, anti amastigotes activity, *In vivo* activity (reduction of parasitemia)	*T. cruzi, L. amazonensis, L. mexicana*	Sülsen et al. (2011)
Chamomilla recutita	(−) α-bisabolol	Cell proliferation (promastigotes)	*L. infantum*	Morales-Yuste et al. (2010)
Xanthium strumarium	Xanthatin	Cell proliferation (bloodstream forms), mitochondrial membrane potential reduction, trypanothione reductase inhibition	*T. b. brucei*	Nibret et al. (2011)
Saussurea costus	Sesquiterpene lactones	Cell proliferation (bloodstream forms)	*T. b. rhodesiense*	Julianti et al. (2011)
Piper aduncum	2′,6′-dihydroxy-4′-methoxychalcone	Cell proliferation (promastigotes), mitochondrial damage, anti-intracellular amastigote activity	*L. amazonensis*	Torres-Santos et al. (1999)
Piper rusbyi	Kavapyrone, Flavokavain	Cell proliferation (promastigotes) *In vivo* activity (lesion size reduction)	*L. amazonensis, L. braziliensis, L. donovani*	Flores et al. (2007)
Piper auritum	Safrole	Cell proliferation (promastigotes), anti-intracellular amastigote activity	*L. major, L. mexicana, L. braziliensis, L. donovani*	Monzote et al. (2010)
Piper regnellii	Eupomatenoid-5	Cell proliferation (promastigotes and axenic amastigotes), anti-intracellular amastigotes Ultrastructural alteration and lipoperoxidation in the cell membrane (epimastigotes and bloodstream forms)	*L. amazonensis Trypanosoma cruzi*	Vendrametto et al. (2010), Pelizzaro-Rocha et al. (2011)
Aframomum sceptrum	Sceptrumlabdalactone B	Cell proliferation (promastigotes and bloodstream forms)	*L. donovani T. b. brucei*	Cheikh-Ali et al. (2011)

In fact, promising results have been obtained by our group and others in the search of plant-derived crude extracts, essential oils, and compounds with activity against pathogenic microorganisms, including *Leishmania* spp., *T. cruzi* (Alviano and Alviano, 2009; Izumi et al., 2011), and *T. brucei* (Gehrig and Efferth, 2008). Useful antiprotozoal phytocompounds can be divided into several categories summarized in **Table 1**.

ANTI-LEISHMANIAL COMPOUNDS

Essential oils are known to possess a wide variety of hydrophobic compounds with antimicrobial potential. The ability to diffuse across cell membranes certainly gives to those molecules some advantage in targeting cellular components, being a valuable research option for the search of bioactive compounds (Bakkali et al., 2008). The *Ocimum gratissimum* essential oil and eugenol, its major component, was tested on the growth, viability, and

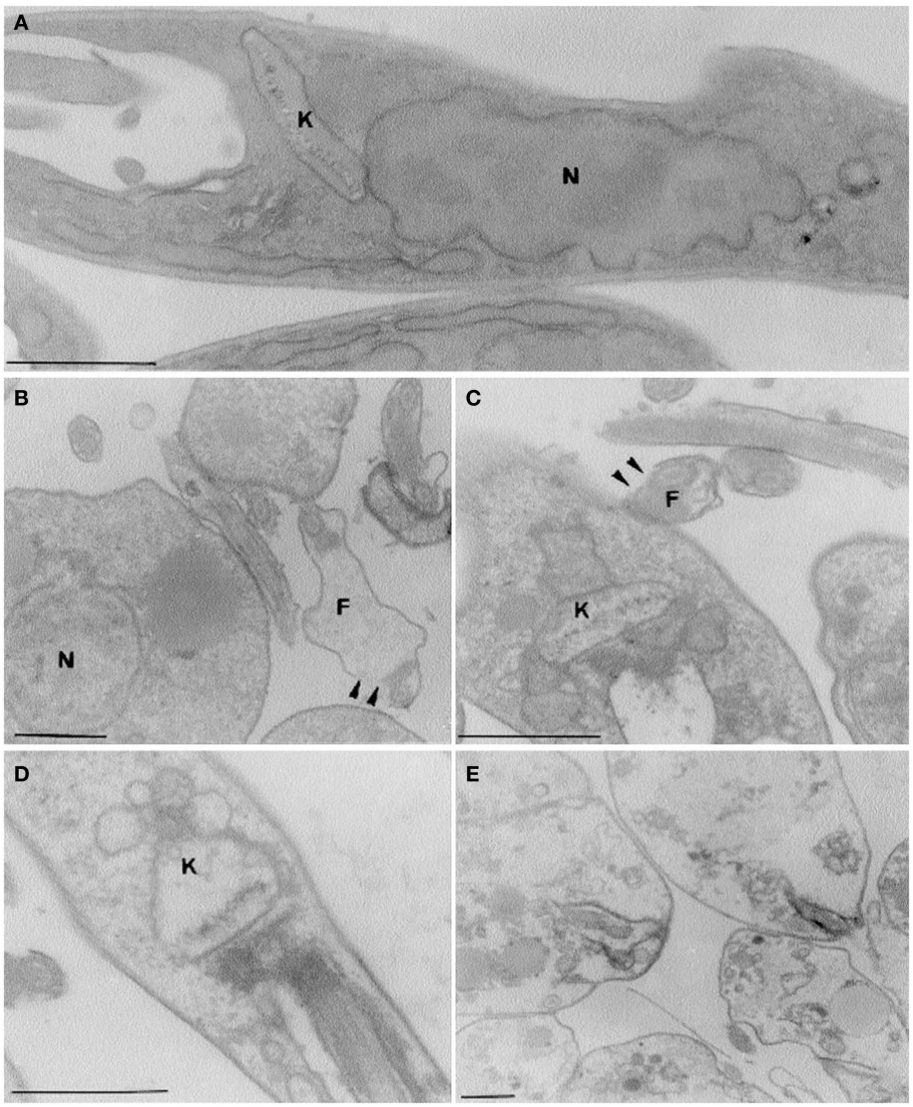

FIGURE 1 | Effects of linalool-rich essential oil (15.0 ng/ml) extracted from *C. cajucara* on the promastigote stage of *L. amazonensis*, as observed by transmission electron microscopy. (A) Control parasites; **(B–E)** parasites treated for 5 **(B)**, 10 **(C)**, 15 **(D)**, and 30 **(E)** min, showing promastigotes with different degrees of damage. Note the disruption of the flagellar membranes [arrowheads in **(B,C)**], the mitochondrial swelling **(C,D)**, and the gross alterations in the organization of the nuclear and kinetoplast chromatins **(C,D)**. In the presence of the essential oil, the parasites were completely destroyed after 30 min of treatment **(E)**. N, nucleus; K, kinetoplast, F, flagellum. Bars, 1 μm. Image reproduced with permission from © Rosa et al. (2003) American Society for Microbiology.

ultrastructural alterations of the amastigote and promastigote forms of *L. amazonensis*, as well as on the interaction of these flagellates with mouse peritoneal macrophages, concomitant with nitric oxide production stimulation by the infected macrophages. Significant mitochondrial alterations occurred at the ultrastructural level of the parasite, such as remarkable swelling, disorganization of the inner membrane, and an increase in the number of cristae after treatment of parasites with *O. gratissimum* essential oil. However, mouse macrophages were unaffected under the same conditions. In addition, nitric oxide production was dramatically stimulated when mouse peritoneal macrophages were treated with 150 μg/ml essential oil, both before and after infection with *L. amazonensis*. Concomitantly, the association indexes were drastically lower in the latter conditions, compared to the control system (Ueda-Nakamura et al., 2006).

The linalool-rich essential oil extracted from the leaves of *Croton cajucara*, has effects on *L. amazonensis* parasites, on the interaction of these flagellates with mouse peritoneal macrophages and on nitric oxide production by the infected macrophages. The median lethal doses and absolute lethal doses of the essential oil and linalool-rich essential oil from *C. cajucara* for promastigotes and amastigotes were very low. Mitochondrial swelling and alterations in the organization of the nuclear and kinetoplast chromatins were observed by electron microscopy when *L. amazonensis* parasites

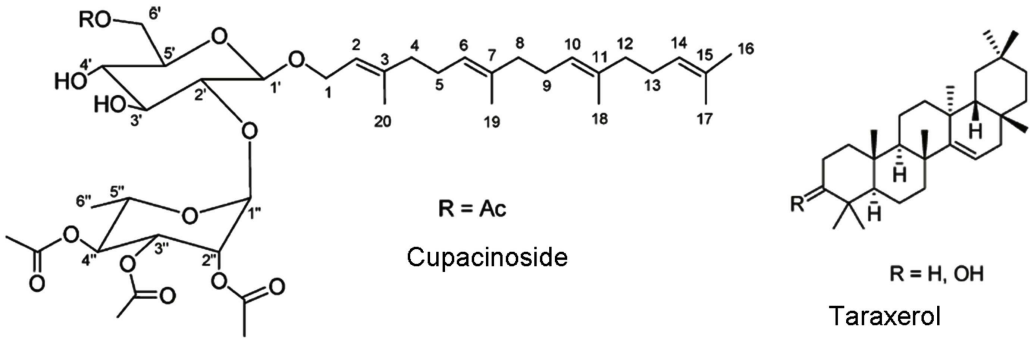

FIGURE 2 | Structure of cupacinoside and taraxerol. Reprinted (adapted) with permission from © Gachet et al. (2011) American Chemical Society.

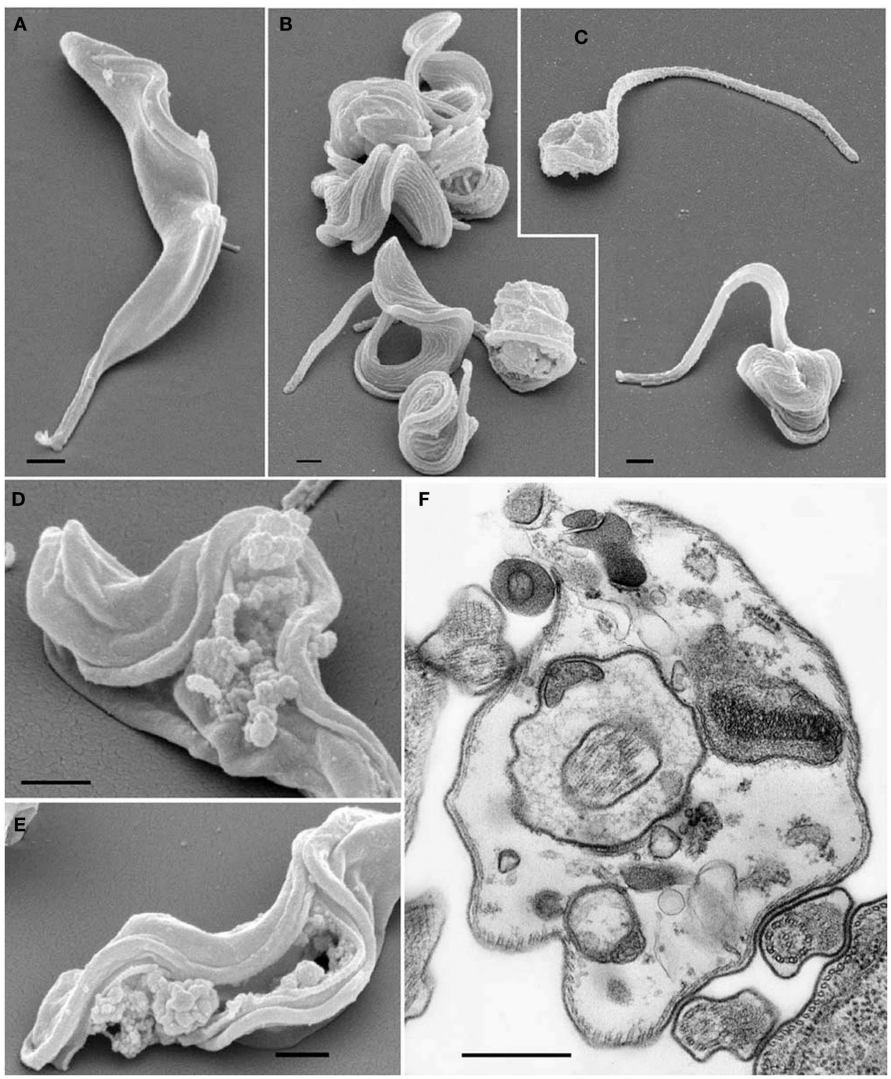

FIGURE 3 | Effects of *Kola acuminate* proanthocyanidin (50 μg) on bloodstream form trypanosomes observed by scanning and transmission electron microscopy. (A) Control parasites showing the normal cell morphology and membrane integrity. **(B,C)** Parasites treated for 6 h showing morphological changes and the rounding up of the cells. **(D,E)** Cells showing disintegrated cell membranes and loss of cytoplasmic contents caused by the effect of the drug. **(F)** TEM of a trypanosome cell confirming the necrotic process of cell membrane disintegration and loss of cytoplasmic contents that led to cell death. Scale bar: 1 μm. Image reproduced with permission from © Kubata et al. (2005) Elsevier.

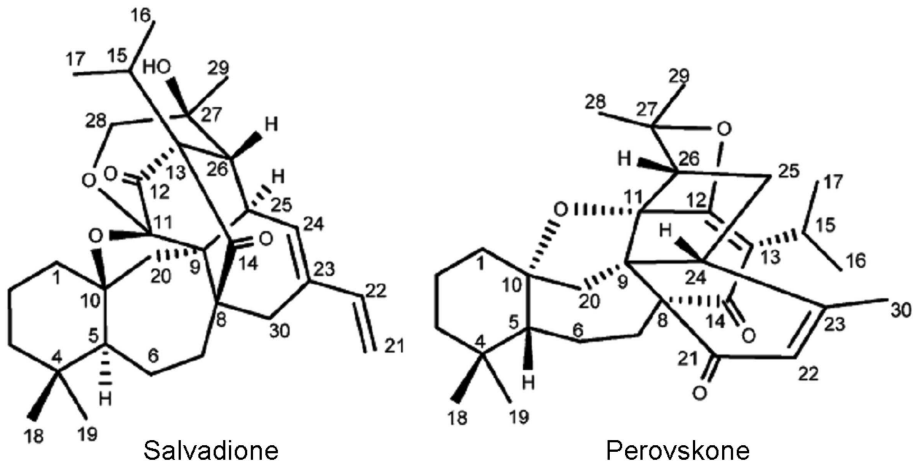

FIGURE 4 | Structure of salvadione and perovskone. Reprinted (adapted) with permission from © Farimani et al. (2011) American Chemical Society.

were treated with the essential oil from *C. cajucara* (**Figure 1**). The viability of mouse macrophages was unaffected by the same concentrations. When the macrophages were pre-treated with *C. cajucara* essential oil, as well as when the macrophages were pre-infected with the parasites and then treated with the essential oil, the association indexes were 50% lower than those for the control system. In addition, the macrophages pre-treated with *C. cajucara* essential oil produced twice the amount of nitric oxide as the untreated macrophages (Rosa et al., 2003).

Piper species have been reported to have activity against *Leishmania* parasites (Torres-Santos et al., 1999; Flores et al., 2007; Sarkar et al., 2008). Our group recently showed that the leaf essential oil from *Piper claussenianum* was able to inhibit the growth of *L. amazonensis* promastigotes with an IC_{50} of 0.0038% (Marques et al., 2010). Trans-nerolidol is a major component of this essential oil. We also examined the effect of the essential oil on the interaction between parasites and host cells and its activity against intracellular amastigotes. The IC_{50} concentration used against promastigotes yielded a 31% reduction in the percentage of infected macrophages and led to a 17% increase in the production of NO by macrophages. The activity of the enzyme arginase may be able to modulate nitric oxide production by macrophages by inhibiting nitric oxide synthase (iNOS). Promastigotes were grown in the presence of the IC_{50} of the essential oil and showed a 62% reduction in arginase activity. (Marques et al., 2011). Monzote et al. (2010) described the anti-leishmanial activity of the essential oil from *Piper auritum*. In that study, essential oil inhibited the growth of promastigotes in all species of *Leishmania* used, with IC_{50} values between 12.8 and 63.3 μg/mL. In addition, piper-oil inhibited the growth of intracellular amastigotes of *L. donovani* at non-toxic concentrations (Monzote et al., 2010).

ANTI-TRYPANOSOMIAL COMPOUNDS

One new diterpene glycoside, cupacinoside, and one known compound, taraxerol (**Figure 2**) from *n*-hexane, and dichloromethane extracts from the bark of *Cupania cinerea* (Sapindaceae) have shown significant *in vitro* activity against one of the etiologic agents of sleeping sickness. Cupacinoside and taraxerol showed IC_{50} values $<10\,\mu M$ against *T. b. rhodesiense*, with taraxerol exhibiting only low cytotoxicity against rat skeletal myoblast cell line (L-6 cells; Gachet et al., 2011).

A proanthocyanidin isolated from *Kola acuminate* seeds was able to inhibit the proliferation and cause the lysis of *Trypanosoma brucei* bloodstream forms *in vitro*. The *in vivo* effect was trypanostatic and prolonged the survival of infected animals that were treated with this substance. Additionally, it was not toxic to the human epidermoid carcinoma cells (KB 3-1), but it caused ultrastructural changes in parasites, such as rupture of the plasma membrane and vesicles and the formation of multivesicular bodies in lysosome-like organelles, when the parasites were treated for 6 h with the compound (**Figure 3**; Kubata et al., 2005).

Salvadione and perovskone (**Figure 4**), two new triterpenoids with rare carbon skeletons isolated from aerial parts and flowers of *Salvia hydrangea* were used in the treatment of leishmaniasis in Iran. These compounds were tested *in vitro* against *T. b. rhodesiense* and exhibited moderated potency with IC_{50} values 4.33 and 15.92 μM, respectively and good selectivity index for L-6 cells (Farimani et al., 2011).

A study using essential oils of cloves (*Syzygium aromaticum*), basil (*Ocimum basilicum*), and a yarrow (*Achillea millefolium*) and the main constituents, eugenol and linalool showed activity on *T. cruzi* bloodstream trypomastigotes and epimastigotes forms. The essential oils inhibited epimastigotes proliferation, caused trypomastigotes lysis and ultrastructural changes in both forms, mainly in the nucleus. In epimastigotes forms was observed shrinkage of the nuclear material with separation of the nuclear membrane and in trypomastigotes forms loss nuclear material and masses of condensed chromatin appeared (Santoro et al., 2007).

Acetyl aleuritolic acid, a terpene isolated from the methanolic extract of stem bark of *C. cajucara* showed significant trypanocidal effect in trypomastigotes of a strain isolated from wild reservoirs (GLT291), genotype TCI as clone Dm28c. was also effective against intracellular amastigotes and did not show a

significant effect on proliferative epimastigotes. This compound was also able to inhibit the activity of trypanothione reductase, an important enzyme in the regulation of redox balance and defense against oxidative stress in this parasite (Campos et al., 2010). Xanthatin, a sesquiterpene lactone, was described by Nibret et al. (2011) as strong trypanocidal agent with an IC_{50} value of 2.63 μg/mL. According to the authors, it seems that the biological activity of the compound is a result of its effect in inducement of apoptosis in trypanosomes as evidenced by a reduction in mitochondrial membrane potential. Furthermore, xanthatin was able to inhibit the two key enzymes involved in the inflammatory process, cyclooxygenase and 5-lipoxygenase, which can be very interesting in diseases that cause this kind of response (Nibret et al., 2011).

CONCLUSION

At a time when there is an urgent need for the efficient treatment of leishmaniasis, Chagas disease, and sleeping sickness, this review shows that plant-derived extracts and compounds exhibit significant antiprotozoal activities *in vitro* and emphasizes the importance of phytoscience in the search for novel anti-leishmanial and anti-trypanosomal therapeutic agents.

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The antimicrobial defense of the Pacific oyster, *Crassostrea gigas*. How diversity may compensate for scarcity in the regulation of resident/pathogenic microflora

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Healthy oysters are inhabited by abundant microbial communities that vary with environmental conditions and coexist with immunocompetent cells in the circulatory system. In *Crassostrea gigas* oysters, the antimicrobial response, which is believed to control pathogens and commensals, relies on potent oxygen-dependent reactions and on antimicrobial peptides/proteins (AMPs) produced at low concentrations by epithelial cells and/or circulating hemocytes. In non-diseased oysters, hemocytes express basal levels of defensins (*Cg*-Defs) and proline-rich peptides (*Cg*-Prps). When the bacterial load dramatically increases in oyster tissues, both AMP families are driven to sites of infection by major hemocyte movements, together with bactericidal permeability/increasing proteins (*Cg*-BPIs) and given forms of big defensins (*Cg*-BigDef), whose expression in hemocytes is induced by infection. Co-localization of AMPs at sites of infection could be determinant in limiting invasion as synergies take place between peptide families, a phenomenon which is potentiated by the considerable diversity of AMP sequences. Besides, diversity occurs at the level of oyster AMP mechanisms of action, which range from membrane lysis for *Cg*-BPI to inhibition of metabolic pathways for *Cg*-Defs. The combination of such different mechanisms of action may account for the synergistic activities observed and compensate for the low peptide concentrations in *C. gigas* cells and tissues. To overcome the oyster antimicrobial response, oyster pathogens have developed subtle mechanisms of resistance and evasion. Thus, some *Vibrio* strains pathogenic for oysters are equipped with AMP-sensing systems that trigger resistance. More generally, the known oyster pathogenic vibrios have evolved strategies to evade intracellular killing through phagocytosis and the associated oxidative burst.

Keywords: innate immunity, invertebrate, mollusk, host pathogen interaction, mode of action, selective pressure, molecular diversity, antimicrobial peptide

INTRODUCTION

The Pacific oyster *Crassostrea gigas* is a marine invertebrate belonging to the family *Ostreidae* (Mollusca, Bivalvia) with a worldwide distribution from Japan to occidental countries in Europe and America (Guo et al., 2008). Oysters are filter-feeders cultured in bays, lagoons, and estuaries (Buestel et al., 2009; Dumbauld et al., 2009) where they are exposed to abundant and changing microbial communities. To date, the oyster-associated microflora, which in some regions of the world includes human pathogens, has been essentially studied for food safety purposes. Although the microbial communities associated to healthy oysters remain poorly studied, they were shown to be abundant and to vary with environmental conditions. This abundance in non-diseased animals could be relevant of a protective function and raises several questions on oyster biology, immunity, and homeostasis: Does an oyster-specific microbial community exist? How does the oyster microflora coexist with immunocompetent cells in the circulatory system? How can the oyster immune system discriminate between pathogens and commensals? In invertebrates, pathogen recognition, and disease control are mediated by an innate immune system, in which reactive oxygen species (ROS) and antimicrobial peptides play a key defense function (Lemaitre and Hoffmann, 2007). In this review, we give a brief summary on oyster environmental and resident microflora and present a state of the art on the antimicrobial defense of *C. gigas* oysters, its regulation, diversity, and its molecular effectors, with a special focus on the antimicrobial peptides and proteins.

OYSTERS AND THE ENVIRONMENTAL MICROFLORA

The health status of marine organisms is uniquely related to their immediate environments, which can contain very high concentrations of microorganisms. When conditions become favorable for multiplication, both the saprophytic and pathogenic microorganisms from their environment are capable of infecting marine organisms (Ellis, 2001). However, under normal conditions, marine invertebrates like oysters maintain a healthy status

by defending themselves against these potential invaders using a repertoire of innate defenses (Bachère et al., 2004; Schmitt et al., 2012b).

THE ENVIRONMENTAL MICROFLORA IN LAGOONS AND ESTUARIES

Human activities are concentrated along the coasts worldwide. As a result, marine coastal systems are under increasing anthropic and environmental pressures. The high densities of human populations along the coasts as well as the human exploitation of the sea, lagoons, and estuaries for recreational activities, fishing, and shellfish farming are important factors that modify marine coastal systems. Moreover, due to the shallow water of these systems, they are highly impacted by seasonal variations and long term climate changes. Thus, both drastic and rapid changes are observed in sea water temperature, salinity, oxygenation, sunlight, pH, and content in organic matter. Those variations, which occur both temporarily and spatially, influence the viability/culturability, the abundance, and the ecology of bacteria (Pruzzo et al., 2005).

For instance, changes in the microflora of lagoons resulting from human activities have been observed following major rain events with a massive input in fecal coliforms and streptococci. It was shown that floods massively drained bacteria entrapped in the sediment of coastal rivers down to the lagoons (Chu et al., 2011). Additional sources of bacterial loads during rainfall events were also attributed to overflow from sewage systems and the failure of septic tanks (Al Bakri et al., 2008; Astrom et al., 2009).

Such natural events not only modify the abundance but also the composition of the coastal microflora by favoring the development of natural inhabitants of coastal sea waters better adapted to a given salinity or temperature. Thus, vibrios, which are autochthonous in riverine, estuarine, and coastal waters throughout both temperate and tropical regions of the world, are highly dependent on environmental parameters such as temperature, salinity, concentration of organic matter and the presence of chitinous organism (e.g., zooplankton), which are in turn controlled by larger-scale climate variability (Lipp et al., 2002). For instance, the prevalence of the human pathogen *Vibrio cholerae* is inversely correlated with salinity (Jiang, 2001), and a statistically significant relationship could be established between the time series for cholera and rainfall anomalies (Constantin de Magny et al., 2008).

Numerous surveys of coastal marine systems have shown that temperature is favoring the spread of vibrios including species pathogenic for marine invertebrates. Thus, as a consequence of ocean warming, *Vibrio*-associated diseases have increased causing mass mortalities in both wild and farmed species of marine invertebrates such as corals (Vezzulli et al., 2010), abalone (Travers et al., 2009), and oysters (Garnier et al., 2007). It is still difficult to determine how far the increase of the *Vibrio* load in marine coastal water contributes to animal disease. Indeed, environmental factors such as temperature also modulate the virulence status of bacteria (Vezzulli et al., 2010) and the physiological status of the animal species cultured in these systems, in particular oysters, whose reproductive status is dependent on an elevated sea water temperature.

OYSTER COMMENSAL MICROFLORA AND HOMEOSTASIS

Animals including marine invertebrates carry numerous species of bacteria and it is becoming increasingly clear that most of these microorganisms constitute a host-specific community. However, we understand relatively little about the mechanisms involved in the reciprocal host–bacterial signaling because of the multiple interactions existing between and among the typically multispecies microbial consortia of animals (Ruby, 1999).

As filtering animals, oysters need high volumes of water for their nutrition and respiration. Consequently, they are in continuous contact with abundant microorganisms from their marine environment, which include both pathogens and commensals. Generally, oyster body surfaces harbor a dense and diverse natural microbiota, dominated by Gram-negative bacteria of the genera *Pseudomonas* and *Vibrionaceae* (Ortigosa et al., 1994). Like the human gut, oyster bacteria-enriched epithelia offer a naturally competitive environment that can prevent the establishment of potential pathogens (Salzman et al., 2007). Surprisingly, in healthy oysters, many organs inside the body, including the hemolymph, are also populated by a natural microbiota. Among others, it is composed of species from the genus *Vibrio*, *Pseudomonas*, *Flavobacterium*, *Cytophaga*, *Bacillus*, and *Micrococcus* (Lauckner, 1983; Paillard et al., 2004).

The existence of a host-specific microflora in oysters is still questioned. The microflora associated to oysters varies according to the environmental microflora. Thus, depuration, which is used after collection and prior to commercialization of oysters, results in a major decrease of coliforms and other transient residents of oyster tissues. However, bacterial communities in oyster hemolymph and surrounding sea water display different composition (de Lorgeril et al., unpublished data), indicating the existence of communities better adapted to oyster hemolymph (see below). Among these, the *Vibrio* species, which have a long co-evolution story with marine invertebrates resist rather efficiently to depuration procedures of edible bivalves, having a worldwide incidence on *Vibrio*-related seafood-borne diseases (Pruzzo et al., 2005). Pruzzo et al. (2005) therefore proposed that this resistance to depuration could indicate that vibrios belong to the persistent microflora of oysters.

Homeostasis is the maintenance of a stable state within the body in response to a changing external environment. Without homeostasis, no life form could adapt to their environment. Therefore, maintaining this equilibrium is essential for any organism to remain healthy and stay alive. As mentioned above, the oyster environment is subjected to drastic and rapid changes. Therefore, oysters must have an efficient system to maintain homeostasis. Oyster homeostasis is maintained through layered defenses. The first line of defense is determined by physical protective barriers that prevent damage of the underlying tissues and infections by pathogenic microorganisms. The main physical barriers are the shell and mucus (Glinski and Jarosz, 1997). However, when pathogens breach these protective barriers, they must confront the oyster immune response. For this, oysters have developed complex mechanisms of recognition and immune regulation to discriminate advantageous microorganisms from pathogenic ones, control infections, and maintain homeostasis.

It is generally assumed that the circulatory system of healthy animals is sterile, whereas the presence of bacteria is considered as infection. The abundant microflora of healthy oysters is therefore puzzling. The coexistence of immunocompetent cells

and microorganisms in the circulatory system of oysters appears intriguing and impressive. It has undoubtedly been decisive for the evolution of the oyster immune system and its interaction with pathogens. One can assume that the oyster microflora plays a protective role by stimulating oyster immunity and/or competing with potential pathogens, and is therefore essential for the maintenance of homeostasis. The production of antimicrobial peptides (bacteriocins) is widespread in bacteria associated to marine organisms (Desriac et al., 2010). Together with the host antimicrobial defenses, which themselves are enhanced upon pathogen recognition (see immune responses section), antimicrobials from bacterial origin likely have a protective effect against pathogens. Thus, as in corals, a dynamic relationship between commensal microorganisms and oysters could select for a holobiont (host and associated microorganisms) better suited for resistance to specific pathogens. This probiotic hypothesis has been referred to as the hologenome theory of evolution (Rosenberg et al., 2007).

INFECTIOUS AGENTS AND DISEASES IN OYSTERS

While oyster-associated microorganisms can be significantly beneficial for the maintenance of oyster homeostasis, some can be highly virulent for given species of oysters resulting in massive mortalities or even the extinction of some oyster populations. Oyster pathogens include protozoans, viruses, and bacteria, which affect all stages of development, from larvae to juveniles and adult oysters (for review see Schmitt et al., 2012b).

Considering bacteria, the difficulties encountered in identifying the pathogenic ones are due to the normal accumulation of a very rich bacterial commensal microbiota composed of different species. Some of these bacteria, mainly opportunistic and pathogenic, eventually colonize and invade the host, depending on the environmental factors influencing host–bacteria interactions (Paillard et al., 2004). As an example, oyster mortalities are frequently associated to the presence of bacterial pathogens of the *Vibrio* genus, particularly strains of *V. splendidus* and *V. aestuarianus* (Le Roux et al., 2002; Garnier et al., 2008). However, it remains unclear whether these bacteria act as primary pathogens or as opportunists (Paillard et al., 2004). Besides, many vibrios potentially pathogenic to humans, inhabitants of coastal waters, are found in bivalves and can be transmitted to humans through their raw consumption. Those human pathogens do not cause disease in oyster and are likely normal residents of the oyster microflora. The ability of *Vibrio* species to persist within oyster tissues is responsible for their high concentration within this host that can act as an environmental reservoir of human pathogens (Pruzzo et al., 2005).

OYSTER IMMUNE DEFENSES

OYSTER IMMUNE CELLS

As marine invertebrates, oysters have an immune system that resembles the innate immune system of vertebrates (for review see Schmitt et al., 2012b). In oysters, both hemocytes (circulating blood cells) and surface epithelia display immune functions, expressing both non-self recognition receptors and immune effectors involved in the control of the resident/pathogenic microflora. Still, to date, most of the studies have been dedicated to the description of hemocyte immune functions.

Oysters possess a semi-open circulatory system where the blood, called hemolymph, flows largely through a system of sinuses. The hemolymph bathes the organs directly providing oxygen and nutrients, and is composed of fluid plasma and hemocytes. The circulatory system contributes to the hemocyte ability to migrate toward connective tissues (**Figure 1**). They migrate by diapedesis from blood sinuses and vessels to the surface body, particularly to surface epithelia such as mantle and gills (Cheng, 1996).

The classification of oyster hemocytes has been the subject of extensive studies since early 1970s. Based on different criteria (morphology, ultrastructure, physicochemical features, biological functions), they have been classified into main two cell types (granular and agranular cells), which are subdivided into several subgroups (Cheng, 1981; Hine, 1999; Bachère et al., 2004). Whereas granulocytes form a distinct group composed of both basophilic and eosinophilic granulocytes, agranular hemocytes are heterogeneous in appearance and ultrastructure, characterized by the absence or the presence of few cytoplasmic granules (Bachère et al., 1988). Three types of agranular hemocytes have been identified: blast-like cells, basophilic macrophage-like cells, and hyalinocytes. However, the distinction between types and/or lineages, the origin, maturation, and the life span of oyster hemocytes has never been demonstrated.

At present, there is considerable information about the role of hemocytes in oyster defense. Hemocytes are capable of non-self recognition, chemotaxis, and active phagocytosis (Cheng, 1981; Bachère et al., 2004). They are implicated in cytotoxic reactions by the production of hydrolytic enzymes (Cheng and Rodrick, 1975), ROS (Bachère et al., 1991a; Lambert et al., 2007) and antimicrobial peptides/proteins (Gueguen et al., 2006, 2009; Gonzalez et al., 2007a,b; Rosa et al., 2011). They also produce soluble factors to protect oyster against infections (Rinkevich and Muller, 1996; Pruzzo et al., 2005). Thus, as immunocompetent cells, infiltrating hemocytes are present in all cavities and tissues of oyster body where they can be able to phagocyte and to destroy infectious agents.

Although less studied for their immune functions, surface epithelial cells also express receptors dedicated to pathogen recognition (Itoh and Takahashi, 2008) as well as hydrolytic enzymes (Matsumoto et al., 2006; Itoh and Takahashi, 2007; Itoh et al., 2007, 2010a; Xue et al., 2010) and antimicrobial peptides/proteins involved in the control of infections (Gueguen et al., 2006; Gonzalez et al., 2007b).

IMMUNE RESPONSES

Recent advance in our knowledge of the oyster immune response has been made through the development of genomic-based approaches (Gueguen et al., 2003; Tanguy et al., 2004; Fleury et al., 2009; Lang et al., 2009; Roberts et al., 2009; Taris et al., 2009; de Lorgeril et al., 2011). The main defense mechanisms described in oysters have been recently reviewed in (Schmitt et al., 2012b). They include: (i) recognition of microbe/pathogen-associated molecular patterns (M/PAMPs) or damage-associated molecular patterns (DAMPs) by both soluble and cellular pattern recognition receptors/proteins (PRRs and PRPs), (ii) hemocyte signaling and activation, (iii) hemocytes mediated reactions (phagocytosis,

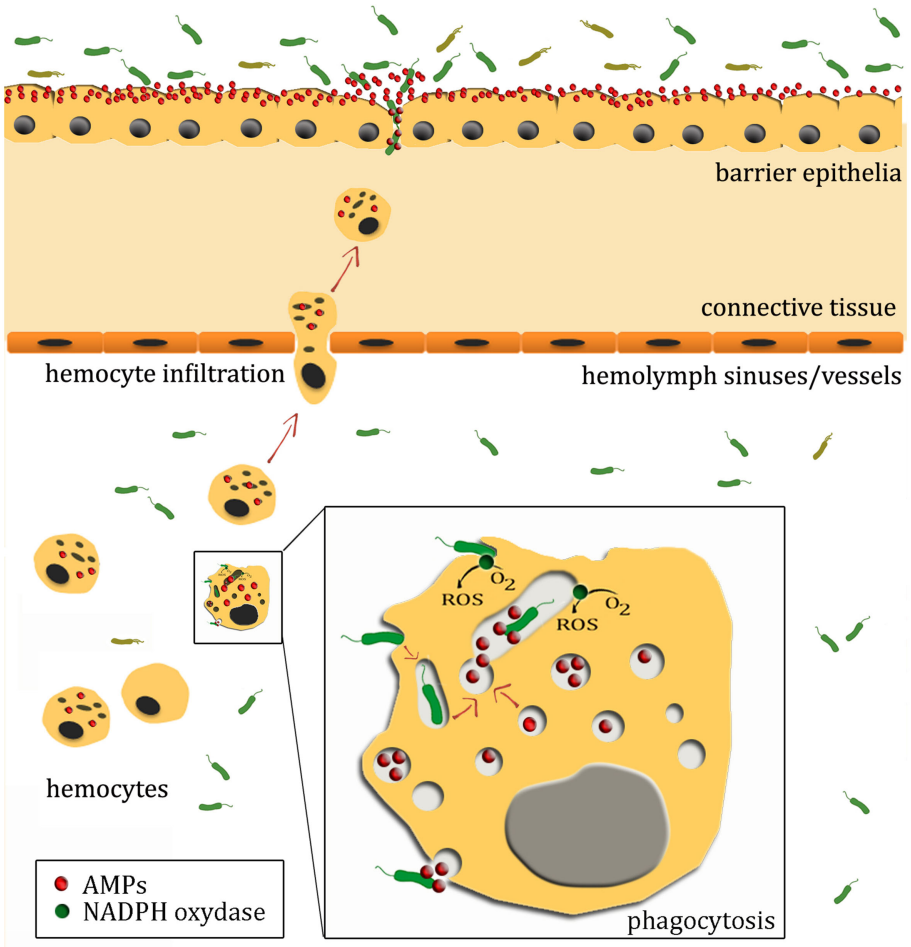

FIGURE 1 | A not-to-scale schematic illustration of oyster antimicrobial defenses. Oyster epithelial surfaces are in contact with an abundant microflora that can offer a competitive environment that prevents the establishment of potential pathogens. In healthy oysters, surface epithelia constitutively express AMPs, which also contribute to prevent invasion by pathogens. Upon wounding or infection, damage- or pathogen-associated molecular patterns trigger hemocyte infiltration to the site of infection. Infiltrating hemocytes serve as vehicles for AMP families that display synergistic activities with AMPs from epithelial surfaces. Upon recognition by pattern recognition receptors/proteins, microorganisms present in tissues and hemolymph can also be phagocytosed and further eliminated by the production of reactive oxygen species (ROS), hydrolytic enzymes, and AMPs. It is still unknown whether AMP-containing hemocyte granules fuse with the phagosome, undergo exocytosis or both.

encapsulation, and tissue infiltration), and (iv) production of reactive species of oxygen and nitrogen and host defense molecules (**Figure 1**).

Recognition is mediated by a series of PRPs and PRRs. In oysters, the known PRPs/PRRs include peptidoglycan recognition proteins (PGRPs), which recognize specifically peptidoglycans (PG) from bacteria (Itoh and Takahashi, 2008), carbohydrate-binding proteins such as the β-1,3-glucan binding protein (βGBP), which binds to β-1,3-glucans from fungi cell walls (Tanguy et al., 2004; Itoh and Takahashi, 2009) and lectins (gigalins, C-type lectins, ficolins, and galectins) (Olafsen et al., 1992; Gueguen et al., 2003; Tasumi and Vasta, 2007; Yamaura et al., 2008). Over expression of C-type lectins and a rhamnospondin-like (L-rhamnose-binding lectin) in *C. gigas* hemocytes has been associated to a successful immune response to an experimental infection with virulent *Vibrio* strains as compared to an infection with an avirulent *Vibrio* (de Lorgeril et al., 2011). Additionally to those classical PRPs/PRRs, other proteins were also described as participating in oyster non-self recognition by their LPS-binding properties. These include the major plasma protein Cu/Zn extracellular superoxide dismutase (*Cg*-EcSOD) and the antimicrobial protein *Cg*-BPI for bactericidal/permeability-increasing (BPI) protein that will be further discussed below (Gonzalez et al., 2005, 2007b).

Intracellular signaling cascades are initiated upon M/PAMP or DAMP recognition. Highly conserved in the animal kingdom, one major signaling cascade identified in oyster is the NF-κB pathway (for review see Schmitt et al., 2012b). In *Drosophila*, it plays a major role in the regulation of the antimicrobial response (Lemaitre and Hoffmann, 2007). In oysters, although many elements of the NF-κB pathway have been identified, its direct role in immunity remains unexplored. Signaling events induce several

defense responses, such as hemocyte activation, migration, and the expression of immune mediators that elicit defense reactions which coordinate the elimination of pathogens and infected cells. Some families of oyster antimicrobial peptides/proteins including big defensins and BPI protein are among the known effectors of oyster immunity that respond to a microbial challenge or an injury (see Regulation of AMP Expression in Healthy and Diseased Animals). Nonetheless, the signaling cascades controlling their gene expression are still unknown.

The control of pathogens relies on a combination of cellular and molecular defenses. Thus, following injury, hemocytes are recruited massively toward the sites of infection where they contribute to a local defense (Cochennec-Laureau et al., 2003; Bachère et al., 2004). Hemocyte migration leads to a local concentration of immune effectors likely to prevent host invasion (Schmitt et al., 2012a). May invasion occur, circulating hemocytes are capable of readily initiating phagocytosis as a major defense reaction in oyster (**Figure 1**). The crucial role of phagocytosis in *C. gigas* immune response has been highlighted by the overrepresentation of transcripts of phagocytosis-related genes in hemocytes of oysters surviving infections by virulent *Vibrio* strains as compared to oysters receiving the same doses of an avirulent *Vibrio* (de Lorgeril et al., 2011). Not surprisingly, phagocytosis is consequently either avoided by extracellular pathogens such as *V. aestuarianus* (Labreuche et al., 2006a,b) or impaired in elimination of intracellular pathogens such as *V. splendidus* (Duperthuy et al., 2011).

To be phagocytosed, the foreign agent is opsonized by plasma molecules and further engulfed within a phagosome. As soon as they come in contact with hemocyte membranes, phagocytosed microorganisms are exposed to the respiratory burst, a major microbicidal reaction in oysters (see below). The phagosome maturation then leads to acidification and fusion with cytoplasmic vesicles/granules (including lysosomes), which in oyster were shown to contain diverse families of antimicrobial peptides/proteins (Gueguen et al., 2009; Schmitt et al., 2012a). The release into the phagosome of this arsenal of microbicidal compounds leads to the rapid neutralization/degradation of the microorganisms non-adapted to intracellular life (**Figure 1**). Not surprisingly, lysosome-related genes (enzymes and transport proteins) have been associated to survival in oysters infected with virulent *Vibrio* strains (de Lorgeril et al., 2011). On the opposite, as far as intravacuolar pathogens are concerned, the success of infection appears to rely on a restricted respiratory burst and/or the development of mechanisms of resistance to the oyster antimicrobial response. This has been observed for two oyster pathogens, *V. splendidus* and *Perkinsus marinus*, which resist to intracellular elimination after phagocytosis (Schott et al., 2003; Duperthuy et al., 2010, 2011).

EFFECTORS OF THE ANTIMICROBIAL RESPONSE

PRODUCTION OF REACTIVE OXYGEN SPECIES

Immediately following phagocytosis, there is a significant increase in the intracellular oxygen consumption (respiratory burst), resulting in the production of a variety of intermediate reactive oxygen species (ROS) (Bachère et al., 1991b; Torreilles and Romestand, 2001; Lambert et al., 2003, 2007).

The production of ROS (superoxides and peroxides) is one major inducible mechanism of the antimicrobial response in oysters (Bachère et al., 1991b; Lambert et al., 2003). It results from the activation of the transmembrane NADPH oxidase, which generates the highly toxic and reactive superoxide anion 0_2^-. The production of ROS can cause serious injuries to the host tissues. Interestingly, parallel to the well known NADPH oxidase, a sequence homologous to another transmembrane enzyme termed DUOX for (DUal OXidase) involved in the production of ROS in *Drosophila melanogaster* gut epithelial cells was evidenced in the *C. gigas* genomic resource database (Fleury et al., 2009). In *C. gigas*, the expression of a DUOX-like gene was associated, together with other genes related to oxidative stress, to a successful response to virulent vibrios as compared to avirulent vibrios (de Lorgeril et al., 2011). Interestingly, DUOX expression was shown to be involved in the tolerance of resident microflora in the gut of *Drosophila* and to be over expressed when the gut bacterial load dramatically increases (for review see Royet, 2011). Therefore, as in *Drosophila*, DUOX, which is expressed in the digestive gland of oysters, could be involved in the epithelial defense against food-borne pathogens and in the maintenance of homeostasis.

To avoid self-damage, the host relies on effective antioxidant defense systems that involve antioxidant enzymes such as a superoxide dismutase (SOD), which converts 0_2^- into hydrogen peroxide H_20_2, and peroxidases (catalase and glutathione peroxidase), which convert H_20_2 into hypochlorous acid in the presence of chloride ions. As a sign of its contribution to an effective immune response in oysters, the *C. gigas* extracellular SOD was shown to be higher expressed in an oyster line selected for its resistance to *in situ* mortalities than in a susceptible line (Fleury and Huvet, 2012). It was also higher expressed in oysters surviving an infection with virulent *Vibrio* strains than in oysters challenged with avirulent vibrios (de Lorgeril et al., 2011). Other antioxidant enzymes are also produced in oysters such as peroxiredoxins, glutathione *S*-transferases, and others (Huvet et al., 2004; Tanguy et al., 2004; Vertuani et al., 2004; Gonzalez et al., 2005; Jo et al., 2008; Green et al., 2009; Park et al., 2009; Itoh et al., 2010b; Yu et al., 2011). As with *Cg-EcSOD*, transcripts of glutathione S-transferases (microsomal glutathione S-transferase, glutathione S-transferase theta and omega class glutathione S-transferase) were more abundant in hemocytes of oysters capable to survive an injection with virulent *Vibrio* strains than in oysters challenged with an avirulent *Vibrio* strain (de Lorgeril et al., 2011).

HYDROLYTIC ENZYMES

After phagocytosis, microorganisms are entrapped in phagosomes that fuse with lysosomes and/or intracellular granules. These granules are characterized by their acidic pH and the presence of a wide variety of hydrolytic enzymes and antimicrobial effectors (**Figure 1**). Among the hydrolytic enzymes, the lysozymes play an important role in microbial destruction due to their lytic properties on the peptidoglycan of the bacteria cell wall (Hancock and Scott, 2000). Oyster lysozymes form a diverse group and are expressed in many tissues, such as hemocytes (CGL-1 or *Cg*_lysoz1), digestive diverticula (CGL-2 or *Cg*_lysoz2), and mantle (CGL-3 or *Cg*_lysoz3) (Matsumoto et al., 2006; Itoh and Takahashi, 2007; Itoh et al., 2007, 2010a; Xue et al., 2010). In

hemocytes, lysozyme transcript abundance was shown to be higher in oysters capable to survive an injection of virulent *Vibrio* strains than in oyster challenged with an avirulent *Vibrio* (de Lorgeril et al., 2011). Recombinant lysozymes from *C. gigas* (CGL-1 and CGL-3) were found to be active only against Gram-positive bacteria (Itoh et al., 2010a).

ANTIMICROBIAL PEPTIDES AND PROTEINS

Antimicrobial peptides and proteins (AMPs) are key host defense effectors found in virtually all kingdoms of life (Yeaman and Yount, 2003). They display multifunctional roles in immunity, including microbicidal activities and selective immunomodulatory effects (Yount et al., 2006; Guaní-Guerra et al., 2010). AMPs are classically described as small cationic (less than 10 kDa), amphipathic, gene-encoded peptides that differ considerably in amino acid sequence and structural conformation (Bulet et al., 2004; Brogden, 2005). Several categories of AMPs have been described based on common structural features or conserved sequence motifs. More recently, other groups of antimicrobial molecules referred to as "non-conventional" have been identified that do not fit into this classical definition. These are (i) anionic peptides, (ii) cationic proteins larger than 10 kDa, and (iii) multifunctional proteins that contain antimicrobial sub-domains that are cleaved under certain conditions and generate fragments that behave as antimicrobial peptides (Brogden, 2005; Yount et al., 2006). At present, several families of AMPs or polypeptides sharing common molecular features with the currently known AMP families have been characterized in oysters. These include defensin-like peptides (defensins and big defensins), proline-rich AMPs, and BPI proteins (**Table 1**).

Structure and classification of C. gigas AMPs

Defensins. Defensins were identified in most plants, fungi, and both invertebrate and vertebrate animals (Bulet et al., 2004). They are the best characterized AMPs to date. The term "defensin" was first introduced by Ganz et al. (1985) after the discovery of antimicrobial cysteine-rich peptides in human neutrophils. All vertebrate defensins are small (3–5 kDa) cationic molecules containing six cysteine residues engaged in three intramolecular disulfide bridges. Vertebrate defensin family falls into three subfamilies, namely the open-ring α- and β-defensins as well as the head-to-tail θ-defensins, which differ in terms of cysteine array and secondary structure (for review see Lehrer and Lu, 2012). Thus, the cysteine pairing of β-defensins differs from that of α- and θ-vertebrate defensins.

Unlike vertebrate defensins, which adopt a three-stranded antiparallel β-sheet structure, the defensins from plants, fungi, and invertebrates are composed of an α-helix linked to an antiparallel two-stranded β-sheet by disulfide bridges, making the so-called cysteine-stabilized α-helix/β-sheet motif (CSαβ; Zhu et al., 2005). They contain from six to eight cysteine residues. This motif is widespread in fungi and in invertebrate defensins, like in arthropods and mollusks (Yang et al., 2000; Bulet and Stöcklin, 2005; Mygind et al., 2005), but the presence of eight cysteine residues was only reported in species of bivalve mollusks (Hubert et al., 1996; Gueguen et al., 2006; Gonzalez et al., 2007a). CSαβ-containing defensins have no evident phylogenetic relationships with the vertebrate defensins and big defensins (Rosa et al., 2011).

CS*αβ*-containing defensins (Cg-Defs). In *C. gigas*, members of the invertebrate CSαβ-containing defensin family were identified by screening EST libraries of oyster immune cells (hemocytes and surface epithelia). A broad diversity of CSαβ-containing defensins was found in *C. gigas* (see diversity section below). Still, three representative members, namely *Cg*-Defm, *Cg*-Defh1, and *Cg*-Defh2, were characterized in more details in terms of structure, antimicrobial activity, and mechanism of action. *Cg*-Defm was identified from the oyster mantle whereas *Cg*-Defh1 and *Cg*-Defh2 were identified from the hemocytes (Gueguen et al., 2006; Gonzalez et al., 2007a). All oyster defensin precursors consist in a hydrophobic signal peptide (prepeptide) immediately followed by the 4.6–4.7-kDa cationic mature peptide (p*I* 8.5–8.7), which contains eight cysteine residues (**Figure 2**). The tridimensional structure of the recombinant *Cg*-Defm was solved (PDB: 2B68; **Figure 3**), showing that the CSαβ motif that characterizes this AMP family is stabilized by four disulfide bridges (cysteine pattern: $C_{1-5}C_{2-6}C_{3-7}C_{4-8}$; Gueguen et al., 2006; **Table 1**). The fourth disulfide bridge, only found in oyster and mussel defensins, has been proposed to be implicated in the stabilization of the mature peptide to the high osmolarity environment found in the sea water (Yang et al., 2000).

Each *Cg*-Def is encoded by a separate gene with different genomic organization. The mantle defensin genes (*Cg-defm*) present two structures (i) two exons separated by a unique intron (Gueguen et al., 2006), a similar genomic organization to that of the mussel and scorpion defensin genes (Froy and Gurevitz, 2003), and (ii) three exons separated by two introns. Hemocyte defensin genes (*Cg-defhs*) only display the latter structure, in which the second intron separates the two last residues of the mature peptide apart from the rest of the sequence (Schmitt et al., 2010a). The number of *Cg-def* gene copies was shown to be highly variable (14–53 copies) among individual oysters (Schmitt et al., 2010a). At the transcriptional level, *Cg*-Defs appear to be constitutively expressed in each specific tissue.

Table 1 | Oyster antimicrobial peptides and proteins (AMPs).

Oyster AMP	Variants	MW (kDa)	p*I*	Localization (expression)
CSαβ-containing defensins	*Cg*-Defm	4.6	8.7	Mantle (constitutive)
	Cg-Defh1	4.7	8.5	Hemocytes (constitutive)
	Cg-Defh2	4.6	8.5	Hemocytes (constitutive)
Big defensins	*Cg*-BigDef1	10.7	9.2	Hemocytes (inducible)
	Cg-BigDef2	9.8*	8.6	Hemocytes (inducible)
	Cg-BigDef3	9.7*	8.8	Hemocytes (constitutive)
Proline-rich peptides	*Cg*-lgPrp	1.8*	12.1	Hemocytes (repressed)
	Cg-stPrp	1.5*	12.0	Hemocytes (repressed)
Bactericidal/ permeability-increasing protein	*Cg*-BPI	50.1	9.3	Hemocytes (inducible)
				Epithelia (constitutive)

MW: observed or theorical (*) molecular weight; pI: theoretical isoelectric point.

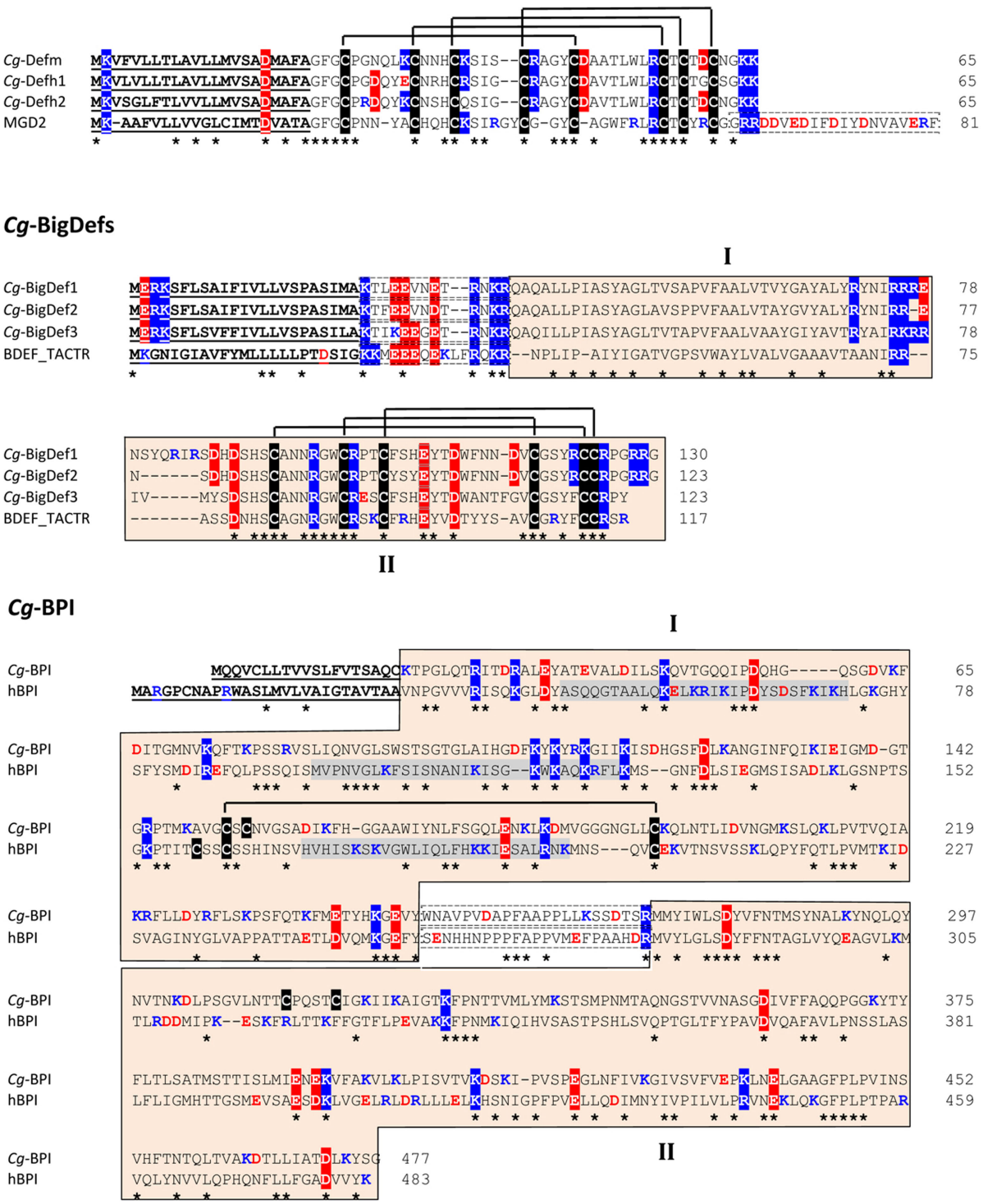

FIGURE 2 | Amino acid alignment of oyster antimicrobial precursors with similar proteins/polypeptides from other species. *Cg*-Def precursors are composed of a 22-residue signal peptide followed by a 43-residue mature peptide. By comparison, the *Mytilus galloprovincialis* MGD2 (GenBank AAD45118.1) carries an additional anionic proregion at C-terminal position. The amino acid sequences displayed here for *Cg*-Defm, *Cg*-Defh1, and *Cg*-Defh2 correspond to GenBank AJ565499, ACQ73009, and ACQ76280, respectively. The cysteine array is based on the 3D structure of *Cg*-Defm (PDB: 2B68). *Cg-BigDef* precursors are composed of a predicted signal peptide (23

(*Continued*)

FIGURE 2 | Continued
residues) followed by 13-residue anionic proregion and the putative mature BigDefs (87–94 residues). Mature BigDefs are multi-domain polypeptides composed of a hydrophobic N-terminal domain (I) and a C-terminal cysteine-rich domain (β-defensin-like domain, II) shown here as orange boxes. A putative cleavage motif (RXKR) for furin-like enzymes separates both domains. Sequences of big defensins from *Crassostrea gigas* (Cg-BigDef1: GenBank AEE92768, Cg-BigDef2: GenBank AEE92775, Cg-BigDef3: GenBank AEE92778) are aligned here with *Tachypleus tridentatus* big defensin (BDEF_TACTR, GenBank: P80957). The cysteine array is based on the 3D structure of the horseshoe crab big defensin (PDB: 2RNG). *Cg-BPI* precursor is composed of a 19-residue signal peptide followed by a 458 amino acid protein. Its amino acid sequence (GenBank AY165040) is aligned here with human BPI (hBPI) sequence (GenBank J04739). The *N*-terminal (domain I) and *C*-terminal (domain II) barrel type domains characterized for hBPI as well as the corresponding sequences in *Cg*-BPI are in orange boxes (domains I and II, respectively). The proline-rich central domain is boxed with a dashed line. The three functional regions of hBPI, which display LPS-binding activity, are highlighted in gray. The conserved disulfide bridge characterized in hBPI 3D structure (PDB: 1BP1) is displayed. Amino acids are numbered on the right. Signal peptides are underlined. Cysteines are highlighted in black and disulfide bridges are displayed as black lines. Conserved residues are shown by an asterisk. Lys/Arg residues are in blue. Asp/Glu residues are in red. Positively and negatively charged residues conserved in more than 50% of the sequences are highlighted in blue and red, respectively. In *Cg*-Def and *Cg*-BigDef alignments, anionic proregions are in dashed boxes. In *Cg*-BPI alignment, the central proline-rich region is in dashed boxes.

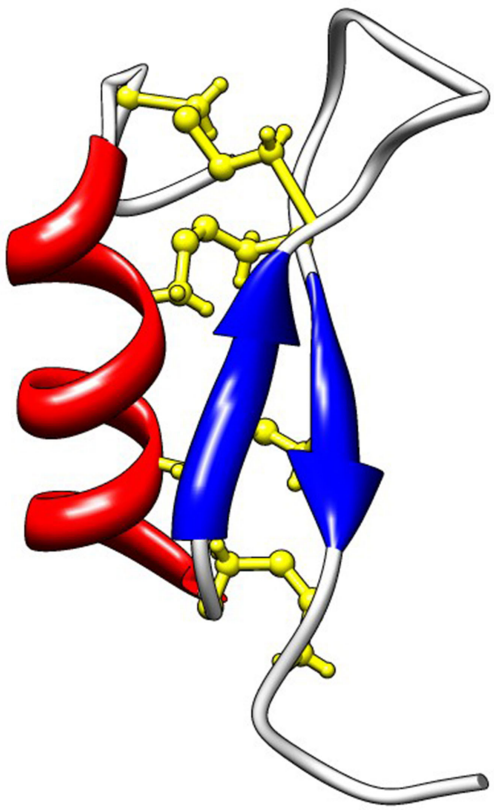

FIGURE 3 | Solution structure of *Cg*-Defm (PDB: 2B68). *Cg*-Defs display the CSαβ motif also found in defensins from plants, fungi, and invertebrates, which is composed of an α-helix (red) linked to an antiparallel two-stranded β-sheet (blue) by disulfide bridges (yellow). In *Cg*-Defs, eight cysteines are involved in four disulfide bridges as follows: $C_{1-5}C_{2-6}C_{3-7}C_{4-8}$.

Until now, native defensins could not be purified from *C. gigas* oysters in sufficient amounts to enable complete biochemical characterization and determine their spectrum of activity. Such data were obtained with recombinant oyster defensins (Gueguen et al., 2006; Schmitt et al., 2010b, 2012a). All oyster defensins were shown to be mainly active against Gram-positive bacteria against which they displayed very low minimal inhibitory concentrations (MIC; 0.01–6 μM) as determined by liquid growth inhibition assay. Conversely, they did not display significant antimicrobial activity against Gram-negative bacteria including oyster pathogens (>40 μM).

Big defensins (Cg-BigDefs). Big defensins (BigDefs) are antimicrobial polypeptides that were initially purified from the hemolymph cells (amoebocytes) of the horseshoe crab *Tachypleus tridentatus* (Chelicerata; Saito et al., 1995). The solution structure of the horseshoe crab BigDef (PDB: 2RNG) revealed a N-terminal globular domain connected to a C-terminal domain containing β-sheet structures and folded by three disulfide bridges whose arrangement is identical to that of vertebrate β-defensins (β-defensin domain: $C_{1-5}C_{2-4}C_{3-6}$; Kouno et al., 2008). The horseshoe crab BigDef is produced as a precursor molecule that is further processed into a 8.6-kDa mature polypeptide (Saito et al., 1995). Big defensin encoding sequences were also evidenced in amphioxus (Cephalochordata) and in several mollusk species including *C. gigas* oysters (for review see Rosa et al., 2011). Oyster big defensins (*Cg*-BigDefs) share 47–55% amino acid sequence identity with the big defensin from horseshoe crab and amphioxus species, and within mollusks, they are 25–28% identical to gastropod sequences and 39–67% identical to bivalve big defensins (Rosa et al., 2011).

Oyster big defensins form a diverse AMP family composed of three representative members, namely *Cg*-BigDef1, *Cg*-BigDef2, and *Cg*-BigDef3. Big defensin precursors are expressed as prepropeptides, which start with a predicted 23-residue signal peptide (prepeptide), followed by a propeptide region of 13 residues and a cationic 94- or 87-residue mature polypeptide of 10.7 kDa (*Cg*-BigDef1), 9.8 kDa (*Cg*-BigDef2), or 9.7 kDa (*Cg*-BigDef3; **Table 1**). The mature polypeptides have theorical *pI* ranging from 8.6 to 9.2. Based on mass spectrometry data, it has been proposed that after elimination of the, the N-terminal glutamine residue of the native *Cg*-BigDef1 is converted into a pyroglutamic acid (Rosa et al., 2011). *Cg*-BigDefs are chimeric molecules that display the structural feature of the horseshoe crab BigDef composed by a hydrophobic N-terminal region and a cationic C-terminal region containing the conserved six cysteine residues (**Figure 2**).

Each of the three *Cg*-BigDefs is encoded by a separate gene whose expression is restricted to hemocytes, both circulating and infiltrating oyster tissues. The genomic organization of *Cg-bigdef1* and *Cg-bigdef2* genes is similar, with two exons interrupted by

a single intron. In contrast, in *Cg-bigdef3*, additional intron and exon are observed upstream the first exon common to the other *Cg-bigdefs*. In all *Cg-bigdef* genes, the β-defensin domain is exclusively encoded by the last exon (Rosa et al., 2011).

The antimicrobial activities of *Cg*-BigDefs are still unknown, but in another bivalve mollusk, *Argopecten irradians*, and in the horseshoe crab *T. tridentatus*, BigDefs were reported to be active against both Gram-positive and Gram-negative bacteria and fungi (Saito et al., 1995; Zhao et al., 2007). Besides, the native horseshoe crab BigDef was shown to display a significant LPS-binding activity (Saito et al., 1995).

Proline-rich AMPs (Cg-Prps). Like defensins, proline-rich AMPs (Prps) have been identified in vertebrates including mammals and amphibians as well as invertebrates such as insects and crustaceans (Scocchi et al., 2011). They form a group of diverse peptides that share some structural features, like a high content in proline and arginine residues (typically from 25 to 50%). They display short Pro-Arg-Pro motifs, which have been proposed to be implicated in their antimicrobial activity. Prps are also characterized by their mode of action, which does not involve the lysis of bacterial membranes but rather the penetration into cells, where they then act intracellularly (Scocchi et al., 2011).

In oysters, a cDNA sequence showing homologies to proline-rich AMPs was identified as expressed in hemocytes by the exploration of *C. gigas* EST libraries (Gueguen et al., 2009). The cDNA sequence encoded a 37-amino acid peptide, which is composed of an N-terminal acidic region (putative propeptide) and a C-terminal cationic proline-rich region, containing two repetitions of a Pro-Arg-Pro motif (**Figure 4**). This highly cationic peptide (theorical *pI* 12.1), which displays the typical features of proline-rich AMPs, was named *Cg*-Prp. A high number of *Cg*-Prp forms were then identified as expressed by oyster hemocytes. They display two lengths, a shorter peptide being evidenced, which derives from the original *Cg*-Prp form by the deletion of a Arg-Pro dipeptide (Schmitt et al., 2010a). Thereby, we renamed the original form as long *Cg*-Prp (*Cg*-lgPrp; 1.8 kDa) and the new form as short *Cg*-Prp (*Cg*-stPrp; 1.5 kDa) (Schmitt et al., 2012a; **Table 1**; **Figure 4**).

Cg-Prps are a multigenic family with genes containing or not an intron. The presence/absence of an intron is observed in the genes encoding both the long and short peptide forms. The number of *Cg-prp* gene copies was shown to vary among individuals from 4 to 18 copies (Schmitt et al., 2010a).

Native *Cg*-Prps could not be purified from oyster hemocyte extracts until now. Therefore, synthetic *Cg*-Prps were used to explore their sprectrum of antimicrobial activity. Both long and short *Cg*-Prps were poorly active against Gram-positive bacteria and were not active against Gram-negative bacteria. However, *Cg*-lgPrp displayed strong synergy with *Cg*-Defs and *Cg*-BPI against both Gram-positive and Gram-negative bacteria (Gueguen et al., 2009; Schmitt et al., 2012a).

Bactericidal/permeability-increasing protein (Cg-BPI). Bacteri cidal/permeability-increasing proteins and lipopolysaccharide-binding proteins (LBPs) are components of the immune system that have been mainly characterized in mammals. Both proteins show 45% sequence identity, sharing basic structural features and residues involved in LPS-binding (Krasity et al., 2011). Human LBP is an acute phase plasma protein constitutively secreted by liver that induces cellular responses (Thomas et al., 2002). In particular, LBP participates in the acute mobilization of circulating neutrophils to sites of tissue injury. In contrast, human BPI is a 55-kDa cationic protein specifically active against Gram-negative bacteria. Stored in neutrophils, human bactericidal/permeability-increasing (hBPI) contributes to the elimination of bacteria and increases the permeability of the bacterial membranes (Levy, 2004). Accumulated extracellularly, it also opsonizes bacteria, enhancing neutrophil phagocytosis (Iovine et al., 1997). The antibacterial BPI also displays LPS-neutralizing properties and suppresses LPS inflammatory activity (Weiss, 2003). By genomic approaches, LBP/BPI-related genes have been found in a number of non-mammalian vertebrate species and several invertebrates like nematodes and mollusks (Krasity et al., 2011).

A homolog of the hBPI protein was identified in *C. gigas* oysters by a screening of a hemocyte EST library (Gonzalez et al., 2007b). As deduced by molecular modeling, *Cg*-BPI displays the typical structural features of hBPI 3D structure (PDB: 2RNG) with an N- and C-terminal β-barrel type domains connected by a proline-rich central domain. In addition, the N-terminal domain of *Cg*-BPI contains the LPS-binding regions characterized in hBPI and the Lys and Arg residues required for LPS-binding. This domain also contains the cysteine bridge of hBPI at conserved position, three extra cysteines being identified (both in the N- and C-terminal domains) whose folding remains unknown. The recombinant *Cg*-BPI was shown to be a monomeric protein (50.1 kDa) and to display both LPS- and Lipid A-binding activities (Gonzalez et al., 2007b). It was highly active against the short-chain LPS *E. coli*

FIGURE 4 | Amino acid alignment of two sequences representative of long and short *Cg*-Prps. Precursors of *Cg*-Prps are composed of a 24-residue signal peptide (underlined) followed by a 19-residue anionic proregion (dashed box), and a 16–18 residue putative mature peptide. In long forms (referred to as *Cg*-lgPrps), two repeats of the Pro-Arg-Pro motif (orange boxes) are found, while in short forms (*Cg*-stPrps), one motif is missing. While Pro-Arg-Pro motifs are common in proline-rich AMPs across species, no further structure similarity has been evidenced until now between oyster Prps and other proline-rich peptides. The amino acid sequences displayed here for *Cg*-lgPrp and *Cg*-stPrp correspond to GenBank FJ669361 and FJ669381, respectively. Amino acids are numbered on the right. Conserved residues are shown by an asterisk. Conserved Lys/Arg and Asp/Glu residues are highlighted in blue and red, respectively. Pro residues are shown in green.

SBS363, against which it displayed bactericidal activity, but was at least 30 times less active against the long-chain LPS *E. coli* ML35. Like hBPI, recombinant *Cg*-BPI permeabilized both the inner and outer membranes of *E. coli* ML35 (Gonzalez et al., 2007b). Like *Cg*-BPI, the recently identified *Cg*-BPI2 was also shown to be exclusively active against Gram-negative bacteria (Zhang et al., 2011).

Diversity of oyster antimicrobial peptides. High levels of sequence diversity were reported to be a characteristic of several genes belonging to the innate immunity system of invertebrates (Schulenburg et al., 2007). Similarly, oyster families of antimicrobials were recently shown to be highly diverse (Schmitt et al., 2010a). Phylogenetic analyses showed that *Cg*-Defs are represented by three distinct groups, of which *Cg*-Defh1, *Cg*-Defh2, and *Cg*-Defm forms are the most representatives. *Cg*-Prp is represented by two major forms, *Cg*-lgPrp and *Cg*-stPrp, but its phylogeny showed more diverse groups of sequences and suggested an ongoing or recent process of neo- or sub-functionalization (Schmitt et al., 2010a). In contrast to both AMPs, *Cg*-BPI was mostly represented by the only one sequence originally identified, but the recently discovery of *Cg*-BPI2 suggests a higher diversity for this antimicrobial (Zhang et al., 2011). Additionally, the *in silico* analysis of the oyster EST database (http://www.sigenae.org/aquafirst/) revealed that *Cg*-BigDefs are a diverse family with several isoforms for the three *Cg*-BigDef1, -2, and -3 forms (Rosa et al., 2011).

Evolutionary analyses showed that *Cg*-Defs and *Cg*-Prps have been subjected to several genetic mechanisms of diversification and that directional selection pressures have shaped their sequence variations. Noteworthy, directional selection driving evolution of invertebrate immune system has been addressed for several genes (Cohuet et al., 2008; Lazzaro, 2008; Roger et al., 2008) and it has been related with the general hypothesis of co-evolution or "arms race" between host and pathogens (Van Valen, 1974).

Specifically, the diversification of AMPs by the accumulation of multiple variants around an initial form has been previously associated to the gain of specific antimicrobial functions (Litman et al., 2005; Rosenfeld and Shai, 2006; Yang et al., 2006). Notably, it has been shown that the different forms of each oyster AMP family could display different antimicrobial potency (Schmitt et al., 2012a). The long *Cg*-Prp forms, which display an extra Pro-Arg-Pro motif in the active region are more potent that the short forms (Schmitt et al., 2012a). When referring to *Cg*-Defs, although the three forms show a similar spectrum of activity, they display different potency against Gram-positive bacteria. In particular, *Cg*-Defh2 is the most potent, showing MIC values 2- to 40-fold lower than *Cg*-Defh1 and *Cg*-Defm against most of the Gram-positive strains tested (**Table 2**; Schmitt et al., 2010a, 2012a). Interestingly, the differences in potency are much higher against the marine strain *B. stationis* (40-fold) than against all other bacteria tested (less than eightfold), which unlikely belong to the oyster environmental microflora and have unlikely co-evolved with the oyster immune system. These evidences tempt us to hypothesize that the diversity of oyster AMPs may have been shaped by the co-evolution between the oyster immune system and species of oyster pathogens, generating new AMP variants with enhanced potency. In support of this hypothesis, the more active defensin forms, *Cg*-Defm and *Cg*-Defh2, display a charged residue under positive selection (Lys and Arg, respectively) where *Cg*-Defh1 displays an uncharged Gly. Because those residues are highly exposed at the surface of oyster defensins, it was inferred they could improve

Table 2 | Antimicrobial activities of *Cg*-Defs, *Cg*-Prps, and *Cg*-BPI against selected microorganisms.

MIC (μM)	*Cg*-Defh2	*Cg*-Defm	*Cg*-Defh1	*Cg*-lgPrp	*Cg*-stPrp	*Cg*-BPI
GRAM-POSITIVE BACTERIA						
Micrococcus lysodeikticus CIP5345	0.01	0.01	0.03	80	>80	>20
Bacilllus megaterium CIP 6620	0.03	0.03	0.06	20	>20	nt
Staphylococcus aureus CIP 103428	0.25	2	2	>20	>20	nt
Staphylococcus aureus SG511	0.12	0.25	0.5	>20	>20	nt
Microbacterium maritypicum CIP 105733T	1	1	2	>20	>20	nt
Brevibacterium stationis CIP 101282	0.1	0.2	4	>20	>20	nt
Staphylococcus haemolyticus	2	2	6	>20	>20	nt
GRAM-NEGATIVE BACTERIA						
Escherichia coli SBS 363	20	20	40	>80	>80	0.7
Vibrio splendidus LGP32 CIP 107715	>40	>40	>40	>40	>40	10
Vibrio aestuarianus LPi 02/41	>40	>40	>40	nt	nt	nt
Vibrio anguillarum ATCC 19264	>40	>40	>40	nt	nt	nt
Vibrio nigripulchritudo CIP103195	>40	>40	>40	>40	>40	nt
FILAMENTOUS FUNGI						
Fusarium oxysporum	nt	9	nt	>200	nt	0.7
Botrytis cinerea	nt	>20	nt	>200	nt	nt
Penicillium crustosum	nt	>20	nt	nt	nt	nt

MIC values (μM) refer to the minimal inhibitory concentration required to achieve 100% growth inhibition. nt: not tested. Modified from Gueguen et al. (2006), Schmitt et al. (2010b).

defensin antibacterial activity by promoting a better binding to bacterial membranes. Therefore, as far as oyster defensins are concerned, diversifying selection is affecting charge distribution, which is known as one major determinant of antimicrobial activity in AMPs. Thus, although solid evidences are still missing in oysters, the selection of specific repertoires of AMPs with enhanced activity may have consequences on the oyster capacity to survive infections, as proposed in other species (Tennessen et al., 2009).

Mechanisms of antimicrobial action and synergism

The mechanisms of action of antimicrobial peptides have been extensively reviewed. Many families of AMPs, which carry a positive net charge and adopt an amphipathic structure, have been shown to be membrane-disruptive. Their physicochemical properties determine their initial electrostatic interaction with the negatively charged bacterial membranes and further insertion into the bacterial membrane lipid bilayer. Several models have been proposed for membrane-disruptive mechanisms of action, ranging from pore formation to detergent-like activities (for review see Brogden, 2005). Still, not all antimicrobials are membrane-disruptive and alternative mechanisms of action are increasingly described in which intracellular molecules involved in vital processes (e.g., enzymes, precursors . . .) are the target of AMPs. Among others, such non-lytic mechanisms of action have been reported in proline-rich AMPs (Scocchi et al., 2011) as well as in CSαβ defensins (Wilmes et al., 2011). The mechanism of action and consequently the target specificity of oyster antimicrobials are very diverse. At present, *Cg*-Defs and *Cg*-BPI are the best described oyster antimicrobials in terms of mechanisms of action.

Cg-Defs are potent inhibitors of the peptidoglycan biosynthesis in Gram-positive bacteria, against which they display strong antibacterial activity (in the nanomolar range). As shown on strains of *Staphylococcus aureus*, antibacterial activity is observed in absence of membrane disruption and results from the high affinity binding of *Cg*-Defs to the cell wall precursor lipid II, which cannot be further incorporated into peptidoglycan biosynthesis and accumulates in the bacterial cytoplasm (Schmitt et al., 2010b). Interestingly, at high concentrations (10 μM and above), *Cg*-Defs can also present membrane-disruptive properties as observed against the Gram-negative *V. splendidus* LGP32 (Duperthuy et al., 2010).

Cg-BPI, as a member of the BPI family, was shown to be both bactericidal and membrane-permeabilizing against Gram-negative bacteria. Its mechanism of action was mainly studied on *E. coli* strains. Bactericidal activity (loss of culturability) against *E. coli* SBS363 was recorded at 1 μM. Besides, a dose-dependent inner membrane permeabilization was evidenced by the extracellular cleavage in a lactose-permease deficient strain (*E. coli* ML35) of a β-galactosidase (a cytoplasmic enzyme) substrate (Gonzalez et al., 2007b).

Cg-Prps and *Cg*-BigDefs remain poorly studied in terms of mechanism of action. It can be speculated that, like many proline-rich AMPs (Kragol et al., 2001; Otvos, 2002; Scocchi et al., 2011), *Cg*-Prps interact with intracellular targets in bacteria. Concerning big defensins, a mechanism of action was proposed for the horseshoe crab BigDef, which is based on the penetration of the hydrophobic N-terminal domain into the microbial cell membrane (Kouno et al., 2009).

As a result of their different mechanism of action, oyster antimicrobials show a diverse range of antimicrobial activities: *Cg*-Defs are mainly active against Gram-positive bacteria, *Cg*-Prps are barely active alone, and *Cg*-BPI is only active against Gram-negative bacteria. Noteworthy, the combination of oyster antimicrobials produces strong synergistic activities that enlarge their spectra of activity (Schmitt et al., 2012a). Synergistic activities between oyster antimicrobials were evidenced between different families such as *Cg*-Defs and *Cg*-Prps (Gueguen et al., 2009) but also *Cg*-Defhs and *Cg*-BPI (Schmitt et al., 2012a). Interestingly, synergies were also shown to occur between forms of the same AMP family, different potencies of synergism being observed (as measured through fractional inhibitory concentration [FIC] indexes) according to the combination of forms (Schmitt et al., 2012a; **Table 3**). It is therefore tempting to speculate that the production of a large number of structurally similar AMPs is evolutionary relevant in that it helps oyster antimicrobials cover a broader spectrum of antimicrobial activities through combinations of peptide isoforms which produce synergistic effects.

The mechanisms underlying the synergistic effects among members of a given family of antimicrobials, as evidenced for *Cg*-Defs and *Cg*-Prps (Schmitt et al., 2012a), are still not understood. However, with the increasing knowledge on oyster antimicrobial

Table 3 | Synergistic activities between *Cg*-Defs, *Cg*-Prps, and *Cg*-BPI against selected bacteria expressed as a fractional inhibitory concentration (FIC index*).

FIC index	Gram-positive bacteria	Gram-negative bacteria	
	M. lysodeikticus **CIP5345**	***E. coli*** **SBS 363**	***V. splendidus*** **CIP 107715**
Cg-Defh1 + *Cg*-Defh2	0.50	0.75	0.75
Cg-Defh1 + *Cg*-Defm	0.75	0.75	0.75
Cg-Defh2 + *Cg*-Defm	0.37	0.50	0.50
Cg-lgPrp + *Cg*-stPrp	2	2	2
Cg-Defh1 + *Cg*-lgPrp	0.35	0.50	2
Cg-Defh2 + *Cg*-lgPrp	0.30	0.28	2
Cg-Defm + *Cg*-lgPrp	0.45	0.75	2
Cg-BPI + *Cg*-lgPrp	nd	0.50	2
Cg-BPI + *Cg*-stPrp	nd	0.75	2
Cg-BPI + *Cg*-Defh1	0.45	0.26	2
Cg-BPI + *Cg*-Defh2	0.23	0.27	2
Cg-BPI + *Cg*-Defm	0.23	0.31	2

Adapted from Schmitt et al. (2012a).

FIC index = [A]/MICA + [B]/MICB, where MICA and MICB are the MICs of peptides A and B alone and [A] and [B] are the MICs of peptides A and B in combination. The MICs for the peptides alone are as given in **Table 2. When MIC values are superior to the highest concentration we tested, we chose this value as the MIC in the calculation of the FIC index. FIC index were interpreted as follows: ≤0.5, strong synergy; 0.5–1 synergy; ≥1; additive effect; =2, no effect; ≥2, antagonism. nd, not determined.*

mechanisms of action, a model can be proposed for the synergism between different families of antimicrobials. Thus, the combination of membrane-active antimicrobials such as *Cg*-BPI with non-lytic peptides such as *Cg*-Defs (and potentially *Cg*-Prps), which interact with an intracellular target, can be highly effective in terms of antimicrobial activity. Thus, *Cg*-BPI, which was shown to permeabilize bacterial membranes (Gonzalez et al., 2007b), likely facilitates the access of *Cg*-Defs to their intracellular target (lipid II), in particular in Gram-negative bacteria where lipid II is located at both the inner and outer leaflets of the cytoplasmic membranes, protected from *Cg*-Def interaction by the inner and outer membranes. Although the existence of an intracellular target for *Cg*-Prps is still speculative, synergy with *Cg*-BPI could also rely on such a combination with a membrane-disruptive antimicrobial. As a consequence of synergism, the peptide concentrations required to kill bacteria are much lower than when peptides are acting alone.

Regulation of AMP expression in healthy and diseased animals

Oyster antimicrobials exhibit a wide variety of expression profiles in hemocyte populations and epithelia, which vary according to the families and the oyster health status. *Cg*-Defhs, *Cg*-Prps, and *Cg*-BigDef3 are expressed in hemocytes of healthy oysters. Besides, *Cg*-Defm and *Cg*-BPI are constitutively expressed in surface epithelia. While *Cg*-Defm is specifically expressed in the mantle, *Cg*-BPI is expressed in a broad series of tissues including mantle, gills, digestive tract, digestive gland diverticula, and gonad follicles. Comparatively, transcripts of *Cg*-BPI and *Cg*-BigDef1 and 2 are barely detected in hemocytes from healthy oysters (Gueguen et al., 2006, 2009; Gonzalez et al., 2007a,b; Rosa et al., 2011; Schmitt et al., 2012a).

After a microbial infection, each one of these antimicrobials follows a different pattern of gene expression. The expression of *Cg*-BPI as well as *Cg*-BigDef1 and 2 is strongly induced by a bacterial challenge in hemocytes, both circulating and infiltrating massively the oyster connective tissues of the gills, gonads, or digestive gland (Gonzalez et al., 2007b; Rosa et al., 2011). On the opposite, *Cg*-Prp gene expression appears down-regulated in hemocytes upon a *Vibrio* infection (Schmitt et al., 2012a). Based on immunocytochemistry data, *Cg*-BPI, *Cg*-Defhs, and *Cg*-Prps would be stored in cytoplasmic granules of oyster hemocytes (Gueguen et al., 2009; Schmitt et al., 2012a). It is still unknown whether *Cg*-BigDefs are also stored in granules, as in horseshoe crab amoebocytes, or secreted into oyster hemolymph. In surface epithelia, genes encoding *Cg*-BPI as well as *Cg*-Defm remain constitutively expressed (Gueguen et al., 2006; Gonzalez et al., 2007b). Whether the antimicrobials are stored or secreted remains to be established.

Interestingly, with the massive infiltration of given hemocyte populations at sites of infection, some AMPs constitutively expressed in oyster hemocytes show a decrease in transcript abundance in circulating hemocyte populations after a *Vibrio* challenge. Thus, it has been shown that upon injection of *V. splendidus* in the adductor muscle of oysters, *Cg*-Defh-expressing hemocytes migrate toward the damaged tissues as evidenced by the concomitant and significant increase of *Cg*-Defh transcript abundance at the *Vibrio* injection site (Schmitt et al., 2012a). As a consequence, hemocytes seem to serve as a vehicle for given peptides like *Cg*-Defhs, driving them to tissues exposed to microorganisms where other peptides like *Cg*-Defm or *Cg*-BPI are constitutively expressed.

How AMPs may participate in oyster defense

The constitutive expression of *Cg*-Defm and *Cg*-BPI in barrier epithelia of healthy oysters probably contributes to the control of their endobiont microbiota, which at a basal level could be beneficial for outcompeting potential pathogens from the environment. Indeed, in mammals, the constitutive expression of AMPs in tissues exposed to microbes regulates the numbers of colonizing microbes by creating an antimicrobial environment that prevents heavy colonization and helps to shape the composition of the colonizing microflora (Salzman et al., 2007; Duerkop et al., 2009; Bevins and Salzman, 2011).

Upon infection, additional families of AMPs expressed by hemocytes, namely *Cg*-BPI, *Cg*-BigDefs, and *Cg*-Defhs, are rapidly and massively colocalized in oyster tissues. While the constitutively expressed *Cg*-Defhs are transported through the migratory behavior of hemocytes, *Cg*-BPI, *Cg*-BigDef1 and 2 are massively transcribed in hemocytes infiltrating tissues (Rosa et al., 2011; Schmitt et al., 2012a). This contributes to colocalize the antimicrobials which in turn can express synergism. As a consequence, by migration of antimicrobial-expressing hemocyte populations and continuous tissue expression, oyster antimicrobials are likely merged in tissues upon exposure to pathogens. For instance, the strong synergy observed between hemocyte defensins (*Cg*-Defh1 and 2) and mantle defensin (*Cg*-Defm) against the oyster pathogen *Vibrio splendidus* LGP32 (**Table 3**) can only take place upon co-localization, which is dependent on hemocyte infiltration of the infected tissue.

Co-localization of oyster antimicrobials not only occurs in barrier epithelia but also in some populations of circulating hemocyte as demonstrated for *Cg*-Defhs and *Cg*-Prps on the one hand, and *Cg*-Defhs and *Cg*-BPI on the other hand (Gueguen et al., 2009; Schmitt et al., 2012a). The subsequent synergism which potentially takes place could be of prime importance in the intracellular elimination of phagocytosed microorganisms. However, data are still missing on the behavior of hemocyte granules after infection. Do they fuse with the phagosome as described for human neutrophil defensins or are they exocytosed in the oyster plasma as shown for human cathelicidin LL-37? In other species of bivalve mollusks such as the mussel, in crustaceans, and in chelicerates, the contact with microorganisms or their cell wall components induces the degranulation of hemocytes and consequently the release of stored host defense molecules (Mitta et al., 2000b; Muñoz et al., 2004; Kawabata, 2011). Comparatively, in insects, the gene expression and secretion of large amounts of AMPs is induced in epithelial cells and in the fat body by the presence of microorganisms (Hoffmann et al., 1999). Because *C. gigas* antimicrobial response seems to be highly based on phagocytic processes (Cheng, 1996; Hine, 1999; Takahashi and Mori, 2000), oyster AMPs could be involved in the elimination of invading pathogens during phagocytosis, as proposed for mussel mytilins (Mitta et al., 2000a) and human neutrophil peptides (HNP; Ganz et al., 1985). Intracellularly, AMPs could act synergistically through the co-localization

of diverse families and forms; they could also display antimicrobial activities in a coordinate manner with additional microbicidal reactions that involve lysosomial enzymes and ROS.

Unlike in many other marine invertebrates including species of bivalve mollusks, *C. gigas* AMPs are produced at low concentrations and have not been purified to homogeneity until now. Unsuccessful attempts to purify native oyster AMPs by the conventional biochemical methods normally used for the purification of cationic gene-encoded AMPs were documented (Bachère et al., 2004). Besides, after a bacterial challenge, increase of AMP concentration in plasma has never been reported. The lack of release of large amounts of AMPs within hemolymph upon infections greatly suggests the absence of a systemic humoral antimicrobial response in *C. gigas* oysters.

In summary, recent studies suggest that the production of a large number of AMPs within the same animal is an evolutionary strategy to increase the spectrum of antimicrobial activities by using combinations of peptide isoforms (Mangoni and Shai, 2009). Through the migratory properties of oyster hemocytes expressing diverse families of antimicrobials and the basal expression of antimicrobials in tissues, synergistic activities could take place and contribute to control microbial invasion. We propose that such a synergy, which was evidenced both within and between families of antimicrobials (Schmitt et al., 2012a), is essential during the oyster immune response, and that the high degree of sequence diversity in oyster antimicrobials may compensate through synergic activities their low concentration in oyster tissues.

How oyster pathogens evade the antimicrobial response

The continuous interplay between hosts and pathogens has evolved over millions of years. Thus, the success of a microbial infection or the achievement of host strategies to overcome diseases is a consequence of an active co-evolutionary process (Peschel and Sahl, 2006). In this way, pathogens have placed diverse mechanisms of resistance against antimicrobial responses. Particularly, several strategies are used by bacteria to evade host antimicrobials. They can resist AMPs by their inactivation by peptidases and proteases, or modify the net anionic charge of their cell envelope to reduce the affinity for AMPs; or even, AMPs can be actively extruded from bacterial cells (Yeaman and Yount, 2003).

Both commensals and pathogens are found in the hemolymph of oysters from which they can be isolated in a culturable state. Among them, many bacterial strains belong to the *Vibrio* genus (Gay et al., 2004). Interestingly, as commented above, the *C. gigas* plasma (cell-free hemolymph) is poor in antimicrobials. This could make oyster plasma adapted to bacterial life. Whether or not AMP-resistance is required for life in plasma is uncertain. However, in a recent study, one mechanism of AMP-resistance in vibrios, which is mediated by the major outer membrane protein OmpU (Mathur and Waldor, 2004; Mathur et al., 2007), was shown to endow the oyster pathogen *V. splendidus* LGP32 with (i) an increased resistance to oyster AMPs and (ii) a growth advantage in oyster plasma (Duperthuy et al., 2010).

While AMP-resistance mechanisms are still poorly documented in oyster pathogens, evasion of the cellular hemocyte reactions appears essentials. Indeed, both bacterial and protozoan parasites have developed strategies to avoid intracellular elimination by oyster phagocytes. Mechanisms of immune evasion have been reported in pathogenic strains of *P. marinus*, *V. splendidus*, and *V. aestuarianus* (Schott et al., 2003; Labreuche et al., 2006b, Duperthuy et al., 2011). Both *P. marinus* and *V. splendidus* invade oyster hemocytes as part of their pathogenic process. Therefore, after hemocyte invasion through subversion of hemocyte membrane receptors (galectins and β-integrins, respectively; Tasumi and Vasta, 2007; Duperthuy et al., 2011), they have developed strategies to resist to and/or suppress hemocyte defense reactions. Thus, *P. marinus* fails to elicit ROS production when entering hemocytes and displays intrinsic resistance to ROS (Schott et al., 2003). Similarly, *V. splendidus* LGP32 is able to manipulate phagosome maturation and to survive in oyster hemocytes by avoiding acidic vacuole formation and ROS production (Duperthuy et al., 2011). Although it is still unknown whether *V. splendidus* LGP32 invades hemocyte types that produce AMPs, by impairing phagosome maturation, it could prevent contact with host AMPs stored in hemocyte cytoplasmic granules.

Unlike *V. splendidus* LGP32, which is also associated to the summer mortality syndrome of oysters, *V. aestuarianus* strain 01/32 is described as an extracellular pathogen (Labreuche et al., 2006a) and evades hemocyte reactions in a totally different manner. The evasion of *V. aestuarianus* 01/32 from oyster immune response relies on the avoidance of phagocytosis by the secretion of inhibitory extracellular products (ECPs; Labreuche et al., 2006a,b).

Thus, bacterial oyster pathogens have developed different strategies that can eventually reduce the probability to encounter oyster AMPs within the hemocytes. In addition, we have evidence that *Vibrio* spp. display high *in vitro* resistance to oyster AMPs (Duperthuy et al., 2010; Schmitt et al., 2010b). From our current knowledge, such a resistance could be conferred by (i) the OmpU-mediated sensing of membrane perturbations and further signaling of the envelope stress response (Mathur and Waldor, 2004; Mathur et al., 2007), (ii) the VexAB-mediated efflux of AMPs (Bina et al., 2008), or (iii) other structural properties such as capsule formation that could also protect vibrios from the oyster antimicrobial response.

CONCLUSION

Knowledge has been acquired on the antimicrobial response of the oyster over the past years. Still, much remains to be explored regarding its role in the oyster defenses and host–pathogen interactions. From our current knowledge, phagocytosis and the oxidative burst would be major reactions of the defense of *C. gigas* oysters against infections. Remarkably in this species, conventional AMPs such as those reviewed in the present article are found at very low concentrations in tissues. This is not a common feature in species of bivalve mollusks nor in invertebrates more generally. It is therefore reasonable to ask whether AMPs play a significant role in the oyster defense. In support of such a role are the major selective pressures that have been shown to shape AMP sequences at positions required for an efficient mechanism of action (electrostatic interactions with bacterial membranes, interaction with intracellular molecular targets . . .). Still, there is no positive correlation evidenced between AMP expression and resistance to disease. We

have shown here how the diversity of sequences and/or expression patterns of AMPs together with their co-localization in surface epithelia, which is mediated by hemocyte infiltration, can give rise to synergistic activities that likely contribute to the control of infections. We therefore believe that in this species, which lives in permanent contact with and is inhabited by a great variety of microorganisms, the diversity of AMPs may compensate for their scarcity in the regulation of the resident/pathogenic microflora, and as a consequence in the maintenance of oyster homeostasis. Future studies on the microbial communities that constitute the oyster microflora and their interaction with the oyster immune system will be required to better understand how oysters discriminate between commensal and pathogenic bacteria and to identify which host factors are involved in the regulation of this microflora.

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Carbohydrate derived fulvic acid: an *in vitro* investigation of a novel membrane active antiseptic agent against *Candida albicans* biofilms

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Carbohydrate derived fulvic acid (CHD-FA) is a heat stable low molecular weight, water soluble, cationic, colloidal material with proposed therapeutic properties. The aim of this study was to evaluate the antifungal activity of CHD-FA against *Candida albicans*, and to characterize its mode of action. A panel of *C. albicans* isolates ($n = 50$) derived from a range of clinical specimens were grown planktonically and as biofilms, and the minimum inhibitory concentrations determined. Scanning electron microscopy was performed to examine ultrastructural changes and different cell membrane assays were used to determine its mode of action. In addition, the role of *C. albicans* biofilm resistance mechanisms were investigated to determine their effects on CHD-FA activity. CHD-FA was active against planktonic and sessile *C. albicans* at concentrations 0.125 and 0.25% respectively, and was shown to be fungicidal, acting through disruption of the cell membrane activity. Resistance mechanisms, including matrix, efflux, and stress, had a limited role upon CHD-FA activity. Overall, based on the promising *in vitro* spectrum of activity and minimal biofilm resistance of the natural and cheap antiseptic CHD-FA, further studies are required to determine its applicability for clinical use.

Keywords: *Candida albicans*, fulvic acid, biofilm, antiseptic

INTRODUCTION

Oral diseases such as oral candidosis, caries, and periodontitis are characterized by microbial biofilms (Coco et al., 2008), which are difficult to control with standard chemotherapeutic approaches and host immune defenses (Ramage et al., 2010). These biofilm consortia have a community structure, dominant metabolic processes and inter-organism interactions. Antimicrobial resistance is a key characteristic of biofilms, which is associated with exopolymeric matrix, increased cell density, persister cells, and up-regulation of efflux pumps (Ramage et al., 2009). Recent studies by our group demonstrated that *Candida albicans* biofilms were refractory to prescription antifungal agents, mouthwashes, and denture cleansers (Jose et al., 2010; Ramage et al., 2011a). In addition, *Candida* biofilms in the oral cavity are associated with inflammation and symptoms such as pain, burning sensation, and altered taste (Samaranayake et al., 2009). Overall, these factors complicate clinical management, therefore, alternative agents that elicit antifungal activity are of clinical interest.

Fulvic acid is a novel antimicrobial molecule that is reported to have antibacterial and antifungal properties (van Rensburg et al., 2000). Moreover, it has been recently reported to be non-toxic in a rat wound model, in addition to having anti-inflammatory properties (Sabi et al., 2011). A recent randomized, double blind, controlled trial indicated that fulvic acid was well-tolerated in a study of eczema (Gandy et al., 2011). This colloidal organic acid is a major constituent of humic acids and has been recognized for its biological significance for many years, yet there is minimal scientific understanding on which to support the audacious claims of its properties. Fulvic acid can be isolated from the environment or produced from the oxidation of coal or lignite. Such preparations contain high levels of heavy metals and potentially toxic elements, making their use in humans unsuitable. Recent innovation has seen the development of carbohydrate derived fulvic acid (CHD-FA), a pure form of fulvic acid produced by a patented process to GMP standards (PA107470/GB), rendering it free of heavy metals and environmental pollutants normally found in fulvic acid from environmental sources.

The purpose of this study was to investigate the antifungal effects of CHD-FA, to determine whether it was active against biofilms and to define its mode of action. We report for the first time a highly effective novel antiseptic agent with exquisite biofilm activity that acts by disrupting cell membranes.

MATERIALS AND METHODS

CULTURE CONDITIONS AND STANDARDIZATION

Candida albicans type strains ATCC 90028, ATCC 10231, 3153A, SC5314, CAF 2, and a range of clinical strains ($n = 45$) were used for sensitivity testing. All working stocks of *C. albicans* were

maintained at 4°C on Sabouraud (SAB; Oxoid, Cambridge, UK) agar. Isolates were propagated in yeast peptone dextrose (YPD) medium (Oxoid), washed by centrifugation, and resuspended in RPMI-1640, as described previously (Ramage et al., 2001).

ANTIFUNGAL SUSCEPTIBILITY TESTING OF PLANKTONIC CELLS AND BIOFILMS

The following antifungal agents were used in the course of this study: CHD-FA [Fulhold, Cape Town, South Africa (CAS: 479-66-3)], voriconazole (VRZ, Pfizer Pharmaceuticals, Sandwich, UK), caspofungin (CSP, Merck Sharp & Dohme, Hertfordshire, UK), amphotericin B (AMB, Sigma, Poole, UK).

Antifungal testing to determine minimum inhibitory concentrations (MICs) of planktonic cells was performed using the CLSI M-27A broth microdilution method (CLSI, 2008). Biofilms were formed and sessile susceptibility testing was performed as previously described in commercially available presterilized, polystyrene, flat-bottomed, 96-well microtiter plates (Corning Incorporated, NY, USA; Ramage et al., 2001). Sessile minimum inhibitory concentrations (SMICs) were determined at 80% inhibition using an XTT [2,3-*bis*(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-caboxanilide] metabolic reduction assay (Pierce et al., 2008). Testing of all planktonic and sessile isolates was performed in triplicate.

For time-kill studies four isolates were selected based on their superior and equivalent biofilm properties. These were grown and treated with CHD-FA, VRZ, CSP, and AMB at 1, 2, and 4 × $SMIC_{50}$ to investigate whether these antifungals work in a dose dependent manner. At 1, 2, 4, 6, 8, and 24 h the biofilms were carefully washed and the metabolic activity of the biofilms assessed (Pierce et al., 2008). Testing of four isolates for each drug and each time-point was performed with six replicates and on two separate occasions.

BIOFILM DISRUPTION AND ULTRASTRUCTURAL CHANGES

Candida albicans biofilms ($n = 40$) were prepared and treated with 2 and 4 × $SMIC_{50}$ for 24 h as these concentrations of CHD-FA were highly effective in the time-kill study above. Following treatment the wells were washed carefully with PBS to quantify the biomass of each biofilm, as previously reported using a crystal violet assay (Jose et al., 2010).

For scanning electron microscopy *C. albicans* 3153A sessile cells were grown directly onto Thermanox™coverslips (Nunc, Roskilde, Denmark) prior to antifungal treatment, whereas planktonic *C. albicans* cells were first pretreated with CHD-FA prior to immobilization on the coverslip. Treatment was performed with all four antifungal agents for 24 h at 1 × $SMIC_{90}$ for both planktonic and sessile cells. These were then fixed in 2% para-formaldehyde, 2% glutaraldehyde and 0.15 M sodium cacodylate, and 0.15% w/v Alcian Blue, pH 7.4, and prepared for SEM as previously described (Erlandsen et al., 2004). The specimens were sputter-coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope. Images were assembled using Photoshop software (Adobe, San Jose, CA, USA).

MECHANISM OF ACTION

We investigated whether CHD-FA interacted with the membrane using a propidium iodide (PI) uptake and ATP release assay. First we standardized *C. albicans* (SC5314) to 5×10^7 cells/ml in RPMI-1640 and treated with CHD-FA at 4% for 10, 20, 30, 40, 50, and 60 min. Following treatment, cells were washed twice with PBS, stained with 20 μM of PI and incubated at 37°C for 15 min to allow the dye to bind to DNA. Fluorescence was then measured at excitation and emission wavelengths 535/617 nm, respectively. Alternatively, cells in RPMI were plated onto an Ibidi μSlide VI (Thistle Scientific, Glasgow, UK) and cultured for 60 min at 37°C before imaging on an inverted Nikon Eclipse TE300 using a ×20 objective lens.

ATP release was also quantified from collected supernatants. These were buffered to pH 7.0 and equal volumes of supernatant were added to ATP assay mix (Sigma-Aldrich, UK), and the assay performed as per the manufacturer's instructions. Luminescence was then measured in a white 96-well plate (Corning Incorporated, NY, USA) using a microtiter plate reader (FLUOStar Omega, BMG Labtech). Membrane assays were carried out in triplicate and repeated at least twice.

We next investigated the contribution of chitin, using a specific inhibitor of the chitin synthesis pathway ± CHD-FA, to delineate its mechanism of action. For planktonic cells CLSI methodologies were employed, with CHD-FA ± nikkomycin Z (0.4 μg/ml), which was incubated for 24 h at 37°C. Sessile cells were grown in the presence of nikkomycin Z for 24 h at 37°C then treated with CHD-FA for another 24 h. Following incubation of both planktonic cells and biofilms the metabolic activity was quantified using the XTT reduction assay (Pierce et al., 2008). This assay was carried out on two individual occasions, using two type and two clinical strains tested in quadruplicate.

RESISTANCE MECHANISMS

The role of heat shock protein 90 (Hsp90) was investigated using a specific inhibitor of the Hsp90 (17-allylamino-17-demethoxygeldanamycin) ± CHD-FA. Planktonic cells and preformed biofilms were treated with CHD-FA ± geldanamycin (12.5 μg/ml; Invivogen, San Diego, CA, USA) for 24 h at 37°C. Following incubation the metabolic activity of the cells was quantified using the XTT reduction assay (Pierce et al., 2008). This assay was carried out on two individual occasions, using two type and two clinical strains tested in quadruplicate.

The role of extracellular matrix was investigated using strains previously described with altered expression of *FKS1* (Nett et al., 2010a). Reference strain (DAY185), *FKS1/fks1*Δ and *TDH3-FKS1* were standardized to 1×10^6 cells/ml in 96-well flat-bottomed plates and biofilms grown for 8 h at 37°C. Biofilms were carefully washed three times with PBS and treated with 0.05 and 0.1% CHD-FA for 24 h. After incubation, biofilms were carefully washed three times with PBS and metabolic activity was measured using the XTT assay (Pierce et al., 2008). This assay was carried out on two separate occasions, in triplicate.

Efflux pump activity of planktonic and biofilm cells was initially assessed using an MC-005,556 (Ala-Nap) fluorescent assay (Ramage et al., 2011b). *C. albicans* SC5314 was prepared as standardized planktonic cells (~5×10^7 cells/ml) in buffer solution [K_2HPO_4 (50 mM), $MgSO_4$ (1 mM), and glucose (0.4%)] at pH 7.0. Planktonic cells were treated with 0.0313 and 0.0625% CHD-FA for 4 and 24 h before being washed with PBS, resuspended in assay

buffer and added to black flat-bottomed microtiter plate (Costar 3603, Corning, NY, USA). Biofilms were grown directly within the plates and treated with CHD-FA for 4 and 24 h. After incubation, biofilms were washed and the buffer solution added. The reaction was initiated by the addition of Ala-Nap at a final concentration of 100 μg/ml. Fluorescence was quantified at 30 s intervals for 1 h at 37°C using a fluorescence plate reader (FLUOstar OPTIMA) at excitation and emission wavelengths of 355/460 nm, respectively. Higher fluorescence values indicated low efflux activity and vice versa. To determine whether efflux pump activity was detrimental to the effects of CHD-FA, an efflux pump inhibitor [EPI; L-Phe-L-Arg-β-naphthylamide (MC-207,110), Sigma-Aldrich] was prepared at a working concentration in distilled water (Ramage et al., 2011b). To determine the effects of MC-207,110 on the MIC of CHD-FA, a checkerboard titration assay was performed. CHD-FA was tested at a range of 0.002–1% in combination with MC-207,110 at a concentration of 64 μg/ml. Viability was assessed using the XTT reduction assay as previously described (Pierce et al., 2008).

STATISTICAL ANALYSIS

Data distribution and statistical analysis was performed using GraphPad Prism (version 4; La Jolla, CA, USA). The non-parametric data was analyzed using the Mann–Whitney *U* test to assess differences between two independent sample groups. Statistical significance was achieved if $p < 0.05$.

RESULTS

CHD-FA EXHIBITS POTENT ANTIFUNGAL ACTIVITY

All planktonic *C. albicans* isolates tested were susceptible to all four antifungal drugs, with MIC_{90} for CHD-FA, VRZ, AMB, and CSP of 0.125% and 0.125 mg/l, respectively (**Table 1**). MIC_{50} and MIC_{90} refer to the MICs of ≥50/90% of isolates inhibited by each antifungal, respectively. When the sessile activity of each compound was compared to planktonic cells in terms of MIC_{90} fold change, CHD-FA was the most effective (2×), followed by caspofungin (4×), amphotericin B (64×) and voriconazole (>2048×). Both planktonic and sessile cells showed comparable MIC's for all 50 strains tested.

The biofilm rate of kill of CHD-FA was assessed, where dose and time dependent killing was observed (**Figure 1Ai**). Greater killing was observed after 1, 2, and 4 h at 4 × $SMIC_{50}$ in comparison to 1 and 2 × $SMIC_{50}$ where a decrease in viability of 65 and 89% was observed. After 24 h treatment no differences were observed between each concentration, with an approximate final 92% kill. When each antifungal was compared at 4 × $SMIC_{50}$ then CHD-FA displayed the quickest killing compared in ascending order to AMB, VRZ and CSP, which after 2 h was 89, 61, 18, and −2%, respectively (**Figure 1Aii**). However, after 24 h the most effective compound was CSP (97%) followed by CHD-FA (91%), AMB (88%), and VRZ (43%).

The ability to disrupt *C. albicans* biofilms was then evaluated, where it was shown that both 0.25% and 0.5% CHD-FA were unable to significantly reduce the biofilm biomass compared to a PBS control ($p = 0.2318$ and 0.1069, respectively; **Figure 1B**).

CHD-FA IS NON-SPECIFICALLY ACTIVE AGAINST CELL MEMBRANES

Deterioration of the general cell structure of both planktonic and sessile cells was observed, of which the latter appear to be crenated (**Figure 2A**). Based on these observations it was hypothesized that the cell membrane may have been destabilized, so membrane permeability assays were performed. ATP was released in a time dependent manner, reaching a maximum after 40 min exposure to CHD-FA (**Figure 2B**). These observations were confirmed through quantification of PI, where uptake was shown to be rapid over the first 20 min, after which this plateaued. Using time-lapse microscopy, uptake of PI was shown to occur rapidly following addition of CHD-FA, with detectable membrane permeability within 10 min of exposure to CHD-FA, with all cells becoming PI-positive and cell growth attenuated (**Figures 2Ci,ii**).

To further elucidate the mechanisms of action we investigated the impact of chitin using its inhibitor nikkomycin (NKM). We hypothesized that by weakening the chitin layer this would enable CHD-FA to have greater activity, if indeed the cell membrane was the target. *C. albicans* planktonic cells treated with 0.0625% CHD-FA in the presence of NKM were found to be significantly more sensitive to CHD-FA ($p = 0.0022$) than NKM free cells (**Figure 2D**), which is demonstrated by a 21% reduction in cell viability. Treatment of *C. albicans* biofilm showed a ∼34% reduction in cell viability, however, this was not statistically significant ($p = 0.1057$). In both planktonic and sessile states, NKM showed no effect on cell viability (data not shown).

Table 1 | Susceptibility profile of *Candida albicans* to four antifungal agents.

	MIC							
***n* = 50**	**VRZ (mg/l)**		**AMB (mg/l)**		**CSP (mg/l)**		**CHD-FA (%)**	
	PMIC	**$SMIC_{90}$ (fold change)**	**PMIC**	**$SMIC_{90}$ (fold change)**	**PMIC**	**$SMIC_{90}$ (fold change)**	**PMIC**	**$SMIC_{90}$ (fold change)**
Range*	0.0625–2	>256 (>128–4096)	0.0625–0.25	2–256 (32–1024)	≤0.0625–0.25	≤0.0625–8 (≤1–32)	0.125	0.25 (2)
MIC_{50}	0.125	>256 (>2048)	0.0625	4 (64)	0.0625	0.25 (4)	0.125	0.125 (1)
MIC_{90}	0.125	>256 (>2048)	0.125	8 (64)	0.125	0.5 (4)	0.125	0.25 (2)

PMIC, planktonic minimum inhibitory concentration; SMIC, sessile minimum inhibitory concentration; $SMIC_{90}$, 90% reduction in viability assessed by XTT; VRZ, voriconazole (VFend® – Pfizer); AMB, amphotericin B (Sigma-Aldrich); CSP, caspofungin (Cancidas® – Merck), CHD-FA, carbohydrate derived fulvic acid (Fulhold).
**MIC range of all isolates.*

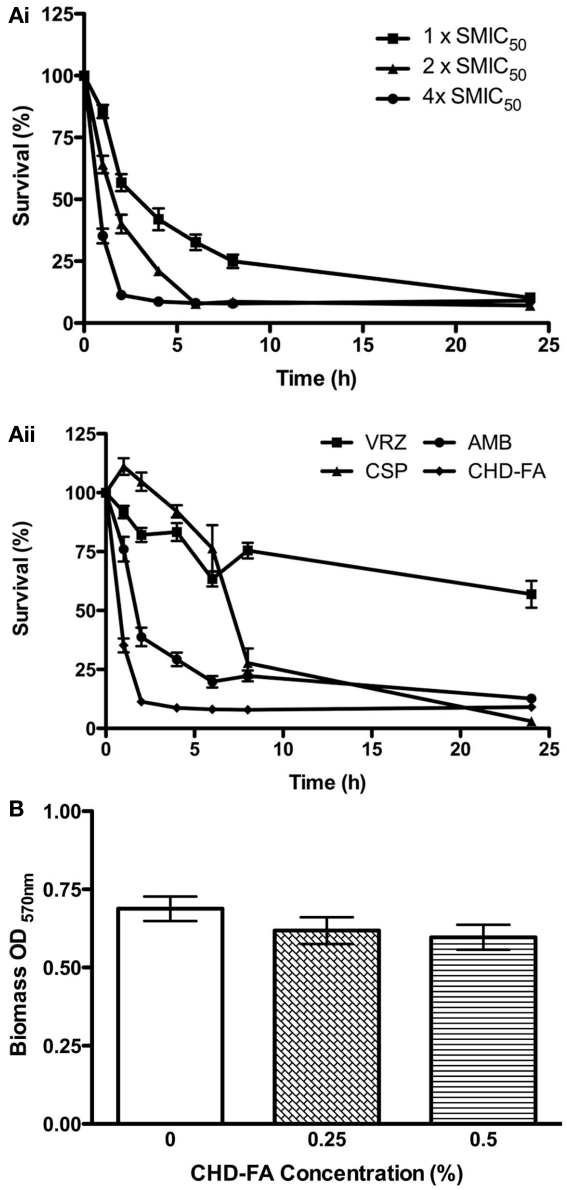

FIGURE 1 | *Candida albicans* biofilm killing by CHD-FA is time and concentration dependent, despite no biofilm disruption. (A) Biofilm time-kill kinetics of CHD-FA at 1, 2, and 4 × $SMIC_{50}$. **(i)** Biofilm time-kill kinetics of VRZ, CSP, AMB and CHD-FA at 4 × $SMIC_{50}$. **(ii)** Standardized *C. albicans* (1 × 10^6 cells/ml) were grown in flat-bottomed 96-well plates for 24 h, washed in PBS and treated with antifungal agents at defined concentrations for 1, 2, 4, 6, 8, and 24 h. Metabolic activity of treated biofilms was then quantified using the XTT assay. Four isolates were used for each assay, and this was performed on two independent occasions in triplicate. **(B)** Standardized *C. albicans* (1 × 10^6 cells/ml) were grown in flat bottomed 96-well plates for 24 h, washed in PBS and treated with 0.25 and 0.5% CHD-FA for 24 h. A negative control was also included. The biomass of the biofilm was then quantified by staining each biofilm with 0.05% w/v crystal violet solution. The biofilms were washed, allowed to air dry, and 100% ethanol applied to destain each biofilm. The biomass was quantified spectrophotometrically by reading at 570 nm in a microtiter plate reader (FLUOStar Omega, BMG Labtech). Forty isolates were tested during this assay, which was performed in triplicate.

THE ROLE OF *CANDIDA ALBICANS* BIOFILM RESISTANCE MECHANISMS

The roles of key resistance mechanisms utilized by *C. albicans* were next investigated to determine whether they were likely to impact the effectiveness of CHD-FA. *C. albicans* susceptibility to 0.0625% CHD-FA (sub-MIC) in the presence of the Hsp90 inhibitor geldanamycin (GDM) was significantly increased in planktonic ($p = 0.0009$) and sessile ($p = 0.0133$) cells (**Figure 3A**), reducing the cell viability by 49 and 7%, respectively. No significant differences were observed for both planktonic and sessile cells treated at MIC levels (0.125%) in the presence of GDM. In both cell states, GDM showed no effect on cell viability (data not shown).

The role of *FKS1* expression in *C. albicans* biofilm resistance was assessed. **Figure 3B** shows that *FKS1/fks1*Δ biofilms were significantly more susceptible to CHD-FA at sub-MIC levels (0.05%) compared to the treated reference strain ($p = 0.0126$), with cell viability being reduced by 40%. In comparison, the over-expressing strain, *TDH3-FKS1*, was less sensitive to 0.05% CHD-FA, with cell survival 10% greater than the reference strain ($p = 0.3095$).

Carbohydrate derived fulvic acid treated planktonic cells exhibited a time and dose dependent up-regulation of efflux pumps compared to untreated cells (**Figure 3Ci**). As the CHD-FA concentration increased (0 to 0.0313 and 0.0625%) there was significant up-regulation of efflux pumps, indicated by low fluorescence, after treatment for 4 h ($p = < 0.0001$), but not after 24 h ($p = 0.2581$ and 0.0625, respectively). For sessile cells a general increase in efflux pump activity was observed compared to planktonic cells. However, no significant increase in efflux pump activity was reported when the biofilms were exposed for 4 h to 0.0313 and 0.0625% CHD-FA ($p = 0.3401$ and 0.0503 respectively). Conversely, 24 h treated biofilms showed a minor down-regulation of efflux pumps compared to the untreated control at these concentrations ($p = 0.0003$ and 0.0019, respectively; **Figure 3Cii**). In addition to efflux pump activity being measured, the effects of an EPI on sensitivity were explored (**Figure 3Ciii**). There was no significant reduction in cell survival when 0.0625% CHD-FA and EPI were used in combination ($p = 0.0569$), despite a 10% reduction in viability. EPI showed no effect on cell viability (data not shown).

DISCUSSION

Many oral diseases are associated with microbial biofilms, which are generally refractory to prescription antibiotics and antifungal agents. Recently we reported that commercial mouthwashes were superior to these and a more favorable chemotherapeutic option for treating oral candidal biofilm infections (Ramage et al., 2011a). However, even with chlorhexidine containing products residual viable biofilm cells are retained, so novel compounds are still urgently required. Here we report for the first time a potential novel antimicrobial agent, CHD-FA, which has potent antifungal activity against *C. albicans* biofilms and other oral biofilms. Furthermore, we propose that this compound is effective through disruption of the cellular membrane and is unaffected by biofilm resistance mechanisms.

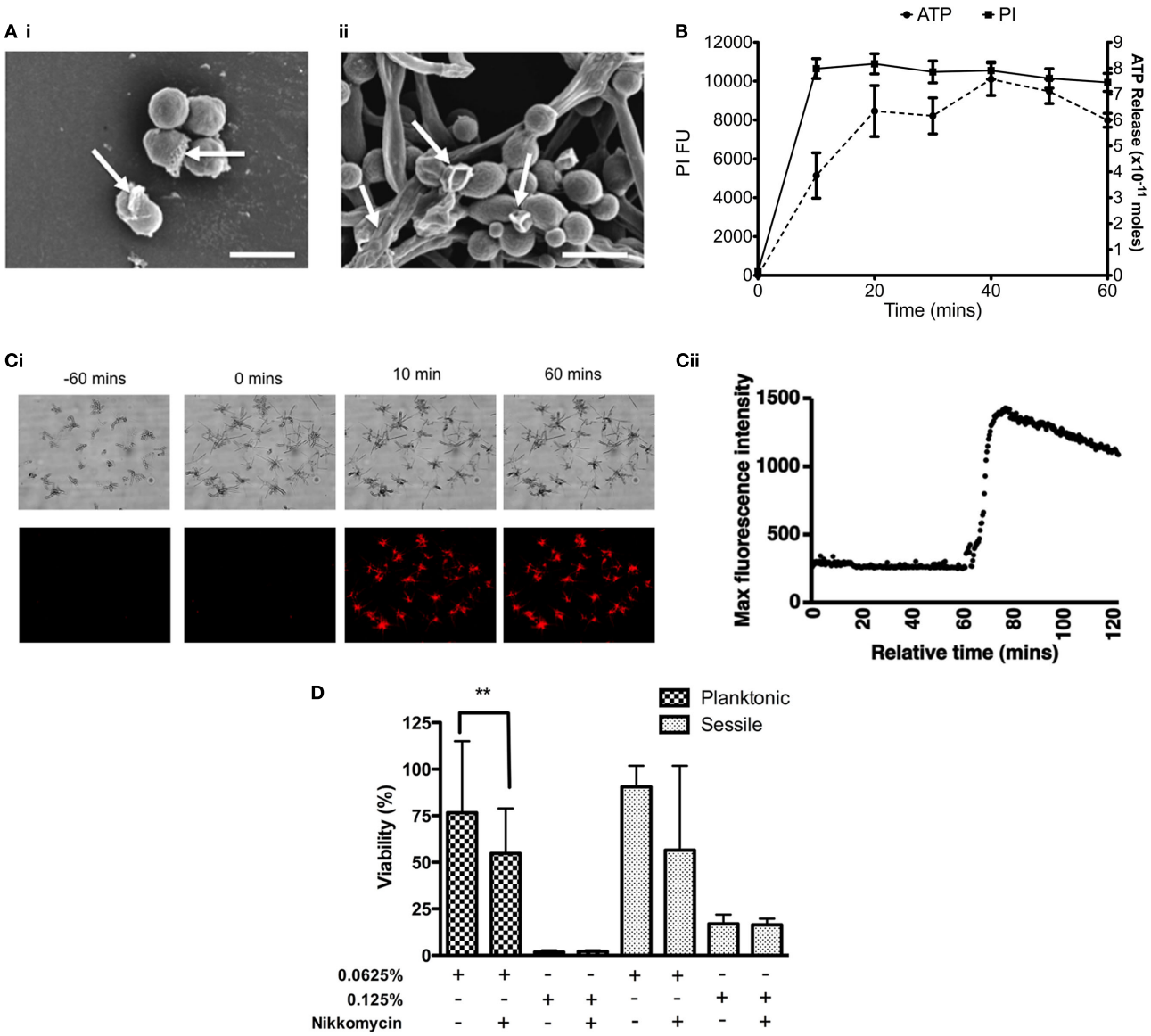

FIGURE 2 | Carbohydrate derived fulvic acid permeabilizes *Candida albicans* cell membranes. (A) Planktonic cells **(i)** and sessile **(ii)** cells were treated at their respective MIC_{90} for 24 h either in solution or on Thermanox™coverslips, respectively. These were then processed and viewed on a JEOL JSM-6400 scanning electron microscope and images assembled using Photoshop software. Note the apparent disruption of the cell wall denoted by arrows on both the planktonic and sessile cells. Scale bars represent 5 μm. **(B)** *C. albicans* (SC5314) planktonic cells (5×10^7 cells/ml) were treated with CHD-FA (4%) for 10, 20, 30, 40, 50, and 60 min. A negative control (no CHD-FA) was also included. For PI experiments the cells were washed by centrifugation, resuspended in PI (20 μM in PBS), and incubated for 15 min at 37°C. These were then transferred to a black 96-well plate for quantification in a fluorescent plate reader (Ex_{485}/Em_{620}). For ATP release the cells were removed by filter sterilization (0.22 μm) and supernatants adjusted to a pH of 7.8 in 10 mM NaOH. The assay, including standards, was performed as per manufacturer's instructions in a white well plate for quantification in a luminescent plate reader. Each assay was performed on at least two independent occasions in triplicate. **(C)** *C. albicans* cells were allowed to grow on an Ibidi μSlide VI for 60 min before 20 μM PI and 2% CHD-FA was added. PI uptake was measured over 60 min using time-lapse microscopy, with images taken every 20 s. Note the sudden uptake of PI into *C. albicans* cells after exposure to CHD-FA for 10 min **(i)**, which was validated through quantifiable data that showed a rapid increase in fluorescence when CHD-FA was added **(ii)**. **(D)** *C. albicans* was grown as planktonic and biofilm cells ± nikkomycin Z (0.4 μg/ml) for 24 h. These were then treated with 0.0625 and 0.125% CHD-FA for 24 h. Negative controls were included. Metabolic activity of treated cells was then quantified using the XTT assay. Four isolates were used for each assay, and this was performed on two independent occasions in quadruplicate. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Initial studies aimed to evaluate the activity of CHD-FA and the three major classes of antifungal agent, namely azoles, polyenes, and echinocandins, using a panel of clinical *C. albicans* strains. This was primarily to assess the anti-biofilm activity of each drug compared to planktonic cells rather than direct antifungal drug comparison. CHD-FA was shown to kill rapidly and

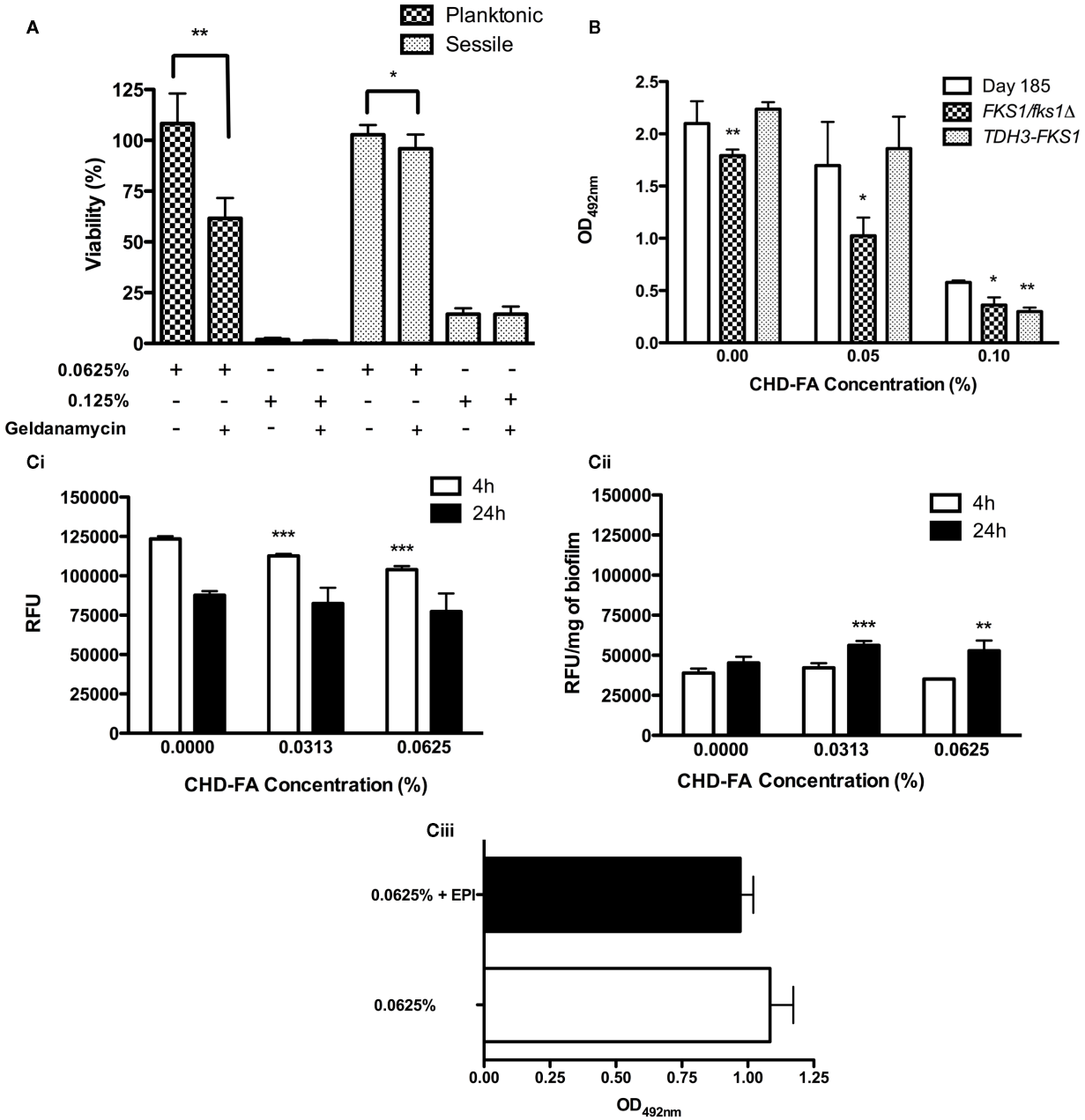

FIGURE 3 | *Candida albicans* biofilm resistance mechanisms play a limited role in CHD-FA sensitivity. (A) *C. albicans* was grown as planktonic and biofilm cells ± geldanamycin (12.5 μg/ml) for 24 h. These were then treated with 0.0625 and 0.125% CHD-FA for 24 h. Negative controls were included. Metabolic activity of treated cells was then quantified using the XTT assay. Four isolates were used for each assay, and this was performed on two independent occasions in quadruplicate. **(B)** *C. albicans* DAY185, *FKS1/fks1*Δ and *TDH3-FKS1* were grown as biofilms for 8 h and treated with 0.05 and 0.1% CHD-FA for 24 h. Negative controls were included. Metabolic activity of treated cells was then quantified using the XTT assay. This was performed on two independent occasions in triplicate. Statistical significance represents the comparison of *FKS1/fks1*Δ to the reference strain DAY185. **(C)** *C. albicans* SC5314 was grown as **(i)** planktonic and **(ii)** biofilm cells and treated with 0.0313 and 0.0625% CHD-FA (sub-MIC levels) for 4 and 24 h. Cells were washed, resuspended in assay buffer and 100 μg/ml of Ala-Nap added. These were then transferred to a black 96-well plate for quantification at 30 s intervals over 60 min in a fluorescent plate reader (Ex_{355}/Em_{460}). For biofilms the relative fluorescence is presented, which is normalized to dry weight of biofilms (RFU/mg). High fluorescence values indicate low efflux activity and vice versa. *C. albicans* 24 h biofilms were treated with 0.0625% CHD-FA ± EPI for 24 h **(iii)**. Biofilms were washed and metabolic activity measured using the XTT metabolic assay with absorbance read at 492 nm. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

be similarly effective against both planktonic and sessile cells for all strains, whereas the existing antifungal agents exhibited increased resistance in the biofilm phenotype. This is in agreement with earlier studies by our group and others who reported

high level azole resistance and decreasing sensitivity of polyenes and echinocandins (Kuhn et al., 2002).

Disruption of the biofilm was also assessed, but despite CHD-FA being highly fungicidal no significant reduction of the biofilm biomass was observed. Nevertheless, given the effectiveness of this compound then it would be possible to combine CHD-FA with a known *C. albicans* biofilm disrupting compound, such as lauroyl glucose (Dusane et al., 2008). Lauroyl glucose is a sugar ester that exhibits antimicrobial and emulsification properties (Ferrer et al., 2005), which contribute to the disruption of preformed *C. albicans* biofilms by up to 45% (Dusane et al., 2008). Therefore, these and other novel biofilm disruption agents could possibly be used to augment the activity of CHD-FA.

The use of SEM demonstrated the appearance of crenated cells, both planktonically and in biofilms. Visually, these appear similar to polyene treated cells, which integrates into and actively disrupts cell membranes, lysing their cytoplasmic contents (Gale, 1963). We therefore hypothesized that CHD-FA activity was potentially based on interaction with the cell membrane; an idea supported from the activity of CHD-FA against bacteria (van Rensburg et al., 2000). Membrane permeabilization assays were therefore used to confirm our hypotheses.

The measurement of intracellular ATP release has been previously described as an appropriate method to measure membrane permeability, as ATP leakage is not observed in viable cells (Smelt et al., 1994). We showed ATP release occurred in a steady manner with maximum release being observed after 40 min CHD-FA exposure, with a slight reduction observed thereafter; a similar phenomenon to a previous report (Smelt et al., 1994). PI uptake was in agreement with ATP release, again showing a time dependent disruption of the cell membrane. We demonstrated that *C. albicans* membrane integrity is compromised rapidly after exposure to CHD-FA, data supported visually from our fluorescent images showing PI uptake in as little as 1 min post-exposure (Ahmad et al., 2011).

To further test our cell membrane hypothesis we weakened the cell wall by inhibiting chitin biosynthesis using the chitin synthase inhibitor NKZ, which has been shown to inhibit all three Chs isozymes in *C. albicans*, and in turn results in a lack of chitin in fungal cells (Kim et al., 2002). We reasoned that if the membrane were the active target then the weakened cell would become hyper-susceptible to CHD-FA. We demonstrated that NKZ in combination with CHD-FA made both planktonic and sessile cells more susceptible to CHD-FA, which is similar to previous work showing that NKZ in combination with azoles increased *C. albicans* biofilm susceptibility (Kaneko et al., 2010).

Our group, amongst others, have shown the importance of biofilms in antimicrobial resistance, a multifactorial process (Ramage et al., 2009). We therefore aimed to evaluate the potential effects of key biofilm resistance mechanisms on CHD-FA. The major regulator of stress in *C. albicans* is Hsp90, which has been shown to potentiate the emergence and maintenance of resistance to azoles and echinocandins in *C. albicans* biofilms, at least in part via calcineurin (Robbins et al., 2011). Our study has also shown that Hsp90 plays a role in stress induced resistance, as planktonic and sessile *C. albicans* cells were more susceptible to CHD-FA at a sub-MIC level when geldanamycin, the Hsp90 inhibitor, is used in combination. Therefore, stress induced resistance mechanisms appear to play a role in *C. albicans* response to CHD-FA, however, this is more apparent in planktonic cells.

We next investigated the role of biofilm ECM, a key component and defining characteristic of fungal biofilms (Baillie and Douglas, 2000). Recent work by the Andes group has shown that ECM β-1,3 glucan, synthesized from Fks1p, is responsible for sequestering antifungal drugs and acting as a "drug sponge" (Nett et al., 2010a,b), therefore conferring resistance on *C. albicans* biofilms. Therefore, we aimed to determine whether CHD-FA was sequestered by ECM β-1,3 glucan in the same way as other classes of antifungal agents. For this we utilized strains used in the previous investigations (Nett et al., 2010b), where it was shown that *FKS1/fks1*Δ biofilm sensitivity to fluconazole was exquisite in comparison to the biofilm resistant parental strain and the ECM β-1,3 glucan over-expressing strain *TDH3-FKS1*, which were highly resistant to fluconazole. In our investigations we have shown the *FKS1/fks1*Δ, *TDH3-FKS1* and the reference strain, DAY185, were equally sensitive at $1 \times MIC_{50}$ to CHD-FA. This is surprising given that Nett et al. (2010a) have shown that ECM impacts other antifungal classes. This suggests in our study that ECM β-1,3 glucan does not sequester CHD-FA, unlike azoles, polyenes, and echinocandins (Nett et al., 2010a).

Finally, we investigated whether efflux pumps are affected by CHD-FA, as these have previously been shown to be expressed in *C. albicans* biofilms (Ramage et al., 2002). In this study we showed efflux pump activity in both planktonic and sessile *C. albicans* cells, but only significant up-regulation after 4 h treatment in planktonic cells at sub-MIC levels of CHD-FA. However, overall efflux pump activity in *C. albicans* biofilms was found to be higher when compared to planktonic cells, which was predicted based on previous literature (Ramage et al., 2002). In order to determine their contribution to potential resistance to CHD-FA we inhibited efflux pump activity using a competitive substrate. Efflux pump inhibitor studies have previously shown, with the use of mutants with single and double deletion mutations, that *C. albicans* have reduced mRNA expression for various multidrug resistant efflux pumps (Ramage et al., 2002). Here we showed that this compound did not alter the MIC. These data indicates that CHD-FA is not impacted by efflux pump activity, despite the fact that these pumps were induced through treatment. This is unsurprising given that CHD-FA acts on the cell membrane and not through an internal cellular mechanism.

CHD-FA is a microbicidal compound that acts non-specifically on the cell membrane. Therefore, it may serve as a potential novel antiseptic agent for the treatment of oral candidosis and other candidal biofilm infections. Increasingly *Candida* biofilms are becoming clinically important (Tumbarello et al., 2007; Ramage et al., 2009), particularly because of inappropriate or misuse of broad spectrum antimicrobials that are ineffective against recalcitrant biofilms (Niimi et al., 2010). In the context of oral health, individuals suffer from a range of oral candidal diseases that cause high levels of morbidity. Moreover, there are many other oral diseases of microbial biofilm origin, such as caries and periodontitis, which would benefit from novel antiseptic molecules, assuming these meet appropriate safety standards. CHD-FA has already shown to have no sign of toxicity in rats and humans, and in

fact was reported to have anti-inflammatory and wound healing properties, possibly through a free radical scavenging mechanism (Gandy et al., 2011; Sabi et al., 2011). Whilst CHD-FA appears to have appropriate biological properties of an antiseptic, further cellular and *in vivo* studies are required.

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The effect of standard heat and filtration processing procedures on antimicrobial activity and hydrogen peroxide levels in honey

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There is increasing interest in the antimicrobial properties of honey. In most honey types, antimicrobial activity is due to the generation of hydrogen peroxide (H_2O_2), but this can vary greatly among samples. Honey is a complex product and other components may modulate activity, which can be further affected by commercial processing procedures. In this study we examined honey derived from three native Australian floral sources that had previously been associated with H_2O_2-dependent activity. Antibacterial activity was seen in four red stringybark samples only, and ranged from 12 to 21.1% phenol equivalence against *Staphylococcus aureus*. Antifungal activity ranged from MIC values of 19–38.3% (w/v) against *Candida albicans*, and all samples were significantly more active than an osmotically equivalent sugar solution. All honey samples were provided unprocessed and following commercial processing. Processing was usually detrimental to antimicrobial activity, but occasionally the reverse was seen and activity increased. H_2O_2 levels varied from 0 to 1017 μM, and although samples with no H_2O_2 had little or no antimicrobial activity, some samples had relatively high H_2O_2 levels yet no antimicrobial activity. In samples where H_2O_2 was detected, the correlation with antibacterial activity was greater in the processed than in the unprocessed samples, suggesting other factors present in the honey influence this activity and are sensitive to heat treatment. Antifungal activity did not correlate with the level of H_2O_2 in honey samples, and overall it appeared that H_2O_2 alone was not sufficient to inhibit *C. albicans*. We conclude that floral source and H_2O_2 levels are not reliable predictors of the antimicrobial activity of honey, which currently can only be assessed by standardized antimicrobial testing. Heat processing should be reduced where possible, and honey destined for medicinal use should be retested post-processing to ensure that activity levels have not changed.

Keywords: honey, Australia, *Staphylococcus aureus*, *Candida albicans*, hydrogen peroxide, heat treatment

INTRODUCTION

Honey has been widely used for thousands of years, not only in food and beverages but also for treating diseases (Blair and Carter, 2005). As a complex natural product, there are a variety of factors that contribute to the antimicrobial activity of honey. The primary antimicrobial component in most honeys is hydrogen peroxide (H_2O_2), which is produced by the bee-derived enzyme glucose oxidase (White et al., 1963). Certain honey types contain additional antimicrobial activity, which has been attributed to various different components including methylglyoxal (MGO), bee defensin-1, and other bee-derived compounds, florally derived phenolics, lysozyme, and other yet undetermined compounds (Estevinho et al., 2008; Irish et al., 2008; Mavric et al., 2008; Adams et al., 2009; Kwakman et al., 2010, 2011). Antimicrobial activity derived from these components has been grouped together and is generally referred to in the literature as "non-peroxide dependent" activity (Blair and Carter, 2005).

Honey is broad-spectrum and active against a range of different bacteria and fungi (Molan, 1992, 2009). Transcriptome and proteome studies on how bacteria respond to treatment have found honey to have a unique and multimodal mode of action (Blair et al., 2009; Packer et al., 2012). In addition, unlike most antibiotics, resistance to honey cannot be induced (Blair et al., 2009). These features make honey an attractive alternative treatment, particularly for topical application to skin and mucosal membranes (English et al., 2004; Chambers, 2006; Molan, 2006b).

Australia has a diverse, unique natural flora, and honey production is a multi-million dollar industry. Although predominantly destined for table use, some antimicrobial Australian honey is also produced and marketed. Recently, a survey of the antibacterial properties of honey derived from a wide range of Australian plants was undertaken that demonstrated the potential of Australian floral sources for the production of medical-grade honey (Irish et al., 2011). As well as finding H_2O_2- and MGO-type activity, this study found some honey samples had antimicrobial activity that was clearly different to known peroxide and non-peroxide activities.

The current study was undertaken to follow up some of the native Australian honeys investigated by Irish et al. (2011) in order to determine whether some of the more promising floral sources would produce consistently active honey, and to assess if new, as yet undefined non-peroxide activities could be identified. During the course of honey testing we were provided with samples that were completely unprocessed, and with aliquots of the same honey samples that undergone heating and filtration procedures used in the honey industry to remove wax and particulate matter and to prevent granulation. Heat treatment is relatively mild (~45°C for up to 8 h) and does not appear to affect MGO levels (Matheson and Murray, 2011) but might affect enzymes such as glucose oxidase or other non-peroxide factors. Since antimicrobial testing is generally conducted on raw, unprocessed honey, but heat and filtration are required to process honey, we were interested to see how antimicrobial activity was affected by these methods. Finally, as the addition of catalase reduced the antimicrobial activity in all samples to insignificant levels and there was no apparent non-peroxide activity, we assessed the levels of H_2O_2 to determine how this correlated with antimicrobial potency.

MATERIALS AND METHODS

HONEY SAMPLES

All honeys were sourced and supplied by Beechworth Honey (Corowa, NSW, Australia) and are listed in **Table 1**. Honey derived from three native Australian floral sources were tested, with five independent samples of each. These included spotted gum (*Eucalyptus maculata*) (samples S1–S5), red stringybark (*Eucalyptus macrorrhyncha*) (samples R1–R5), and yellow box (*Eucalyptus melliodora*) (samples Y1–Y5). One mixed sample of canola/red stringybark (R6) and one pure sample of canola honey (*Brassica napus*) (C1) were also included. It should be noted that the identified plants only represent the major source of honey for that sample; honeys are rarely derived from only one species, and other floral sources may contribute to any one batch. An artificial honey (7.5 g sucrose, 37.5 g maltose, 167.5 g glucose, and 202.5 g fructose in 85 mL sterile water) was used to simulate osmotic factors due to the high sugar level in honey. Comvita UMF® 18+ manuka honey (Te Puke, New Zealand) was used as a positive control in the phenol equivalence assay.

HONEY TREATMENT

"Unprocessed" and "processed" versions of each honey sample were supplied and tested. The unprocessed samples were supplied directly as they had been obtained from beekeepers and had not undergone any heating or filtration. Processed aliquots of each of the same honey samples had been subjected to standard commercial treatment at Beechworth Honey, which involved heating the bulk honey purchase to 45°C for 8 h and filtering with a 100 μm filter.

Immediately prior to antimicrobial tests, all honey samples were diluted and filtered through a 0.2 μm fliter (Millipore) in the laboratory to eliminate contaminating micro-organisms. For the assessment of non-peroxide activity, catalase (Sigma-Aldrich, USA) was added to samples at a final concentration of 2800 U/mL prior to testing.

ASSESSMENT OF ANTIBACTERIAL ACTIVITY

The antibacterial activity of honey samples was assessed using the standard method described by Allen et al. (1991a). This measures the inhibition of *Staphylococcus aureus* strain ATCC 25923 (Oxoid, Hampshire, UK) by honey in an agar well diffusion assay and reports activity as equivalence to dilutions of phenol. Briefly, an 18 h culture of *S. aureus* grown in tryptone soy broth (TSB) was adjusted to 0.5 at A_{540} nm (approx. 5×10^7 cells/ml). One hundred and fifty mL of molten, cooled nutrient agar (BD Difco, USA) was seeded with 100 μL of the prepared *S. aureus* culture and poured into a large square bioassay plate (245 × 245 mm; Corning). Plates were stored inverted at 4°C for use the next day, when wells were cut into the agar with a sterile 8 mm diameter cork borer. Each well was numbered, in duplicate, using a quasi-Latin square, which enabled duplicate samples to be placed randomly on the plate.

Fifty percent (w/v) of each honey sample, including the Comvita and the artificial honey, were prepared fresh for each assay in sterile deionized water, and incubated at 37°C with shaking at 200 rpm for 30 min to aid mixing. Diluted honey samples were then filter sterilized through 0.2 μm pore filters (Millipore) and mixed with equal volumes of either sterile deionized water for total activity testing, or freshly prepared 5600 U/mL catalase solution (Sigma-Aldrich, USA) for non-peroxide activity testing, to give a final concentration of 25% (w/v) honey. Aliquots of 100 μL of each solution were placed into wells of the assay plates.

Phenol (Sigma-Aldrich, USA) standards of 2, 3, 4, 5, 6, and 7% were prepared from a 10% w/v solution that was freshly made every four weeks in sterile deionized water and stored at 4°C. Aliquots of 100 μL of each phenol dilution were placed in duplicate wells of the assay plates. Artificial honey, sterile deionized water and catalase solution were included as negative controls. The plates were incubated at 37°C for 18 h.

The diameter of each zone of inhibition was measured using Vernier calipers. The mean diameter of the zone of inhibition around each well was squared, and a phenol standard curve was generated with phenol concentration against the mean squared diameter of the zone of inhibition. The activity of each honey sample was calculated using the standard curve. To account for the dilution and density of honey, this figure was multiplied by 4.69 (based on a mean honey density of 1.35 g/mL, as determined by Allen et al. (1991b), and the activity of the honey was then expressed as the equivalent phenol concentration (% w/v). Each honey sample was tested on at least three separate occasions, and the mean phenol equivalence was recorded.

ASSESSMENT OF ANTIFUNGAL ACTIVITY

As there is no standardized method for assessing the antifungal activity of honey, this was done using the CLSI (formerly NCCLS) microdilution method with some modifications as described by Irish et al. (2006). This method, which has been developed to assess minimum inhibitory concentrations (MICs) of antibiotics, was used to assess the MIC of each honey against *Candida albicans* ATCC 10231 (Oxoid, Hampshire, UK). Briefly, honey samples were prepared by the addition of RPMI-1640 medium (Sigma-Aldrich, USA) to make 50% (w/v) stock

Table 1 | Antibacterial activity, antifungal activity, and hydrogen peroxide concentrations of honey samples before and after heat and filtering processes.

Honey ID	Honey type	Antibacterial activity (% phenol equivalence against *S. aureus*)			Antifungal activity (MIC % [w/v] honey against *C. albicans*)			Hydrogen peroxide concentration (μM)		
		Unprocessed	Processed	Change following processing[a]	Unprocessed[b]	Processed[b]	Change following processing[a]	Unprocessed	Processed	Change following processing (% remaining)[a]
		Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
R1	Red stringybark	14.9 ± 0.9	11.9 ± 1.2	−3.0	28.3 ± 0.6**	18.0 ± 2.7**	−10.3*	526 ± 24.7	553 ± 31.8	26 (105)
R2	Red stringybark	21.2 ± 1.1	14.0 ± 0.4	−7.2*	19.0 ± 0.0**	20.0 ± 0.0**	1.0	796 ± 1.2	739 ± 20.0	−57 (93)
R3	Red stringybark	14.1 ± 0.9	11.4 ± 0.9	−2.7*	19.3 ± 0.6**	18.0 ± 1.4*	−1.3	1017 ± 10.6	627 ± 3.5	−390* (62)
R4	Red stringybark	No activity	No activity	–	35.0 ± 0.0**	35.0 ± 0.0**	0.0	0	0	–
R5	Red stringybark	No activity	No activity	–	34.3 ± 0.6**	38.3 ± 2.1	4.0*	0	0	–
R6	Canola/Red stringybark	12.0 ± 1.6	No activity	−12.0*	24.3 ± 1.2**	28.5 ± 0.7**	4.2*	916 ± 1.8	321 ± 9.5	−595* (35)
Y1	Yellowbox	No activity	No activity	–	35.0 ± 0.0**	37.3 ± 4.0	2.3	0	0	–
Y2	Yellowbox	No activity	No activity	–	38.0 ± 0.0**	39.0 ± 1.0	1.0	0	0	–
Y3	Yellowbox	No activity	No activity	–	29.0 ± 0.0**	27.5 ± 2.1	−1.5	543 ± 7.7	279 ± 9.4	−265* (51)
Y4	Yellowbox	No activity	No activity	–	33.3 ± 1.2**	33.3 ± 1.2**	0.0	645 ± 7.1	0	−645* (0)
Y5	Yellowbox	No activity	No activity	–	31.7 ± 0.6**	34.7 ± 0.6**	3.0*	488 ± 1.8	0	−488* (0)
S1	Spotted gum	No activity	No activity	–	33.0 ± 1.0**	29.0 ± 2.9**	−4.0	151 ± 1.2	122 ± 7.1	−29 (81)
S2	Spotted gum	No activity	No activity	–	30.3 ± 0.6**	38.7 ± 2.9	8.4*	284 ± 7.6	0	−284* (0)
S3	Spotted gum	No activity	No activity	–	35.7 ± 2.1**	40.7 ± 2.1	5.0*	0	0	–
S4	Spotted gum	No activity	No activity	–	36.3 ± 1.2**	38.0 ± 1.0*	1.7	0	0	–
S5	Spotted gum	No activity	No activity	–	30.3 ± 0.6**	36.3 ± 2.3	6.0*	426 ± 19.4	0	−426* (0)
C1	Canola	No activity	No activity	–	38.3 ± 0.6**	42.5 ± 1.0$^{\infty}$	4.2*	754 ± 5.2	0	−754* (0)
	Artificial honey	No activity	No activity		40.7 ± 2.9	40.7 ± 0.6	–	0	0	–

[a] *Values in red indicate drop in activity or H_2O_2 concentration following processing; * Denotes significant change $p < 0.05$.*

[b] *Significantly different MIC to artificial honey: Lower: * $p < 0.05$; ** $p < 0.01$; Higher: $^{\infty}$ $p < 0.05$.*

solutions and incubated at 37°C with shaking at 200 rpm for 30 min to aid mixing. The diluted honey solutions were filter sterilized through 0.2 μm pore filters (Millipore) and further diluted with RPMI-1640 medium in 96-well U-bottomed microtitre plates to give final honey concentrations in 1% (w/v) increments from 10 to 50%. Artificial honey was included as an osmotic control.

C. albicans cultures were prepared by picking five colonies from an overnight yeast peptone dextrose agar plate and suspending them in 5 mL 0.85% saline. Transmittance of the culture was measured at 530 nm and adjusted to 0.8–0.88. Adjusted cultures were diluted 1:50 in sterile 0.85% saline, then further diluted 1:4 in RPMI-1640 medium, to achieve a working concentration of 5×10^3 to 2.5×10^4 cfu/mL. Twenty-five μL of the diluted culture was added to each well of the microtitre plate, resulting in a final inoculum of 0.5 to 2.5×10^3 cfu/mL. Growth controls (no honey added) and sterility controls (RPMI-1640 medium only and honey solution only) were included in each plate. Following incubation at 35°C for 24 h, the MIC was recorded as the lowest concentration of honey that prevented growth, which was assessed visually. Each honey sample was tested in duplicate and the assays were repeated on at least three separate occasions, with the mean MIC recorded.

HYDROGEN PEROXIDE ASSAY

The concentration of H_2O_2 in honey samples was determined using a colorimetric assay that has previously been used to measure H_2O_2 in honey (Kwakman et al., 2010). Fifty percent (w/v) honey solutions were made by the addition of sterile deionized water to the honey samples. Samples were incubated at 37°C with shaking at 200 rpm for 30 min to aid mixing. The diluted honey solutions were filter sterilized through 0.2 μm pore filters (Millipore, USA) and further diluted to 25% (w/v) by mixing with either sterile deionized water or 5600 U/mL catalase solution (Sigma-Aldrich, USA). Aliquots of 40 μL of each honey sample were added to wells of a 96-well flat-bottomed microtiter plate in triplicate. H_2O_2 (Sigma-Aldrich, USA) standards ranging from 2.1 to 2200 μM were made by 2-fold serial dilutions and 40 μL of each standard was added to the plates. Sterile deionized water and catalase solution were also included as negative controls.

The reagent mixture consisting of 50 μg/mL of *O*-dianisidine (Sigma-Aldrich, USA) and 20 μg/mL H_2O_2 type IV (Sigma-Aldrich, USA) in 10 mM phosphate buffer (pH 6.5), was freshly made from stock solutions of 1 mg/mL stock of *O*-dianisidine and 10 mg/mL of horseradish peroxidise type IV. One hundred and thirty-five μL of this reagent mixture was added to wells of the microtiter plate containing the honey samples and H_2O_2 standards prepared as outlined above. Following incubation for 5 min at room temperature, reactions were stopped by the addition of 120 μL 6 M H_2SO_4. The color of the reaction was measured by absorbance at 560 nm using a Multiskan Ex plate reader (Thermo Scientific, USA), and H_2O_2 concentrations were calculated using a standard curve derived from the H_2O_2 standards. Each honey sample was tested in triplicate and assays were repeated on three separate occasions, giving a total of nine readings per honey sample.

STATISTICAL ANALYSIS

Statistical analysis of the data was performed using IBM SPSS Statistics 19 software. Differences between the activity of the honey samples and the artificial honey were evaluated using the independent samples *t*-test. This test was also used to compare the activity of the different honey types. Correlation analysis was done using Spearman's Rank Correlation with an online tool available at http://www.wessa.net (Wessa, 2011).

RESULTS

ANTIBACTERIAL ACTIVITY OF RAW AND PROCESSED HONEY SAMPLES

Four out of the 17 unprocessed honey samples (35%) had detectable antibacterial activity against *S. aureus* (**Table 1**). Activity in these samples was eliminated following the addition of catalase, and none exhibited detectable non-peroxide activity (data not shown). The four active honeys were red stringybark samples R1, R2, R3, and R6, and these had phenol equivalence values ranging from 12.0 to 21.2% (w/v). Following heat treatment and filtration, antibacterial activity was detected in only three red stringybark samples, and these were all significantly lower than the corresponding unprocessed honey samples. Activity was completely lost from sample R6, the red stringybark/canola blend (**Table 1**).

ANTIFUNGAL ACTIVITY OF RAW AND PROCESSED HONEY SAMPLES

All of the unprocessed honey samples had significantly higher antifungal activity than the artificial honey sample ($p < 0.05$; **Table 1**), however, this was also reduced to insignificant levels following catalase treatment (data not shown). The majority of the unprocessed honey samples had high MICs, corresponding to low antifungal activity, with 12 of the 17 honeys exhibiting MICs >30% (**Table 1**). Only two of the honeys (R2 and R3) had MICs <20% and three of the honeys (R1, R6, and Y3) had MICs <30%.

Most of the processed honey samples also had lower antifungal activity than the unprocessed honeys, and only nine samples remained more active than the artificial honey (**Table 1**). However, in red stringybark sample R1 the reverse was seen, and the processed sample was significantly more active than its unprocessed counterpart.

EFFECT OF PROCESSING ON THE PRODUCTION OF HYDROGEN PEROXIDE

H_2O_2 was measured in the honey samples before and after processing to determine how this was affected by heat processing. Eleven of the 17 unprocessed honey samples produced detectable H_2O_2 (**Table 1**). Four of the unprocessed red stringybark honey samples (R1, R2, R3, and R6) had among the highest H_2O_2 concentrations, which was consistent with the high antibacterial and antifungal activity seen in these samples However, H_2O_2 was also produced in unprocessed yellow box (Y3, Y4, and Y5), spotted gum (S1, S2, and S5) and canola honey (C1) samples that did not have any detectable antibacterial activity.

Correlation analysis using Spearman's Rank Correlation indicated that H_2O_2 production and antibacterial activity in the unprocessed honey samples was positively correlated (rho = 0.64; $p = 0.005$). However, this appeared to be driven by the samples

with no H_2O_2 production, which also showed no antibacterial activity (samples R4, R5, Y1, Y2, S3, and S4). When these were removed from the analysis, correlation was lost (rho = 0.59, $p = 0.056$). In contrast, in the processed samples H_2O_2 production and antibacterial activity were strongly correlated across the entire dataset (rho = 0.77; $p = 0.0002$), and this remained (albeit reduced) when processed samples without H_2O_2 were excluded (rho = 0.88; $p = 0.02$). These results suggest one or more components are present in the unprocessed honey samples that modulate the inhibition of bacteria by H_2O_2, and these are sensitive to heat processing.

Antifungal activity was strongly correlated with H_2O_2 production in both the processed and the unprocessed samples ($p < 0.005$). However, this correlation was again lost when samples with no H_2O_2 production were removed from the analysis (rho = −0.53, $p = 0.096$; and rho = −0.71, $p = 0.11$ for unprocessed and processed samples, respectively).

Detectable H_2O_2 production remained in only six of the 17 honey samples following processing (**Table 1**). Red stringybark honeys R1, R2, R3, and R6 had the highest levels of H_2O_2, however, processing affected their H_2O_2 production differently, with a significant reduction seen in samples R3 and R6 but no significant change in samples R1 and R2. H_2O_2 was no longer produced by the majority of the processed yellow box and spotted gum samples, and it was undetectable in samples Y4, Y5, S5, and C1, which all had high H_2O_2 levels before processing yet had no detectable antibacterial activity. Overall, while it was apparent that heating the honeys negatively affected H_2O_2 production, the extent to which this happened varied in the different honey samples. Within the subset of samples where H_2O_2 was produced, there was no correlation between the amount produced before and after processing (rho = 0.46; $p = 0.15$).

DISCUSSION

There is increasing recognition of the value of medicinal honey, both as a high-value product that can be produced commercially in many parts of the world, including in rural and resource-poor settings, and as a potently active medicine that is effective against antibiotic-resistant pathogens. However, the parameters surrounding the reliable production of medicinally active honey remain poorly understood. Antimicrobial assays are usually performed on raw, unprocessed honey that is diluted and filtered to eliminate microorganisms prior to testing but is not heat-treated (Irish et al., 2011), but this may not be accurate if the honey must be heated subsequently to filter out particulate debris. In the current study, we assessed the antimicrobial properties of a number of independent samples of three common Australian honeys and investigated the effect of mild processing using heating and filtering methods that are routine for commercial honey production. In addition, as the current microbiological tests for antimicrobial activity are relatively labor-intensive, we analyzed whether floral source or H_2O_2 levels might be useful predictors of antimicrobial activity.

RED STRINGYBARK: A USEFUL FLORAL SOURCE FOR MEDICINAL HONEY?

Honey produced from native Australian flora has the potential for therapeutic use, firstly because a number of floral sources produce active honey (Lusby et al., 2005; Irish et al., 2011), and secondly because many Australian native forests occur in relatively remote areas that are likely to be free from pesticides and pollutants that could be introduced into the honey during production (Feás and Estevinho, 2011). Among the honeys selected for this study, some red stringybark samples displayed antibacterial activity at a potentially therapeutically useful level (**Table 1**; Molan, 1999). In a large-scale survey undertaken by Irish et al. (2011), different red stringybark samples had similar, relatively high antibacterial activities. However, in the current study there were large variations in activity among the different red stringybark samples. Similarly, although Irish et al. found Australian spotted gum honeys had antibacterial activity [median of 18.9% (w/v) phenol equivalence], none of the spotted gum samples in the current study showed activity. The current findings, and those from other studies (Allen et al., 1991a; Al-Jabri et al., 2003; Irish et al., 2011), indicate that while floral source is an important determinant of antimicrobial activity, it remains difficult to use this to predict the antimicrobial properties of a given honey sample. Therefore, while this work suggests that red stringybark could be a useful floral source for the production of medically active honey, the inconsistency among samples means individual samples still need to be screened for activity.

HYDROGEN PEROXIDE PRODUCTION IS NOT ALWAYS SUFFICIENT FOR ANTIMICROBIAL ACTIVITY

As antimicrobial activity was reduced to insignificant levels when the honey samples were treated with catalase it was assumed that the production of H_2O_2 was responsible for most or all of the observed activity. In honey, glucose oxidase, which is secreted from the hypopharyngeal glands of bees, breaks down glucose to form gluconic acid and H_2O_2. Lack of free water and an acidic pH renders glucose oxidase inactive, but activity is restored when the honey is diluted with water, providing a slow, sustained release of H_2O_2, at sufficient levels to produce an antimicrobial effect but not high enough to damage mammalian tissues (Bang et al., 2003). In the current study, honey was diluted four-fold, which is optimal for H_2O_2 production from most honey types (Brudzynski et al., 2011).

Although there was a high level of correlation between the level of H_2O_2 produced by honey samples and their level of antibacterial and antifungal activity, which is consistent with other reports (White et al., 1963; Taormina et al., 2001; Brudzynski, 2006), this was lost once samples without any detectable H_2O_2 were excluded from the analysis; the only exception being the processed honey samples and their antibacterial activity. This suggests that H_2O_2 alone may not be sufficient for antimicrobial activity: honey samples with little or no H_2O_2 have a correspondingly low ability to inhibit bacteria and fungi, but if present, the level of H_2O_2 and the degree to which the honey is antimicrobial do not necessarily correlate. Indeed, some samples, such as canola honey C1 and yellowbox honey Y4 had particularly high H_2O_2 levels (754 and 645 μM, respectively) yet no antibacterial activity and very low antifungal activity, while sample R1 had 526 μM H_2O_2, but was among the most active of the honey samples.

Other studies have found that the level of H_2O_2 present in honey is more than 900-fold lower than expected based on the level of antimicrobial activity, and it has been suggested that there are one or more synergents present in honey that augment the action of H_2O_2 (Molan, 2006a; Kwakman et al., 2010; Brudzynski et al., 2011). It is possible that these synergents do not occur in samples where H_2O_2 was produced but little or no activity was seen. Alternatively, there may be other, as yet undefined compounds present in the inactive honey samples that interfere with the antimicrobial activity of H_2O_2. An interesting area of further study would be to compare the components present in honey samples with very different levels of antimicrobial activity but similar H_2O_2 levels (e.g., red stringybark sample R2 vs. canola honey C1), which may allow these possible synergents or agonists to be identified.

STANDARD PROCESSING REDUCES THE ANTIMICROBIAL PROPERTIES OF HONEY BUT EFFECT VARIES AMONG SAMPLES

Processed honey samples had on average lower antifungal and antibacterial activity. Average antibacterial levels in the active samples (R1, R2, R3, and R6) dropped from 15.6 to 9.3% phenol equivalence. A significant reduction was seen in all but sample R1, and in sample R6, the red stringybark/canola blend, activity was lost altogether. Similarly, the average antifungal MIC changed from 31 to 33%, and for the canola honey C1, the MIC became significantly higher than for the artificial honey (**Table 1**; $p < 0.05$), indicating that this honey has less antifungal activity than an osmotically equivalent sugar solution. The change to antifungal activity following processing varied considerably among the different samples: only seven of the 17 samples dropped significantly in activity, nine were unchanged and in sample R1 the activity level significantly increased, with the MIC changing from 28.6 to 18 % (w/v) post-processing.

Heating above physiological temperatures is generally detrimental to enzymes, and a previous study on glucose oxidase in honey found that heating at 50°C for 20 min significantly reduced enzyme activity (Schepartz and Subers, 1964; White and Subers, 1964). Although most honey samples tested in the current study produced less H_2O_2 after heat treatment, with some dropping to zero, in others there was no significant difference before or after treatment, and overall there was no correlation between the level of H_2O_2 across the different honey samples pre- and post-heat treatment. Of interest is that while high levels of H_2O_2 were seen in some of the unprocessed samples that had no detectable antibacterial activity, only the active red stringybark samples (R1, R2, and R3) retained high ($>500\,\mu M$) H_2O_2 levels post-processing. With only these three stably active samples, the current dataset is too small to derive robust conclusions. However, it is possible that the stability of H_2O_2 production is important in determining the activity of a honey sample, and a honey that loses the ability to produce H_2O_2 following standard heat processing could lose useful therapeutic activity, even if H_2O_2 levels prior to processing were high. Further investigation of this is warranted as a test to predict antibacterial activity based on H_2O_2 stability would be very helpful to the honey industry, and H_2O_2 levels alone appear to be a poor indicator of final activity levels.

The level of glucose oxidase in honey can vary depending on the health of the bees and the quality of their diet (Pernal and Currie, 2000; Alaux et al., 2010). However, the amount of H_2O_2 produced in a given honey sample is not determined by glucose oxidase alone, as honey can also contain catalase, peroxidases, and antioxidants such as gallic acid and caffeic acid that can degrade H_2O_2 or interfere with its ability to damage microbial cells (Weston, 2000; Al-Mamary et al., 2002; Sroka and Cisowski, 2003; Yao et al., 2003; Pyrzynska and Biesaga, 2009). In addition, it was recently reported that MGO directly modifies some proteinacious compounds in honey and if present this may also affect glucose oxidase activity (Majtan et al., 2012). The final level of H_2O_2 in a given honey sample therefore depends on various components, which can be present and active to varying degrees. Since any of these may be affected by honey processing, it is not unexpected that the different honey samples responded quite differently to heat treatment.

All commercial table honey is filtered to remove particulate debris, and heating up to 45°C is regularly used to increase the rate of filtration, but it is important to recognize that even relatively mild heat processing can reduce antimicrobial activity. Honey viscosity does not change appreciably above ~30°C (Matheson and Murray, 2011) and lower processing temperatures may be possible without a significant increase in inconvenience. Other studies have noted a reduction in enzymes, antioxidants and other phytonutrients following processing (Blasa et al., 2006; Turkmen et al., 2006; Ropa, 2010), and again this can vary considerably among samples. Minimal processing is therefore advisable for honey produced for medicinal purposes, and samples should be tested post-processing to ensure antimicrobial activity is not significantly reduced.

HONEY: A COMPLEX NATURAL PRODUCT

The complexity of natural products, including honey, makes them very difficult to standardize and this can affect their acceptance in clinical medicine. However, this complexity also has benefits. Unlike conventional antibiotics it appears to be difficult for microorganisms to become resistant to the effects of honey, probably due to the action of the various active components in honey on multiple microbial targets (Blair et al., 2009). An increasing interest in honey has led to recent studies that have begun to unravel how honey affects microbes at the cellular and molecular levels (Blair et al., 2009; Brudzynski et al., 2011; Kwakman et al., 2011; Kwakman and Zaat, 2012; Packer et al., 2012). New, advanced statistical methods for analyzing complex relationships may also help us to understand this complex process (Reshef et al., 2011). As well as developing a wider acceptance of selected honeys in conventional antimicrobial therapy, further studies could reveal lead compounds for the development of novel antimicrobials, which are urgently required.

CONCLUSIONS

We conclude from this study that floral source and H_2O_2 levels, while important in determining the antimicrobial properties of honey, cannot be used to reliably predict whether

a given honey sample will have antibacterial or antifungal activity. In general, processing with heat and filtration reduces H_2O_2-based activity but this varies in different honey samples. The most active honey samples produced high levels of H_2O_2 both before and after heating, suggesting H_2O_2 stability could be a useful indicator of antimicrobial activity, but further research with a greater number of samples is required to support this observation. The potentially detrimental effects of even mild heating should be taken into account when processing and testing honey destined for medicinal use.

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Food applications of natural antimicrobial compounds

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In agreement with the current trend of giving value to natural and renewable resources, the use of natural antimicrobial compounds, particularly in food and biomedical applications, becomes very frequent. The direct addition of natural compounds to food is the most common method of application, even if numerous efforts have been made to find alternative solutions to the aim of avoiding undesirable inactivation. Dipping, spraying, and coating treatment of food with active solutions are currently applied to product prior to packaging as valid options. The aim of the current work is to give an overview on the use of natural compounds in food sector. In particular, the review will gather numerous case-studies of meat, fish, dairy products, minimally processed fruit and vegetables, and cereal-based products where these compounds found application.

Keywords: antimicrobial compounds, food preservation, natural compounds, essential oils, shelf-life extension

INTRODUCTION

Many food products are perishable by nature and require protection from spoilage during their preparation, storage, and distribution to give them desired shelf life. The demand for minimally processed, easily prepared, and ready-to-eat fresh food products, globalization of food trade, and distribution from centralized processing pose major challenges for food safety and quality. Food products can be subjected to contamination by bacteria and fungi. Many of these microorganisms can cause undesirable reactions that deteriorate flavor, odor, color, sensory, and textural properties of foods. Microbial growth is a major concern because some microorganisms can potentially cause food-borne illness. In packaged foods, growth and survival of common spoilage and pathogenic microorganisms such as *Listeria monocytogenes, Escherichia coli* O157, *Salmonella, Staphylococcus aureus, Bacillus cereus, Campylobacter, Clostridium perfringens, Aspergillus niger,* and *Saccharomyces cerevisiae* are affected by a variety of intrinsic factors, such as pH and presence of oxygen or by extrinsic factors associated with storage conditions, including temperature, time, and relative humidity (Singh et al., 2003; Lòpez-Malo et al., 2005; Rydlo et al., 2006).

To prevent growth of spoilage and pathogenic microorganisms in foods, several preservation techniques, such as heat treatment, salting, acidification, and drying have been used in the food industry (Davidson and Taylor, 2007; Farkas, 2007). Numerous efforts are conducted to find natural alternatives to prevent bacterial and fungal growth in foods. In recent years, because of the great consumer awareness and concern regarding synthetic chemical additives, foods preserved with natural additives have become very popular. To inhibit growth of undesirable microorganisms in food, the antimicrobials can be directly added into the product formulation, coated on its surface or incorporated into the packaging material. Direct incorporation of active agents into food results in an immediate but short-term reduction of bacterial populations, while the antimicrobial films can maintain their activity for a long period of time (Appendini and Hotchkiss, 2002; Hanušová et al., 2009).

Main natural compounds are essential oils derived from plants (e.g., basil, thyme, oregano, cinnamon, clove, and rosemary), enzymes obtained from animal sources (e.g., lysozyme, lactoferrin), bacteriocins from microbial sources (nisin, natamycin), organic acids (e.g., sorbic, propionic, citric acid) and naturally occurring polymers (chitosan). In this context, plant essential oils are gaining a wide interest in food industry for their potential as decontaminating agents, as they are Generally Recognized as Safe (GRAS). The active components are commonly found in the essential oil fractions and it is well established that most of them have a wide spectrum of antimicrobial activity, against food-borne pathogens and spoilage bacteria (Gutierrez et al., 2008, 2009). The antimicrobial activity of plant essential oils is due to their chemical structure, in particular to the presence of hydrophilic functional groups, such as hydroxyl groups of phenolic components and/or lipophilicity of some essential oil components (Dorman and Deans, 2000). Usually, the compounds with phenolic groups as oils of clove, oregano, rosemary, thyme, sage, and vanillin are the most effective (Skandamis et al., 2002). They are more inhibitory against Gram-positive than Gram-negative bacteria (Mangena and Muyima, 1999; Marino et al., 2001).

Allyl-isothiocyanate is the major antimicrobial component of mustard and horseradish oil. It has been found to be more effective against Gram-negative bacteria with less or no effect on lactic acid bacteria. Although its antimicrobial activity varies widely (Delaquis and Mazza, 1995), the volatile compound particularly inhibits *E. coli* (Nadarajah et al., 2002; Muthukumarasamy et al., 2003).

The use of bacteriocin-producing lactic acid bacteria or their more or less purified bacteriocins has been also receiving increased interest. Bacteriocins are small bacterial peptides that show strong antimicrobial activity against closely related bacteria. Nisin is a polypeptide produced by *Lactococcus lactis* spp. It has

been approved as a food additive with GRAS status in over 50 countries worldwide. It has a relatively broad spectrum of activity against various lactic acid bacteria and other Gram-positive bacteria. Moreover, it is particularly effective against heat-resistant bacterial spores of *Clostridium botulinum* and against food-borne pathogens such as *L. mococytogenes, S. aureus,* or *B. cereus* (Brewer et al., 2002; Lopez-Pedemonte et al., 2003; Sobrino-Lopez and Martin-Belloso, 2006). Use of nisin in conjunction with ethylenediamine tetra-acetic acid (EDTA) may increase the effectiveness. Moreover, the effect of nisin can be enhanced by using exposure to chelating agents, sub-lethal heat, osmotic shock and freezing, because these treatments make the cell wall of Gram-negative microorganisms more permeable and therefore more susceptible to the nisin (Gálvez et al., 2007).

The enzymes represent another group of natural compounds that found application in food as valid preservatives. Lysozyme for example, is a lytic enzyme found in foods, such as milk and eggs, which can hydrolyze β-1,4 linkages between N-acetylmuramic acid and N-acetylglucosamin (Cunningham et al., 1991). Commercially, lysozyme has been used primarily to prevent late blowing in semi-hard cheeses, caused by *Clostridium tyrobutyricum*. It is well known that lysozyme is bactericidal against Gram-positive bacteria, whereas it is essentially ineffective against Gram-negative bacteria, owing to the presence of a lipopolysaccharide layer in the outer membrane. It has been recognized since the 1960's that susceptibility of Gram negative bacteria to lysis by lysozyme can be increased by the use of membrane disrupting agents, such as detergents and chelators (EDTA) (Vaara, 1992; Shelef and Seiter, 1993; Branen and Davidson, 2004).

Organic acids and their salts are widely used as chemical antimicrobial agents because their efficacy is generally well understood and cost effective. The most effective organic compounds are acetic, lactic, propionic, sorbic, and benzoic acid. Their antimicrobial effect is based on the increase in proton concentration thereby lowering the external pH. Organic acids may affect the integrity of microbial cell membrane or cell macromolecules or interfere with nutrient transport and energy metabolism, causing bactericidal effect (Ricke, 2003). Production of organic acids had been possible before the discovery of microorganisms, with lactic acid first being commercially produced by fermentation in 1880, but the majority of the organic acids produced were being chemically extracted or synthesized from other chemicals. Mixtures of acids could exert a wider antimicrobial activity than a single organic acid (Theron et al., 2010).

Among the natural antimicrobials, chitosan also received considerable interest for commercial applications. It has been used in medical, food, agricultural, and chemical industry, mainly due to its high biodegradability and antimicrobial properties. The biological activity of chitosan depends on its molecular weight, degree of deacetylation and derivatisation, such as degree of substitution, length, and position of a substitute in glucosamine units of chitosan, pH of chitosan solution and the target organisms (No et al., 2007). It is commercially produced from crab and shrimp shell wastes, with different deacetylation grades and molecular weights and, hence, it possess different functional properties, like emulsification ability, dye binding, and gelation. Chitosan has also been documented to possess a film-forming property for use as edible film or coating, to decrease water vapor and oxygen transmission, diminish respiration rate and increase shelf life of fruit (Jiang and Li, 2001).

This review focuses on the use of natural compounds to control microbiological and physicochemical shelf life of main food categories, such as meat, fish, dairy products, minimally processed fruit and vegetables and cereal-based goods. The information is mostly based on case-studies dealing with application of active compounds to prevent microbial proliferation occurring in packaged food during storage.

MINIMALLY PROCESSED FOOD AND VEGETABLES JUICES

Minimally processed products are one of the major growing segments in food retail establishments. However, fresh-cut fruit and vegetables are widely studied because of the difficulties in preserving their fresh-like quality during prolonged periods. The goal of fresh-cut products is to deliver convenience and high quality. Taking into account the pressure of consumers about the use of synthetic chemicals, natural compounds have been suggested as a valid preservation technique (**Table 1**). Dipping, impregnation, coating, and spraying are the different ways of applications of active agents to fresh-cut fruit and vegetables but among them, the most recent results on the application of active compounds to ready-to-eat fruit and vegetables deal with coating systems. In the follow, some relevant examples are reported.

Malic acid in combination with various stabilizing compounds was used by Raybaudi-Massilia et al. (2009) for fresh-cut apples. As reported by Bico et al. (2009), the combined effect of chemical dip and/or edible coating and/or controlled atmosphere (CA) on quality of fresh-cut banana was investigated. Banana slices were dipped into a solution containing 1% (w/v) calcium chloride, 0.75% (w/v) ascorbic acid, and 0.75% (w/v) cysteine and/or combined with a carrageenan coating and/or combined with CA (3% O_2 + 10% CO_2). Dipping combined with CA treatment prevented product weight loss and increased polyphenol oxidase activity; regarding microbiological quality, the combined strategies prevented microbial growth after 5 days of storage at 5°C. The antimicrobial effects of propionic, acetic, lactic, malic, and citric acid against *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on whole red organic apples and lettuce were also clearly demonstrated by Park et al. (2011).

Raybaudi-Massilia et al. (2008) investigated the combined effects of malic acid and essential oils of cinnamon, palmarosa, and lemongrass (0.3 and 0.7%) and their main active compounds (eugenol, geraniol, and citral, 0.5%) on microbiological and physicochemical shelf-life of fresh-cut "Piel de Sapo" melon (*Cucumis melo L.*). The active compounds were incorporated into an alginate-based edible coating. Melon pieces were inoculated with a *S.* Enteritidis (10^8 CFU/ml) culture before applying the coating. The incorporation of essential oils or their active compounds into the edible coating prolonged the microbiological shelf-life by more than 21 days. Pure citral and citron essential oil were added in the syrup of industrial ready-to-eat fruit salads stored at 9°C. Both citral (25–125 ppm) and citron essential oil (300, 600, 900 ppm) were able to prolong the microbial

Table 1 | Relevant examples of natural active agents applied to minimally processed products.

Products and storage conditions	Natural compounds	Main results	References
Fresh-cut papaya stored in trays at 4°C	A microencapsulated beta-cyclodextrin and trans-cinnamaldehyde complex (2 g/100 g) incorporated into a multilayered edible coating made of chitosan and pectin	The coating improved the microbiological and physicochemical quality of fresh-cut papaya. It extended the shelf-life up to 15 days compared to the control (<7 days)	Brasil et al., 2012
Cabbage packaged under vacuum, air and two MAP (100% N_2 and 100% CO_2)	Dipping treatment with acetic, lactic, and malic acids (1% and 2%)	Some pathogens inoculated on cabbage were significantly reduced by treatment with 2% acetic, 1% lactic, and 2% malic acids	Bae et al., 2011
Minimally processed broccoli packaged in multilayered polyolefin bags and stored at 5°C for 18 days	Edible coating based on chitosan and carboxymethyl-cellulose	The coatings retard product weight loss, browning, and yellowing, reduced stem hardening, microbial growth, and improved total chlorophyll and ascorbic acid retention	Ansorena et al., 2011
Ready-to-eat lettuce and carrots packaged in oriented polypropylene bags and stored at 4°C	Dipping treatment with oregano and thyme	The solution containing oregano recorded a significantly lower initial total viable count level than the water treatment on carrots. The sensory panel found essential oil treatments acceptable for carrots throughout storage, but no for lettuce rejected for overall appreciation by day 7	Gutierrez et al., 2009
Strawberries packaged under passive and active MAP with high (80% O_2, 20% CO_2) and low (65% N_2, 30% CO_2, 5% O_2) percentage of oxygen	Solution of 1% chitosan	The chitosan coating inhibited growth of microorganisms and significantly affected the microbiological stability of the strawberries, above all when the samples were packaged under active MAP	Campaniello et al., 2008
Fresh-cut mushrooms packed in polyethylene bags and then stored at 4°C	Coating containing 5, 10, and 20 g of chitosan/L	At 4°C for 15 days, 20 g/L chitosan coating inhibited growth of total bacteria, yeasts, and moulds	Hesham, 2008

shelf-life. Citron essential oil doubled the time needed for the wild microflora to reach concentrations able to produce a perceivable spoilage in condition of thermal abuse (9°C). The same essential oil showed a strong inhibition against *L. monocytogenes*, but exerted limited effects on the survival of *S.* Enteritidis and *E. coli* (Belletti et al., 2008). Generally, when applying bioactive coatings containing essential oils to fruits and vegetables, one of the limiting factors is the impact of such components on the sensory characteristics of the coated products, mainly due to the great amount of volatile compounds which mask the natural flavor of fruits and vegetables. The use of compatible essential oil-foodstuff could also be a good alternative.

Natural volatile compounds such as methyl jasmonate, ethanol, tea tree oil, and garlic oil were applied on fresh-cut tomato stored at 5°C for 15 days. Ethanol combined with methyl jasmonate was more effective in suppressing microbial proliferation than each single compound. In addition, this combination preserved firmness and color better than the other antimicrobial preservatives. Moreover, methyl jasmonate let keep higher content of lycopene, ascorbic acid and phenolic compounds (Ayala-Zavala et al., 2008). Rojas-Graü et al. (2007) investigated the effect of lemongrass, oregano oil, and vanillin incorporated in apple puree-alginate edible coating, on the shelf-life of fresh-cut "Fuji" apples. During 21 days of storage at 4°C, the coating with vanillin (0.3% w/w) was the most effective in terms of sensory quality. All the other studied antimicrobial coatings significantly inhibited growth of psychrophilic aerobes, yeasts, and moulds. The antimicrobial effect of essential oils against *L. innocua* inoculated into apple pieces before coating was also tested. A release system of antimicrobial volatiles was adopted by encapsulation of garlic oil in β-cyclodextrin and tested on microbial growth and sensory quality of fresh-cut tomato (Ayala-Zavala and González-Aguilar, 2010). Grape-fruit seed extract was used as antimicrobial compound into a coating of sodium alginate to prolong the shelf-life of minimally processed kiwifruits. The combination of an active compound to an alginate-based coating delayed microbial growth, whereas the sole dipping treatment was inefficient. The combined use of modified atmosphere packaging (MAP) and coating treatments further prolonged the shelf-life up to 13 days (Mastromatteo et al., 2011a).

As reported by Krasaekoopt and Mabumrung (2008), the effectiveness of chitosan incorporated in the edible methyl cellulose coating on the microbiological quality of fresh-cut cantaloupe was evaluated. During storage at 10°C for 15 days, applications of 1.5 and 2% chitosan in the coating reduced growth of some pathogens, mesophilic aerobic bacteria (3.3 log cfu g^{-1}),

psychrotrophs (3.9 log cfu g^{-1}), lactic acid bacteria (3.1 log cfu g^{-1}), yeasts and molds (1.1 log cfu g^{-1}), and total coliforms (3.8 log cfu g^{-1}). An edible coating containing chitosan was also applied on carrot sticks to maintain quality and prolong the shelf-life (Simões et al., 2009). The coating application preserved the overall visual quality, the microbial proliferation and reduced surface whiteness during storage. While the content of total phenolics markedly increased in coated carrot sticks stored under moderate gas levels, it was controlled under low O_2 and high CO_2 levels. Film based on methyl cellulose incorporating chitosan and chitosan/methyl cellulose film incorporating vanillin were applied to fresh-cut cantaloupe and pineapple to control microbial quality during storage at 10°C (Sangsuwan et al., 2008). Chitosan/methyl cellulose film and vanillin films provided an inhibitory effect against *E. coli* in fresh-cut cantaloupe. The chitosan/methyl cellulose film rapidly reduced the number of *S. cerevisiae* inoculated on the products. For fresh-cut pineapples coating with vanillin was more efficient than chitosan/methyl cellulose in reducing the number of yeasts by 4 logs in six days. An edible coating with soy or wheat gluten protein as a carrier of thymol and calcium chloride was applied on strawberry by Atress et al. (2010). Treating fruits did not exhibit any change in fruit appearance until 9 days of storage. All treatments maintained ascorbic acid content, firmness, total sugar and reduced the total colony, moulds and yeasts compared to the control. The effect of sodium hypochlorite, peroxiacetic acid, acidified sodium chlorite and carvacrol on microbiological, sensory, and nutritional quality of fresh-cut jalapeno peppers stored at 5°C during 27 days was evaluated (Ruiz-Cruz et al., 2010). All sanitizers (except carvacrol) maintained microbiological and overall quality of jalapeno peppers during 27 days. Carvacrol, active ingredient of oregano essential oil, maintained shelf life for only 17 days and reduced sensory acceptability of fresh-cut produce. However, carvacrol-treated samples retained the highest levels of photochemical and antioxidant capacity.

Juices are food very susceptible to yeasts attack. *Pichia anomala, S. cerevisiae*, and *Schizosaccharomyces pombe* caused the most diffuse problems. Generally, heat treatment (pasteurization), aseptic packaging or use of weak acids exclude yeast spoilage. As alternative to these traditional artificial preservatives the use of natural compounds was proposed in the literature (Valero and Salmerón, 2003; Belletti et al., 2010). Clary, sage, juniper, lemon, and majoram essential oil were chosen to preserve apple juice, as being efficient in *in vitro* test and not containing phenolics but alcohol terpenoids, linalool (clary sage), and terpinen-4-ol (marjoram), the cyclic monoterpenes α-pinene (juniper) and limonene (lemon) (Tserennadmid et al., 2011). The anti-yeast effects of these essential oils were good in the acidic pH range optimal for yeasts growth. Synergism or additive effects were recorded by combining the different active compounds. The most interesting result of the study of Tserennadmid et al. (2011) was recorded with lemon essential oil. In fact, experiments with lemon given to apple juices showed that the "open" storage time at ambient temperature could be prolonged and a novel, refreshing taste could be achieved.

Although the influence of smell-taste of some active agents is known, it has not often been evaluated to a sufficient degree. One solution to the above-mentioned problem may be the use of combinations of different food preservation systems that would give the benefits of each of them while at the same time appreciably reducing the amount of antimicrobial required. For this reason, the application of moderate heat treatments and/or the preservation of the foodstuff in cold, refrigerated temperatures may play a key role. By using this method, a stable and, from a microbiological viewpoint, safe food can be produced without any loss in sensory quality. In this context various aroma compounds and citron essential oils containing citral, β-pinene, limonene, linalool, and α-pinene, combined with mild heat treatment, were used to inhibit the growth of *S. cerevisiae* in non-carbonated soft drinks (Belletti et al., 2007).

DAIRY PRODUCTS

Fresh dairy products are ready-to-eat foods easily contaminated by undesirable microorganisms. Some of them are spoilage microorganisms which may produce unwanted visual appearance and diminish the commercial value of cheese, other ones are pathogens that affect product safety. Moreover, fungal spoilage can also occur. Recently, some studies have recorded the efficacy of natural compounds, alone or in combination with other preservation methods, when directly applied to milk (Cava et al., 2007) or to cheese by spraying, immersing, or dusting the products. Antimicrobials may also be spread onto the packaging materials that come in contact with the cheese or incorporated into the plastic films used for packaging (Conte et al., 2007). A brief overview of some recent examples of natural active agents applied to cheese is reported.

The effectiveness of lysozyme and EDTA on microbiological shelf life of mozzarella cheese was studied by Sinigaglia et al. (2008). Mozzarella was packaged in a brine that contained lysozyme (0.25 mg mL^{-1}) and different amounts of EDTA (10, 20, and 50 mmol L^{-1}), and stored at 4 ± 1°C for 8 days. The packaging system significantly inhibited growth of coliforms and *Pseudomonadaceae*, without affecting the typical lactic acid bacteria. Conte et al. (2011) also evaluated the effects of lysozyme and EDTA in burrata cheese packaged under MAP (95:5 CO_2:N_2), thus demonstrating that these compounds were valid to prolong cheese shelf life, especially at high lysozyme concentrations.

Different release systems containing nisin and natamycin were also used in various works to create an additional hurdle for spoilage microorganisms in dairy products. For example, edible coatings made of galactomannans incorporating nisin were tested against *L. monocytogenes* in Ricotta cheese. The system not only help in retarding the growth of *L. monocytogenes* but also help in the maintenance of water content, therefore reducing cheese weight loss (Martins et al., 2010). Cao-Hoang et al. (2010) used sodium caseinate-based films for incorporating nisin to be active in mini red Babybel cheese. The active films affected *L. innocua* inoculated on cheese surface. As reported by Ture et al. (2011) natamycin was incorporated into wheat gluten and methyl cellulose biopolymers and tested against *A. niger* and *P. roquefortii* inoculated on surface of fresh kashar cheese. In a study conducted by Fajardo et al. (2010) natamycin was also used in combination with chitosan. In particular, the effects of the chitosan coating containing natamycin (0.50 mg/mL) on semi-hard

cheese were assessed. The natamycin coated samples presented a decrease of moulds and yeasts compared to the control after 27 days of storage. The effects of nisin, natamycin, and their combination into a cellulose polymer matrix were also studied by dos Santos Pires et al. (2008). Films efficacy was first evaluated *in vitro* and then on sliced mozzarella cheese. Best effects were found when the two compounds were applied together on cheese.

As regards the applications of essential oils, numerous examples are reported in the literature (**Table 2**). The addition of different concentrations of lemon extract in the brine of mozzarella and in a gel solution made up of sodium alginate was evaluated by Conte et al. (2007). Shelf-life tests were run at 15°C to simulate thermal abuse. An increase in shelf-life of all active packaged mozzarella cheese was observed, confirming that the investigated substance may exert an inhibitory effect on the microorganisms responsible for spoilage, without affecting the typical dairy microbial population. As reported by Otaibi et al. (2008), three essential oils, thyme, marjoram and sage, were added to concentrated yoghurt (labneh) at concentrations of 0.2, 0.5, and 1.0 ppm. The better concentration of each essential oil was 0.2 ppm that allowed obtaining a shelf-life up to 21 days. Singh et al. (2011) also added essential oils in yogurt. The anise volatile oil and its oleoresin added to yogurt (prepared from buffalo's milk) (stored up to 20 days at 4 ± 1°C) at various concentrations (0.1–1.0 g L^{-1}) were effective in controlling spoilage microorganisms. Eleven essential oils were evaluated *in vitro* for their antibacterial properties against *Vancomycin-resistant Enterococci* and *E. coli* O157:H7 (Selim, 2011). The most active essential oils against bacteria were thyme oil, eucalyptus, juniper, and clove oils. Furthermore, their effects were evaluated against the same microbial groups experimentally inoculated in Feta soft cheese and stored at 7°C for 14 days. The addition of thyme oil at concentrations of 0.5 and 1% caused a significant reduction in microbial growth. On Feta cheese inoculated with *E. coli* O157:H7 and *L. monocytogenes* oregano and thyme (0.1 or 0.2 and 0.1 ml/100 g) combined with MAP were also tested by Govaris et al. (2011). In the control Feta microbial strains survived up to 1 month of storage. On the contrary, in Feta cheese treated with oils a significant reduction of microbial growth was found. The use of chitosan, lemon, and sage extract in Fior di latte cheese was assessed by Gammariello et al. (2010). Different concentrations of active substances were added during processing cow's milk. Lemon extract and chitosan showed a good compromise between the antimicrobial effectiveness and the sensory impact caused by their addition, thus promoting a satisfactory shelf life increase (129%). In contrast, the addition of sage extract

Table 2 | Relevant examples of natural active compounds applied to dairy products.

Products and storage conditions	Natural compounds	Main results	References
West African soft cheese	Treatment with eucalyptus oil and lemongrass oil	The treatment of eucalyptus oil 75% plus 25% lemon grass exerted a positive impact on the nutritional, sensory, and microbial values of West African soft cheese	Belewu et al., 2012
Ricotta cheese stored under modified atmosphere at 4°C	Coating with a chitosan/whey protein edible film	The chitosan/whey protein film slowed detrimental phenomena. The viable numbers of lactic acid bacteria and mesophilic and psychrotrophic microorganisms were significantly lower in the chitosan/whey protein coated cheese, compared to the control	Di Pierro et al., 2011
Traditional Minas Serro cheese	Nisin	Nisin was effective in reducing *S. aureus* count in Serro cheese. A reduction of 1.2 and 2.0 log cycles in *S. aureus* count was observed from the 7th day of ripening for cheese containing 100 IU mL^{-1} and 500 IU mL^{-1} of nisin, respectively, compared with control sample	Pinto et al., 2011
Fresh cheese Tosèla	Antimicrobial compounds produced by six strains of non-starter lactic acid bacteria. In particular, *Lactobacillus paracasei* NdP78 was also found to produce a bacteriocin	Cheese showed higher concentrations of lactobacilli (7.90 log CFU/g) and streptococci (6.10 log CFU/g), lower development of coliforms and staphylococci than control cheese	Settanni et al., 2011
Caprese salad packaged under MAP (65% N_2, 30% CO_2, and 5% O_2)	Dipping with thymol (400 ppm)	The combined use of thymol and MAP decreased the coliform populations from 5.65 to 4.23 log CFU/g and extended the microbiological shelf-life from 3.77 to 12 days. It also decreased the concentration of *Pseudomonadaceae*	Bevilacqua et al., 2007
Gorgonzola cheese	Natamycin-incorporated film in the production process of cheese	Films with 2 and 4% natamycin presented satisfactory results for *P. roquefortii* inhibition	de Oliveira et al., 2007

negatively affected the sensory properties, thus making the cheese unacceptable.

Pires et al. (2009) developed a microbial sachet incorporated with allyl-isothiocyanate. Its efficiency was tested against yeasts, molds, *Staphylococcus* sp. and psychrotrophic bacteria in sliced mozzarella cheese stored at 12°C ± 2°C. A reduction of 3.6 log cycles was observed in yeasts and molds counts in the mozzarella packed with the antimicrobial sachet over 15 days of storage time. The sachet also showed an antibacterial effect on *Staphylococcus* sp.; however, psychrotrophic bacteria were very resistant. A new dairy product "Karishcum" obtained by adding *Curcuma Longa* (Curcumin or Turmeric) to classic Karish cheese at a rate of 0.3% (w/v) was realized in a study conducted by Hosny et al. (2011). A primary experiment was done to determine the correct percentage of Curcumin addition to cheese milk to get a good taste and a long shelf life. The behavior of pathogenic bacteria in the artificially contaminated product during cold storage for 14 days, revealed that addition of the extract (0.3%) determined a reduction of bacterial counts of about 1 log of *S.* Typhimurium and two log of *P. aeurogenosa* and *E. coli* 0157:H7.

MEAT-BASED PRODUCTS

Spoilage of meat products contributes to deterioration of texture and change in flavor and color. The use of natural antibacterial compounds, such as extracts of spices and herbs, essential oils, organic acids, salts, and bacteriocins is reported in the literature to improve the shelf life of meat (Jamilah et al., 2008; Jałosńska and Wilczak, 2009). Some applications are reported in **Table 3**.

Mastromatteo et al. (2011b) suggested the combined use of natural antimicrobials, such as lemon and thymol, and MAP to improve the shelf life of reduced pork back-fat content sausages. In particular, the application of thymol and thymol-MAP limited the development of *Pseudomonas* spp., responsible for sausages' unacceptability. The use of bay essential oil combined to MAP without oxygen (20% CO_2—80%N_2) was suggested to control *L. monocytogenes* and *E. coli* growth and also to extend the shelf life of naturally contaminated ground chicken meat (Irkin and Esmer, 2010). Moreover, the addition of essential oils of marjoram and rosemary to beef patties formulated with mechanically deboned poultry meat at a concentration of 200 mg/kg reduced lipid oxidation and improved the sensory characteristics

Table 3 | Relevant examples of natural active agents applied to meat-based products.

Products and storage conditions	Natural compounds	Main results	References
Fresh minced beef patties packaged under MAP	Thymol (250, 500, 750 mg/Kg)	Better effects on product quality were obtained for sample with increased amount of thymol, under MAP conditions (shelf life about 7 days)	Del Nobile et al., 2009a
Minced beef mixed with soy-protein stored at 4°C	Sage essential oil (0.1, 0.3, and 0.5%)	The highest concentration of essential oil controlled development of main microorganisms	Ahmed and Ismail, 2010
Meat-balls stored at 10°C	0.2% of cranberry, rosemary, and lovage extracts	Rosemary extract was the most effective on product shelf life (13.3 days)	Jałosńska and Wilczak, 2009
Sausages stored at 4°C under vacuum conditions	Sodium lactate (0%, 0.6%, 1.2%, 1.8%) as alternative to nitrite	Sodium lactate improved the microbiological quality, extended shelf life, and exhibited a better antimicrobial effect than nitrite	Bingöl and Bostan, 2007
Fresh sausage	Oregano and marjoram essential oil	Addition of oregano and marjoram essential oil exerted a bacteriostatic effect	Busatta et al., 2007, 2008
Broiler chicken wings stored at 4°C	Dipping treatment for 10 min with chlorine dioxide, lactic acid, and fumaric acid	Samples treated with lactic acid alone showed the most effective reduction on *E. coli* and mesophylic bacteria	Hecer and Guldas, 2011
Fresh chicken meat stored under MAP at 4°C	Treatments with nisin and EDTA, alone or in combination	Chicken was better preserved under treatments with 500 IU/g of nisin and 50 mM of EDTA, even up to 24 days	Economou et al., 2009
Fresh beef	Organic acids (citric, lactic, acetic, and tartaric)	Organic acids promoted a significant shelf life extension	Jamilah et al., 2008
Fresh chicken sausage stored at 4°C	Rosemary or Chinese mahogany (500, 1000, 1500 ppm)	Chinese mahogany and rosemary improved meat quality	Liu et al., 2009
Turkey-bologna stored at 4°C	Coating with gelatin containing Nisaplin and Guardian	Both Nisaplin film and Guardian film effectively inhibited *L. monocytogenes*	Min et al., 2010
Meat pieces	Combined application of oregano essential oil and acetic acid	Combination of essential oils and organic acids inhibited microbial growth and proliferation of pathogens such as *S. aureus*	de Souza et al., 2009

(Mohamed and Mansour, 2012). To reduce microbial growth and to preserve the oxidative stability of mortadella, a Bologna-type sausage, the addition of orange dietary fiber (1%), rosemary essential oil (0.02%) and thyme essential oil (0.02%), combined with specific storage conditions, showed very desirable effects (Viuda-Martos et al., 2010, 2011). Antioxidant and antibacterial effects of rosemary, orange, and lemon extracts was also investigated in cooked Swedish-style meat-balls. Results indicated that significant advantages were obtained using rosemary and citrus extracts in rancidity-susceptible meat products; however, only rosemary slightly reduced lactic acid bacteria (Fernàndez-Lòpez et al., 2005). Ayachi et al. (2007) reported that the addition of a mixture of organic acids (sodium lactate 90% and sodium acetate 10%) at different concentrations (from 0 to 20 g/Kg) on Marguez sausages, made with lamb and beef, significantly reduced microbial cell loads during storage at 8°C.

Chitosan (0.5% and 1%) added individually or in combination with nitrites (150 ppm) as ingredients was tested to protect fresh pork sausages from microbial spoilage. Its application as active coating was demonstrated (Bostan and Isin-Mahan, 2011). Soultos et al. (2008) found chitosan active against total viable count, lactic acid bacteria, *Pseudomonas* spp., *B. thermosphacta*, *Enterobacteriaceae*, yeasts, and moulds.

Krisch et al. (2010) compared the antimicrobial effect of commercial herbs, spices and essential oils (fresh and dried garlic, onion, thyme, marjoram, and oregano) in minced pork. While fresh spices showed weak or no inhibition on viable cells of minced pork, some effects of essential oils were observed. Best shelf life values were obtained for pork meat added with garlic and marjoram oil. Dipping of thyme and oregano oil in concentrations of 0.1 and 0.3% were carried out to improve the shelf life of meat-based products (Karabagias et al., 2011). The combination of dipping and MAP extended the shelf life to about 22 days, against 7 days of the control sample packaged in air.

A relevant preservation effect for fresh chicken breast meat, stored at 4°C, was obtained by dipping meat in oregano oil, prior to packaging under MAP (Chouliara et al., 2007). Fratianni et al. (2010) also proposed use of thyme and balm essential oils to decrease the natural microflora of chicken breast meat. In particular, balm essential oil significantly limited growth of *Salmonella* sp., whereas thyme essential oil effectively inhibited growth of *E. coli*. It was also widely demonstrated that dipping with lactic acid, clove oil and vitamin C can exert significant advantages over dipping with lactic acid alone to improve shelf life of buffalo meat steaks. In particular, use of clove oil along with lactic acid provided synergistic antioxidant and antimicrobial effects; the inclusion of vitamin C also stabilized product color (Naveena et al., 2006). Ntzimani et al. (2010) highlighted that combined use of EDTA, lysozyme, rosemary, and oregano oil extended shelf life of semi-cooked coated chicken fillets stored under vacuum packaging at 4°C to more than 2 weeks.

Effects of pork chops dipped in organic acids, such as ascorbic acid (500 ppm) and citric acid (250 ppm) individually or in combination, packaged under MAP and vacuum and stored at 1°C were studied by Huang et al. (2005). Ascorbic acid dipping reduced psychrotrophic microbial count, while ascorbic and citric acids improved lipid stability. The obtained results were enhanced by packaging under MAP conditions.

As regards bacteriocins, the combined use of lactoferrin and nisin on naturally contaminated Turkish-style meat-balls was proposed. Treatment with lactoferrin alone and in combination with nisin significantly reduced spoilage bacterial counts (total aerobic bacteria, coliform, *E. coli*, total psychrophilic bacteria, *Pseudomonas* spp., yeast and molds) and extended shelf life to 10 days (Colak et al., 2008). The synergistic antimicrobial activity of lysozyme, nisin, and EDTA against *L. monocytogenes* and meat-borne spoilage bacteria in ostrich patties packaged in air and vacuum was observed by Mastromatteo et al. (2010a). In particular, the antimicrobial treatment was effective for controlling growth of lactic acid bacteria even if it was not effective against Gram-negative bacteria.

FISH-BASED PRODUCTS

Fresh fish is a highly perishable product due to its biological composition. The main cause of deterioration is the activity of spoilage seafood microorganisms that provoke loss of essential fatty acids, fat-soluble vitamins and protein functionality, production of biogenic amines, and formation of off-odors (Gram and Dalgaard, 2002).

Literature widely demonstrates that treatments with natural compounds are effective preservation methods for fish products (**Table 4**). Different effects are generally exerted depending on the active agent used and on the characteristics of the raw material. Some examples are provided hereinafter. Erkan et al. (2011) proposed the use of thyme (1%) and laurel essential oil (1%) to extend the shelf life of bluefish by about 3–4 days. The quality of hot smoked rainbow trout packaged under vacuum and treated with thymol and garlic oil (1%) was also improved (Erkan, 2012). Kykkidou et al. (2009) demonstrated that combination of MAP and thyme essential oil (0.1%) resulted in a significant shelf life extension of fresh Mediterranean swordfish fillets. In particular, the addition of thyme essential oil extended product shelf life (13 days) if compared to the control (8 days), whereas its combination to MAP conditions further prolonged product shelf life (about 20.5 days). Goulas and Kontominas (2007) also showed that oregano essential oil (0.8 %) extended shelf life of sea bream fillet by more than 17 days. Similar results were also reported by Pyrgotou et al. (2010) on rainbow trout where the similar combined strategies reduced the cell load of main spoilage microorganisms. Corbo et al. (2008) highlighted the possibility to extend the microbial acceptability limit of fresh fish burgers by using a mix of thymol, grapefruit seed extract, and lemon extract. The mixture of the three natural compounds prolonged the sensory quality without compromising the flavor of fish. Each antimicrobial compound was first tested *in vitro* against the main fish spoilage microorganisms (Corbo et al., 2009). In a subsequent work, thymol, grapefruit seed extract, and lemon extract were used in combination to MAP to demonstrate that MAP further enhanced the effects of the natural active compounds. (Del Nobile et al., 2009b). Min et al. (2009) demonstrated the antimicrobial and antioxidant activity of purple rice bran extract against catfish patties.

Table 4 | Relevant examples of natural active agents applied to fish-based products.

Products and storage conditions	Natural compounds	Results	References
Rainbow trout fillets packaged under vacuum	Oregano essential oil (0.2, 0.4%)	The combination of oregano (0.2%) and vacuum resulted in a significant shelf life extension of trout fillets (11–12 days) if compared to the control packaged in air (5 days)	Frangos et al., 2010
Rainbow trout fillets packaged with oxygen absorber	Oregano essential oil (0.4%)	The antimicrobial compound improved the sensory shelf life	Mexis et al., 2009
Fish burgers packaged under vacuum	Rosemary extract 0.4% and 0.8%	Rosemary extract, in combination with vacuum packaging was effective in controlling microbial growth and biochemical changes	Uçak et al., 2011
Fried mullet fish fillets	Edible coating solution mixed with thyme (2.5, 5%) and marjoram (2.5, 5%)	Thyme and marjoram have strong effects against Enterobacteriaceae	Yasin and Abou-Taleb, 2007
Sea bass fillets packaged under different MAP	Thyme essential oil (0.2%)	Essential oil improve the quality of sea bass fillets when used in combination with 60% CO_2-30% N_2-10% O_2, providing a shelf life of 17 days as compared to 6 days of the control samples	Kostaki et al., 2009
Cooked blue swimming crab meat	Sodium acetate dipping treatments (1, 1.5, and 2%)	Shelf life of product dipped in 2% for 2 min was 12 days compared to the control (6 days)	Lohalaksanadech and Sujarit, 2011
Peeled shrimps packaged under MAP	Coating with thymol (500, 1000, 1500 ppm)	Shelf life of about 14 days for the active coating (1000 ppm) packaged under MAP compared to the sample in air (5 days) was obtained.	Mastromatteo et al., 2010b

Coatings enriched with essential oils were also proposed in literature as valid technique to improve quality of fish products. As example, Ojagh et al. (2010) reported that the use of a coating with chitosan and cinnamon essential oil improved trout fillet shelf life (16 days vs. 10 days of the control) and in particular it enhanced texture, odor, and color. Similar results were also obtained for trout fresh fillets coated with gelatin enriched with cinnamon oil (1%, 1.5%, and 2%). In particular, experimental data indicated that the active coating can be suitable for preserving the fillets and maintain quality to an acceptable level (Andevari and Rezaei, 2011).

To control quality of northern snakehead fish fillets at refrigeration temperature cinnamon, coatings with nisin and EDTA, alone and in combination were used (Lu et al., 2010).

Acetic acid, glucono-delta-lactone and chitosan were tested, individually and in combination, to inhibit microbial growth in surimi. The results showed that microbial proliferation was successfully inhibited by packaging the fish ball in 1% chitosan dissolved in 1% of acetic acid (Kok and Park, 2007). Shirazinejad et al. (2010) evaluated the use of lactic acid alone and in combination with nisin for reducing microorganisms on chilled shrimp. Best results against *Pseudomonas spp*. were obtained for samples treated with the mixture of lactic acid and nisin.

CEREAL-BASED PRODUCTS

Shelf life of bread is generally limited due to the staling phenomenon and fungi spoilage, in particular moulds. Among strategies aimed to improve quality of bread, some effects were reported by using different natural compounds. In particular, it was reported that chitosan coating improved bread quality by inhibiting microbial growth and retarding oxidation and staling. A reduced microbial proliferation was obtained for bread coated with chitosan during storage at room temperature (No et al., 2007).

Rehman et al. (2007) reported different applications of citrus peel essential oils in bread. Results demonstrated that the oils influenced sensory characteristics and delayed microbial growth. Maximum inhibitory effect against moulds and bacteria was achieved by spraying peel essential oil. The combination of MAP and mustard oil was proposed for wheat and rye bread artificially inoculated with moulds (Suhur and Nielsen, 2005).

Breads prepared from wheat flour by adding different additives were also evaluated by Latif et al. (2005). The studies on colony count in bread at different storage time showed that treatment containing 0.32% of suhanjna, 3% of lecithin, and 0.1% of ascorbic acid proved to be most effective. The different combination of the three selected natural additives improved bread

shelf life. In particular, lower cell loads of yeasts and moulds were observed for bread with lecithin and ascorbic acid (Latif and Masud, 2006). An active packaging with cinnamon essential oil combined with MAP was tested to increase the shelf life of gluten-free sliced bread. Results showed that the active packaging is better than MAP to increase product shelf life because it inhibited microbial growth while maintaining the sensory properties of the gluten-free bread (Gutiérrez et al., 2011).

Natural active compounds were also applied to fresh pasta that is a product easily perishable for its high water content. Del Nobile et al. (2009c) proposed the use of different natural antimicrobial compounds such as thymol, lemon extract, chitosan, and grapefruit seed extract at different concentrations (2000 mg/kg and 4000 mg/kg) to improve the microbiological stability of refrigerated amaranth-based fresh pasta. Results pointed out that chitosan were the most successful among the investigated compounds in slowing down the spoilage, whereas lemon extract was the less effective. In a subsequent work, the antimicrobial activity of chitosan in combination with different MAP was tested. It was found that among the tested MAP conditions, the combination of 30:70 N_2:CO_2 extended the shelf life beyond two months (Del Nobile et al., 2009d). The antimicrobial activity of chitosan against the main microorganisms of fresh pasta was also reported by Costa et al. (2010). In particular, statistically significant differences were found between the shelf life of pasta with chitosan packaged under MAP conditions in a low barrier film made up of polypropylene and in a multilayer high barrier film made up of polyethylene terephthalate, ethylene-vinyl alcohol, and polyethylene.

To improve the shelf life of yellow alkaline noodle Rosyid et al. (2011) tested the antibacterial activity of ethanol and water extracts of six types of leaves against the principal spoilage microbial groups of this product. Results highlighted that ethanol extracts of aromatic leaf, *Murraya koenigii* L., added in yellow alkaline noodle contributed to improve shelf life better than the other extracts. Budka and Khan (2010) demonstrated that essential oils from basil, thyme and oregano exhibit bactericidal properties against *B. cereus* in rice-based foods. The antioxidant and antimicrobial activity of different natural compounds (anise, black cumin, rosemary and sage) were also tested to increase shelf life of some bakery products. Preliminary results showed that both Gram-positive and Gram-negative bacteria were sensitive to all tested essential oils and phenolic compounds (Basuny et al., 2012).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Most food products require protection against microbial spoilage during storage. Consumers demand safe natural products. This drives the search of food authorities and researchers for mild preservation techniques to improve microbial quality and safety without causing nutritional and organoleptic losses. In this context natural compounds are gaining a great interest from research and industry, due to the potential to provide quality and safety benefits, with a reduced impact on human health. In addition, utilization of natural active agents promotes the accepted criteria of food sustainability. The numerous experimental applications of essential oils, enzymes, bacteriocins, chitosans, and organic acids to various fresh perishable foods demonstrate that they are well suited to be utilized as preservatives in foods and could be often valid alternatives to synthetic food additives. However, there is a need to search for new sources of antimicrobial substances, including plant metabolites. Natural products have been the most successful source of drugs ever. Historically, the most important natural sources have been plants. Research progressed along two major lines: ethnopharmacology (medicinal herbs, substances of abuse, ordeal poisons) and toxicology (poisonous plants, venomous animals, arrow, and fish poisons). These strategies have produced many valuable drugs and are likely to continue to produce lead compounds. It must be stated that traditional medicines have not been found by systematic research but by a combination of coincidence and observation, and at best by trial and error. In order to further promote the application of natural active compounds at industrial level, some factors are of striking importance. First of all it is necessary to have a good understanding of the mechanism by which antibacterial agents operate. For many natural compounds these information are still lacking. Better understanding of the modes by which antimicrobials can control microorganisms should provide solid grounds for engineering new and upgraded derivatives with optimized potency and stability. Further research is still necessary for specific case-study because it is well demonstrated that the combination of more than one active agent not always amplifies the antimicrobial effects. Very often, the combined use of some natural essential oils did not induce synergistic effects. So, generally specking some considerations must be taken into account before using antimicrobials in food preservation. One of them is the possible existence of interactions between compounds and food components. Moreover, also the adoption of active compounds under MAP conditions could exert different effects depending on the product.

In the specific case of essential oils, despite their great potential, their use in food preservation remains limited mainly due to their intense aroma and toxicity problems. Several authors have reported changes in the organoleptic properties of the food when these oils are used. To minimize the required doses and improve the effectiveness of active coatings enriched with essential oils, interesting options would be micro- and nanoencapsulation of active compounds. In addition, the use of combinations of different food preservation systems, such as the use of proper temperature, could represent another solution to the above-mentioned problem. As regards toxicity, the ingestion of high doses of essential oils can induce serious problems. Thus, it is necessary to find a balance between the effective compound dose and the risk of toxicity. It is also worth noting that the use of essential oils remains expensive, so from an economic point of view this preservation strategy needs further enhancement. Moreover, more specific ISO standards are also necessary to assess the legal aspects to set out the definition, the general rules for their use, the requirements for labeling and the maximum levels authorized.

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Antifungal, cytotoxic, and immunomodulatory properties of tea tree oil and its derivative components: potential role in management of oral candidosis in cancer patients

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Candida albicans forms oral biofilms that cause disease and are difficult to treat with conventional antifungal agents. Tea tree oil (TTO) is a natural compound with reported antimicrobial and immunomodulatory activities. The aims of the study were to evaluate the antifungal efficacy of TTO and key derivatives against *C. albicans* biofilms, to assess the toxicological effects of TTO on a clinically relevant oral cell line, and to investigate its impact on inflammation. TTO and its derivatives were examined against 100 clinical strains of *C. albicans*. Planktonic minimum inhibitory concentrations (MICs) were determined using the CLSI M-27A broth microdilution method. Sessile MICs were determined using an XTT reduction assay. Inhibition, time-kill, and mode of action studies were performed. OKF6-TERT2 epithelial cells were used for cytotoxicity and cytokine expression assays. Planktonic *C. albicans* isolates were susceptible to TTO, terpinen-4-ol (T-4-ol), and α-terpineol, with an MIC_{50} of 0.5, 0.25, and 0.25%, respectively. These three compounds also displayed potent activity against the 69 biofilm-forming strains, of which T-4-ol and α-terpineol displayed rapid kill kinetics. For all three compounds, 1 × MIC_{50} effectively inhibited biofilm growth when *C. albicans* were treated at 0, 1, and 2 h post adhesion. By scanning electron microscopy analysis and PI uptake, TTO and derivative components were shown to be cell membrane active. TTO and T-4-ol were cytotoxic at 1 × MIC_{50}, whereas at 0.5 × MIC_{50} T-4-ol displayed no significant toxicity. Transcript and protein analysis showed a reduction of IL-8 when treated with TTO and T-4-ol. These data provide further *in vitro* evidence that TTO and its derivative components, specifically T-4-ol, exhibit strong antimicrobial properties against fungal biofilms. T-4-ol has safety advantages over the complete essential oil and may be suitable for prophylaxis and treatment of established oropharyngeal candidosis. A clinical trial of T-4-ol is worthy of consideration.

Keywords: tea tree oil, oral candidosis, oral cancer, antifungal agent, terpinen-4-ol

INTRODUCTION

Many cancer therapies have a profound negative impact on oral health, causing serious complications (Dreizen, 1990). These include both non-infectious and infectious side-effects with potential for systemic spread (Wingard, 1990). Oral fungal infections, caused predominantly by *Candida* species, are common in patients suffering from cancer at all stages of the disease, especially those receiving palliative care (Sweeney et al., 1998; Sweeney and Bagg, 2000; Davies et al., 2006). *Candida albicans* is known to form complex biofilms (Ramage et al., 2005; Ganguly and Mitchell, 2011) and other *Candida* spp. also have this ability to varying degrees, though it is strain-dependent (Silva et al., 2009). Such biofilms form on the oral epithelium or on the surfaces of intra-oral prostheses (Williams et al., 2011), and can result in pseudomembranous or erythematous candidosis.

The effectiveness of most antifungal agents is significantly reduced if yeasts are in a biofilm as opposed to the planktonic state (Kuhn and Ghannoum, 2004; Lamfon et al., 2004; d'Enfert, 2006). Furthermore, in recent years there has been growing concern about the increasing prevalence of infections caused by yeasts that are resistant to commonly used antifungal drugs (Bagg et al., 2003), with the emergence of strains of *C. albicans* that are resistant to the azole antifungals (Casalinuovo et al., 2004), in addition to the inherently reduced drug susceptibility of many non-albicans yeasts that can be selected through over-use of antifungal drugs (Davies et al., 2002; Bagg et al., 2003). There is a need to identify new methods of preventing and treating oral candidosis among immunocompromised patients, including those with cancer, both to improve treatment of established infections and to limit further development of drug resistance. Given the extensive evolutionary

interaction that exists between plants and microorganisms it is unlikely that resistance would be cause for concern, nevertheless, clinically we would anticipate natural compounds to augment existing antifungal agents opposed to direct replacement.

One agent which merits consideration is tea tree oil (TTO; Hartford and Zug, 2005; Carson et al., 2006), which has recently reported to have minimal impact on developing resistance (Hammer et al., 2012). TTO is produced as a distillate of leaves of the *Melaleuca alternifolia* shrub, which grows in New South Wales, Australia. It is a complex mixture of essential oils, comprising approximately 100 components, most of which are monoterpenes, sesquiterpenes, and their related alcohols (Carson et al., 2006). TTO has been shown to possess a number of therapeutic properties, including anti-inflammatory activities (Hart et al., 2000; Koh et al., 2002; Pearce et al., 2005) and there is current interest in its possible anti-tumor properties (Bozzuto et al., 2011). However, it is best known for its antimicrobial activity against a wide spectrum of microorganisms, for example *Staphylococcus aureus* (including MRSA; Thompson et al., 2008; Kwiecinski et al., 2009), a range of oral bacteria (Hammer et al., 2003b), and certain viruses, including herpes simplex and influenza viruses (Carson et al., 2001; Garozzo et al., 2011). TTO also has potent activity against many fungi (Hammer et al., 2003a, 2004), including some azole-resistant yeasts (Mondello et al., 2003; Bagg et al., 2006) and there is some evidence for its efficacy in treating fluconazole refractory oral candidosis in AIDS patients. This raises the possibility of using TTO preparations for the prevention and treatment of oral candidal infections. Oral care products are now available containing TTO (Soukoulis and Hirsch, 2004), including an alcohol-free mouthwash. However, hypersensitivity reactions to TTO have been reported (Knight and Hausen, 1994; Mozelsio et al., 2003; Hammer et al., 2006; Rutherford et al., 2007) and the palatability of the agent as an oral preparation is poor. Moreover, given the complex chemical composition of TTO, which results in batch-to-batch variability, then the ability to accurately interpret its clinical utility is limited. Some of the individual components of TTO are believed to have antimicrobial properties (Mondello et al., 2006) and may be more appropriate for development into oral care products with respect to safety and consistency.

This study had three aims. The first was to evaluate the efficacy of TTO and two of its key derivatives [terpinen-4-ol (T-4-ol) and α-terpineol] against biofilms formed by a clinically diverse panel of *C. albicans* isolates. Secondly, the toxicological effects of TTO and derivative components were assessed by means of a clinically relevant oral cell line. Finally, the study aimed to investigate further the previously reported anti-inflammatory effects of TTO.

MATERIALS AND METHODS

STRAINS AND GROWTH CONDITIONS

One-hundred clinical strains of *C. albicans* were used in the course of this study, which were isolated from a wide variety of patient groups, including those with denture stomatitis (Glasgow Dental School, $n = 26$), those receiving palliative care for advanced cancer (Accord Hospice, Paisley, $n = 30$), neonates (Royal Hospital for Sick Children, Glasgow, $n = 36$), and those with bloodstream infections (Glasgow Royal Infirmary, $n = 8$). All strains were maintained routinely on Sabouraud dextrose (SAB) agar (Oxoid, Basingstoke, UK) stored at 4°C, and stored indefinitely on Microbank beads (Prolab Diagnostics) at −80°C.

ANTIFUNGAL COMPOUNDS

Tea tree oil and seven HPLC-grade derivatives of the oil obtained from Sigma-Aldrich (Poole, Dorset) were used in the study. The derivatives included terpinen-4-ol (T-4-ol), α-terpineol, 1-8-cineole, terpinolene, α-terpinene, γ-terpinene, and *p*-cymene. Stock concentrations of each component were prepared in RPMI-1640 AutoMod™ medium (Sigma, UK), containing 0.5% (v/v) Tween® 80 (ICI Americas, Inc.). Each compound was prepared as a 4% v/v solution for testing and serially diluted for both planktonic and sessile susceptibility testing. All procedures were carried out in a laminar flow cabinet (Hera Safe laminar flow cabinet, Kendro, Model K515).

SUSCEPTIBILITY TESTING AND BIOFILM INHIBITION

Antifungal testing to determine planktonic minimum inhibitory concentrations (PMICs) was performed using the CLSI M-27A broth microdilution method (Clinical Laboratory Standards Institute, 2008). For sessile susceptibility testing, *C. albicans* biofilms were formed as previously described on polystyrene, flat-bottomed, 96-well microtiter plates (Corning Incorporated, Corning, NY, USA; Ramage et al., 2001). These were treated with a range of concentrations of each antifungal compound (0.0625–2%) in RPMI-1640 containing 0.5% (v/v) TWEEN 80®. After 24 h challenge the sessile minimum fungicidal concentrations (SMFCs) were determined as a 50% inhibition compared to the untreated control using a XTT reduction assay, as described previously (Ramage et al., 2001). For time-kill studies *C. albicans* biofilms ($n = 3$) were grown for 24 h, then exposed to TTO, T-4-ol, or α-terpineol at a concentration of $2 \times MIC_{90}$ for 2, 5, 15, and 60 min. Metabolic activity was quantified by the XTT assay and biofilm viability assessed as a relative reduction in absorbance in comparison to untreated controls. For inhibition, *C. albicans* isolates ($n = 4$) were plated in 96-well microtiter plates for biofilm growth (1×10^6 cells/ml) then treated at 0, 1, 2, or 4 h after adhesion with TTO, T-4-ol, and α-terpineol at 0.5 and $1 \times MIC_{50}$. The cells were then incubated for a further 24 h at 37°C. The levels of biofilm inhibition were compared to an untreated control using a crystal violet biomass assay, as previously described (Mowat et al., 2007). All experiments outlined above were performed on two independent occasions with a minimum of three clinical isolates in triplicate.

MODE OF ACTION

We investigated whether TTO or T-4-ol interacted with the membrane using a propidium iodide (PI) uptake assay, as previously reported (Sherry et al., 2012). PI is a fluorescent dye that diffuses through the membranes of dead cells and is incorporated within nuclear DNA, therefore increased fluorescence directly correlates with reduced membrane integrity. *C. albicans* (3153A) was grown in an overnight broth as described previously, and standardized to 1×10^7 cells/ml in RPMI-1640 and treated with TTO, T-4-ol, or α-terpineol at a concentration of $2 \times MIC_{50}$ for 10, 20, 30, 40, 50, and 60 min. Following treatment, cells were centrifuged at 10,000 rpm to remove the compounds, washed with

PBS, stained with 20 μM of propidium iodide (PI; Sigma-Aldrich, UK), and incubated at 37°C for 15 min to allow the dye to bind to DNA. One-hundred microliters of each sample were transferred to a black microtiter plate (Corning Incorporated, NY, USA) and fluorescence measured using a microtiter plate reader (FluoStar Omega, BMG Labtech) at excitation and emission wavelengths 535/617 nm, respectively. Raw data were corrected for background fluorescence and the assay was carried out in triplicate. In addition, the physical effect of these compounds was assessed microscopically using scanning electron microscopy (SEM). *C. albicans* was adhered to Thermanox™coverslips (Nunc Inc., Thermo Fisher Scientific) for 2 h within 24 well tissue culture plates then treated with TTO and T-4-ol at 2 × MIC_{50} for 24 h. The cells were fixed using 2% w/v para-formaldehyde, 2% v/v glutaraldehyde, 0.15 M sodium cacodylate, and 0.15% Alcian Blue (pH 7.4), and prepared for SEM as previously described (Erlandsen et al., 2004). The fixed and dried biofilm samples were sputter-coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope.

ORAL EPITHELIAL CELL LINE

OKF6-TERT2 epithelial cells were used as previously described (Ramage et al., 2012). This is an immortalized human oral keratinocyte cell line provided by the Rheinwald Laboratory (Brigham and Woman's Hospital, Boston). These cells were immortalized by forced expression of telomerase, and resemble primary oral keratinocytes in studies of cytotoxicity or inducible cytokine and beta-defensin expression (Dongari-Bagtzoglou and Kashleva, 2003). The cells were cultured in keratinocyte serum-free medium (KSFM) supplemented with 100 IU penicillin, 100 μg/ml streptomycin, 25 μg/ml bovine pituitary extract (BPE), 0.2 ng/ml epidermal growth factor (EGF), and 0.3 mM $CaCl_2$ (0.4 mM total Ca^{2+}). Primary periradicular fibroblasts (PRF, kind gift of Professor Colin Murray, University of Glasgow) were cultured in standard Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU penicillin, and 100 μg/ml streptomycin. Both cell lines were passaged at approximately 90% confluence, using 0.05% Trypsin EDTA. Experiments were used with cells between passages 5 and 10.

TOXICITY STUDIES

TTO and T-4-ol dilutions were prepared in KSFM or DMEM with 0.25% (v/v) Tween® 80 for OKF6 or PRF cells, respectively. To assess cytotoxicity, cells were seeded at 1×10^5 cells per well per ml in KSFM or DMEM in a 24 well culture plate and grown until 90–100% confluent. Medium was then removed and cells exposed to 0.5 and 1 × MIC_{50} of each compound for 2 min, then cells were washed gently (×3) in Hanks balanced salt solution. The viability of the cells was assessed using an XTT assay (0.25 mg/ml XTT and 1 μM of menadione in KSFM or DMEM), in which the cells were incubated in 5% CO_2 at 37°C for 2 h, and the absorbance quantified at 490 nm in an automated microtiter plate reader (Tecan Sunrise, Jencons, UK). Viability was calculated based on unexposed control cells.

CYTOKINE EXPRESSION STUDIES

OKF6 cells were grown in 12 well tissue culture trays were seeded and grown as described above and treated with TTO and T-4-ol at 0.5 × MIC_{50} for 2 min, washed then stimulated with zymosan (50 μg/ml). Media and zymosan controls were also included. Supernatants were collected after 4 and 24 h and RNA extracted using TRIzol® according to the manufacturer's instructions (Invitrogen, Paisley, Scotland). Total RNA was treated with DNase I (Promega, Southampton, UK) at 37°C for 30 min. RNA quality and quantity was assessed using a NanoDrop™spectrophotometer ND-1000 (Labtech International Ltd., Ringmer, East Sussex, UK), prior to cDNA production using Superscript II Reverse Transcriptase (Invitrogen). qPCR was then performed using an MxPro MX3000P Quantitative PCR machine (Stratagene, Amsterdam, Netherlands) with the following primers: IL-8 (F: 5′-CAGAGACAGCAGAGCACACAA-3′; R: 5′-TTAGCACTCCTTGGCAAAAC-3′; 170 bp), and the housekeeping gene GAPDH (F: 5′-GGTGGTGAAGACGCCAGT-3′; R: 5′-CAAGGCTGAGAACGGGAAG-3′). Each duplicate PCR reaction contained the following: 0.5 μl of 1 μg/μl of cDNA, 0.5 μl forward and reverse primer (10 nmol), 0.25 μl ROX reference dye, 12.5 μl SYBR® Green (Invitrogen), and 10.5 μl dH_2O. PCR reaction conditions were as follows: 95°C – 10 min, 40× (94°C – 30 s, 58°C –30 s, 72°C – 30 s) and 72°C – 10 min. Analysis was performed using the Mx3000P software (Stratagene) and gene expression normalized to β-tublin gene according to the $2^{-\Delta\Delta CT}$ method. To assess IL-8 protein levels an ELISA was performed on retained supernatants. ELISA kits (R&D Systems, Abingdon, UK) were used in accordance with the manufacturer's instructions. All assays were optimized and validated prior to use, with standards and samples performed in at least duplicate.

STATISTICAL ANALYSIS

For assessing the statistical significance of observed changes in metabolic activity and biomass of *C. albicans* and IL-8 protein levels in oral keratinocyte culture fluids (data that conformed to a near normal distribution) a one-way ANOVA with Bonferroni multiple comparison post-test was performed. ANOVA and *post hoc* tests were also used on natural log transformations (to normalize) of proportional data for the analysis of IL-8 mRNA abundance in these cells. $p < 0.05$ was considered significant. The analyses were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, CA, USA).

RESULTS

TTO AND DERIVATIVE COMPONENTS ARE EFFECTIVE AGAINST *CANDIDA ALBICANS* BIOFILMS

The planktonic *C. albicans* isolates tested were susceptible to TTO, T-4-ol, and α-terpineol, with an MIC_{50} of 0.5, 0.25, and 0.25%, respectively, and an MIC_{90} of 1.0, 0.5, and 0.5%, respectively (**Table 1**). There was minimal variation in susceptibility as determined by the concentration ranges for the 100 strains tested. These three compounds also displayed potent activity against the 69 strains capable of forming biofilms, with the $SMIC_{50}$ for T-4-ol and α-terpineol showing no change when compared to median effective planktonic concentration ($SMFC_{50} = 0.25\%$). However, the concentration of TTO required to show a similar effect was double that required to inhibit planktonic cells ($SMFC_{50} = 1\%$). All other compounds tested showed relatively poor activity against the isolates tested.

Table 1 | Susceptibility profile of *Candida albicans* to tea tree oil and derivative components.

	Planktonic minimum inhibitory concentrations ($n = 100$)			Sessile minimum inhibitory concentrations ($n = 69$*)		
	MIC_{50} range (%)	MIC_{50} (%)	MIC_{90} (%)	$SMFC_{50}$ range (%)	$SMFC_{50}$ (%)**	Sessile/planktonic MIC_{50} ratio
TTO	0.125–1.0	0.5	1.0	1–4	1	2
T-4-ol	0.0625–0.5	0.25	0.5	<0.25–>1	0.25	1
α-Terpineol	0.125–0.5	0.25	0.5	<0.25–1	0.25	1
1,8-Cineole	0.5–>1	1	>1	<2–>2	2	2
Terpinolene	0.5–>1.0	>1	>1	2–>2	>2	>2
α-Terpinene	2–>2	>2	>2	4–>4	>4	>2
γ-Terpinene	2–>2	>2	>2	4–>4	>4	>2
ρ-Cymene	2–>2	>2	>2	4–>4	>4	>2

**69 of the 100 strains tested were able to form biofilms.*

***Sessile minimum inhibitory concentration at which a 50% reduction in biofilm metabolic activity was achieved.*

The biofilm rate of kill for the three most effective components (TTO, T-4-ol, and α-terpineol) at $2 \times SMIC_{50}$ was performed, where time dependent killing was observed (**Figure 1**). TTO was shown to reduce the viability of the biofilms within 2 min by 44%, but after 5, 15, and 60 min this had declined only slightly to 46, 56, and 65%, respectively. However, both T-4-ol and α-terpineol displayed comparable and more effective killing, reducing the viability rapidly after 2 min by approximately 55%. This anti-biofilm activity remained, reducing biofilm viability by approximately 61, 75, and 94% after 5, 15, and 60 min, respectively. Both T-4-ol and α-terpineol showed significantly better activity than TTO only at 60 min ($p < 0.001$).

Inhibition of biofilm formation was assessed for these three compounds at 0.5 and $1 \times MIC_{50}$ by assessing biofilm biomass. It was shown for all compounds that $1 \times MIC_{50}$ was able to inhibit biofilm growth effectively when treated at 0, 1, and 2 h post adhesion (8.5−12% biomass compared to control). At 4 h post adhesion, by which time cells had began to form hyphae, treatment with TTO, T-4-ol, and α-terpineol further suppressed biofilm proliferation by 71, 74, and 82% that of the control (**Figure 2A**). No significant differences were observed between the treatments at each time point. Treatment with $0.5 \times MIC_{50}$ was overall less effective at inhibiting biofilm growth most notably with TTO, which displayed a time dependent reduction in inhibition, showing a 44% inhibition of cells treated at 4 h (**Figure 2B**). Both T-4-ol and α-terpineol were significantly more effective against 1 and 2 h cells ($p < 0.05$), inhibiting biofilm formation by >80%. However, this inhibition was reduced against the 4 h cells (54 and 58%, respectively).

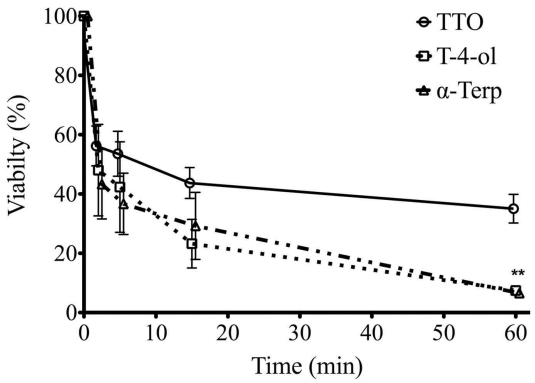

FIGURE 1 | *Candida albicans* biofilm killing by TTO, T-4-ol, and α-terpineol is time dependent. Standardized *C. albicans* (1×10^6 cells/ml) were incubated in flat-bottomed 96-well plates for 24 h, washed in PBS, and treated with $2 \times SMIC_{50}$ at 2, 5, 10, 15, and 60 min. Metabolic activity of treated biofilms was then quantified using the XTT assay by reading at 492 nm in a microtiter plate reader (FluoStar Omega, BMG Labtech). Three isolates were used for each assay, and this was performed on two independent occasions in triplicate. $^{**}p < 0.001$. Error bars represent the ±standard error of the mean.

TTO AND DERIVATIVE COMPONENTS ARE CELL MEMBRANE ACTIVE

Scanning electron microscopy analysis of TTO and T-4-ol treated cells was performed. It was noted that compared to the control (untreated) cells (**Figure 3Ai**), both compounds had ruptured the cells, allowing the cell contents to leak out, giving a punctured appearance (**Figures 3Aii,iii**). The cell damage was shown to be more extensive for T-4-ol treated cells. Given this appearance we hypothesized that cell membrane integrity had been compromised. We therefore undertook PI uptake experiments, as previously reported (Sherry et al., 2012). For TTO, PI uptake was shown to be relatively slow, with maximal fluorescence obtained at 30 min (**Figure 3B**). In comparison, for T-4-ol fluorescence was shown to increase in a time dependent manner up to 40 min, twice that of TTO, after which time this reached a plateau. These data show similar kinetics to the time-kill data presented.

TTO AND TERPINEN-4-ol ARE BIOLOGICALLY ACTIVE AGAINST MAMMALIAN CELLS

The effect of a short exposure (2 min) of TTO and T-4-ol on cellular toxicity was investigated. It was shown that TTO and T-4-ol were toxic to both fibroblast and epithelial cells at $1 \times MIC_{50}$, reducing the viability of PRF to approximately 12% and OKF6

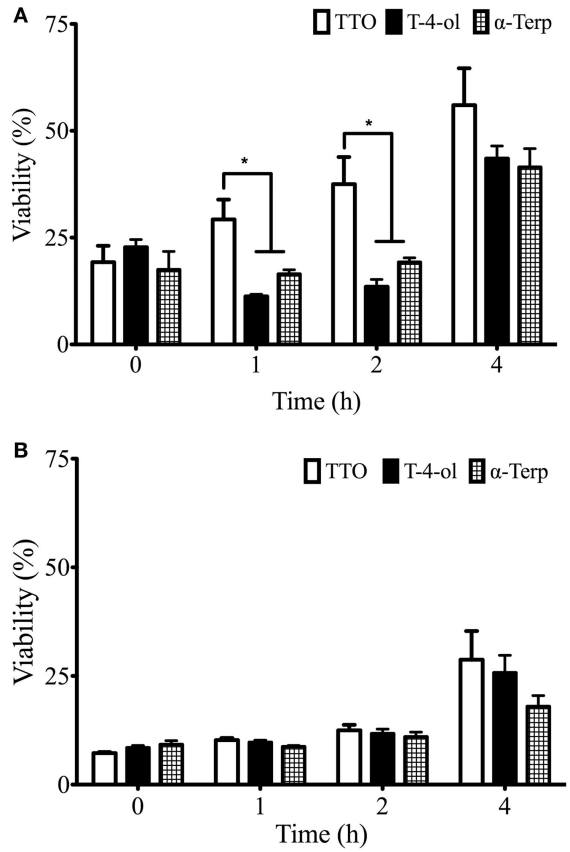

FIGURE 2 | *Candida albicans* biofilm development is inhibited by TTO, T-4-ol, and α-terpineol. Standardized *C. albicans* (1×10^6 cells/ml) were added to a flat-bottomed 96-well plate and treated with **(A)** 0.5 and **(B)** $1 \times SMIC_{50}$ for 0, 1, 2, and 4 h post incubation. Biofilms were subsequently allowed to develop for 24 h. Resultant biofilms were quantified using a crystal violet assay read at 570 nm in a microtiter plate reader (FluoStar Omega, BMG Labtech). Four isolates were used for each assay, and this was performed on two independent occasions in triplicate. $*p < 0.05$. Error bars represent the ±standard error of the mean.

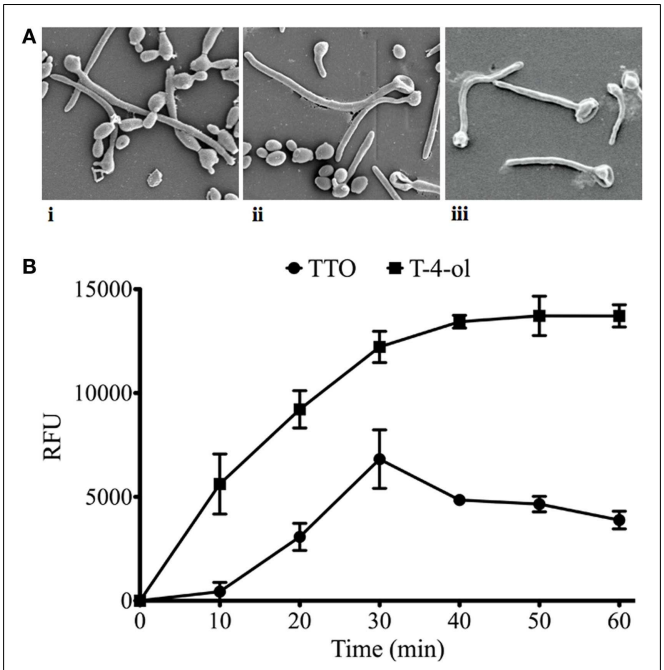

FIGURE 3 | Tea tree oil and T-4-ol are cell membrane active. (A) Adherent cells (3153A) were adhered for 2 h on Thermanox™coverslips and were **(i)** untreated (control), or treated with **(ii)** TTO, or **(iii)** T-4-ol at $2 \times MIC_{50}$ for 24 h. These were then processed and viewed on an SEM. **(B)** *C. albicans* (3153A) planktonic cells (5×10^7 cells/ml) were treated with TTO, T-4-ol, or α-terpineol at a concentration of $2 \times MIC_{50}$ for 10, 20, 30, 40, 50, and 60 min. The cells were washed by centrifugation, resuspended in PI (20 μM in PBS), and incubated for 15 min at 37°C. These were then transferred to a black 96-well plate for quantification in a fluorescent plate reader (Ex_{485}/Em_{620}). Each assay was performed on at least two independent occasions in triplicate. Error bars represent the ±standard error of the mean.

cells to 35 and 15%, respectively (**Figure 4A**). At $0.5 \times MIC_{50}$ T-4-ol displayed no toxicity (98% viable) against PRF compared with TTO (63% viable). Both 0.5× T-4-ol and 0.5× TTO were non-toxic to OKF6 cells (>100% viable).

Given that the concentration of $1 \times MIC_{50}$ is cytotoxic, all subsequent work was performed with $0.5 \times MIC_{50}$. Transcriptional expression of IL-8 was assessed by qPCR, using zymosan as a potent cell inflammatory agonist. Generally, greater IL-8 mRNA levels were observed at 4 h compared to 24 h. TTO and T-4-ol pre-treatment of the cells had no effect on the induction of the IL-8 gene when compared to the control (**Figure 4B**). In zymosan-stimulated cells pre-treatment with TTO did not reduce IL-8 expression significantly at either 4 or 24 h ($p > 0.05$). Although T-4-ol showed up to a twofold reduction in IL-8 expression in cells at both 4 and 24 h, though this reduction was not statistically significant ($p > 0.05$).

Analysis of IL-8 protein levels after 4 and 24 h stimulation with zymosan showed that neither TTO or T-4-ol were able to significantly affect IL-8 levels in culture fluids ($p > 0.05$) of untreated and zymosan-stimulated oral keratinocytes (data shown for zymosan only). Nevertheless, a significant (50%) reduction in IL-8 expression was observed in culture fluids of TTO treated zymosan-stimulated cells ($p < 0.05$), and a modest reduction of approximately 14% of IL-8 was noted for T-4-ol treated zymosan-stimulated cells (**Figure 4C**). These compounds had no effect on the IL-8 ELISA (data not shown).

DISCUSSION

Increasing use of conventional antifungal agents, such as azoles, in parallel with larger groups of susceptible individuals (aging population and more common immunosuppressive therapies) has resulted in the emergence of multidrug-resistant *Candida* strains (Sanglard and Odds, 2002; Akins, 2005; Cannon et al., 2007; Niimi et al., 2010). Non-compliance due to toxic side-effects and palatability may also exacerbate this escalating trend. Consequently, there is a demand for novel therapies to manage these infections. Our current and previous data investigating the effect of TTO on a wide variety of yeast species clearly demonstrate that it is effective against *C. albicans* planktonic and biofilm cells (Bagg et al., 2006).

Given the complex chemical composition of TTO we aimed to investigate key individual components to assess specific activities.

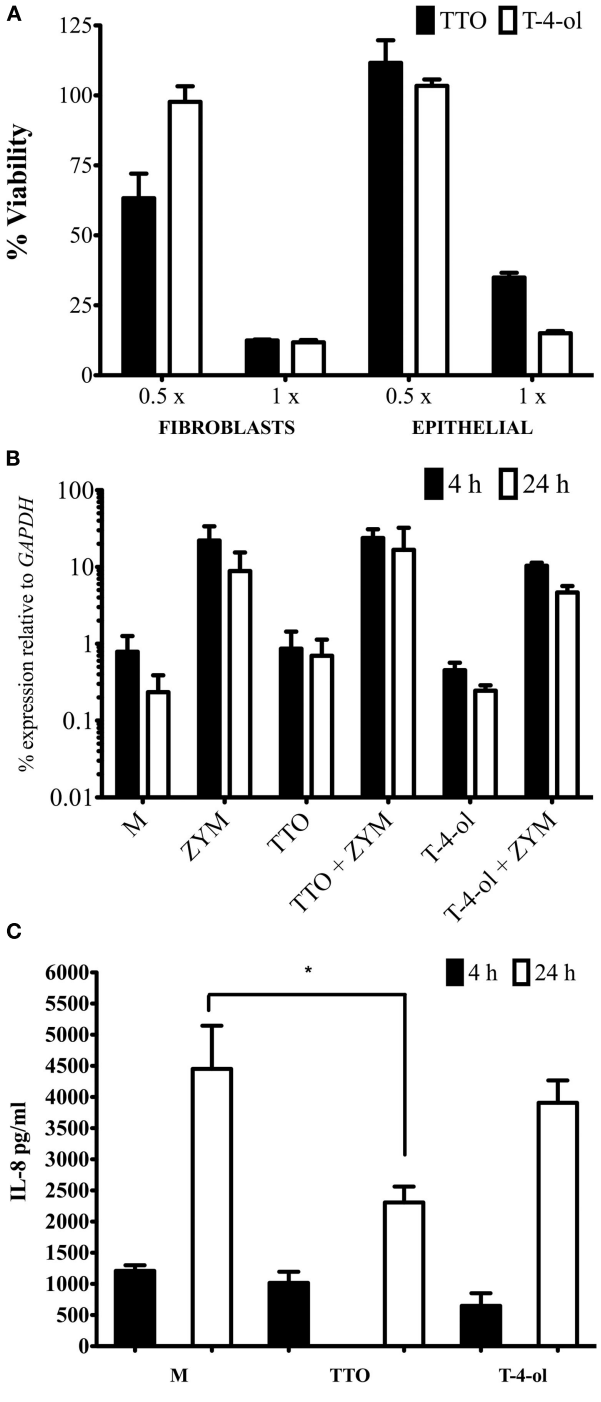

FIGURE 4 | Tea tree oil and T-4-ol are biologically active against mammalian cells. (A) PR fibroblast and OKF6 epithelial cells were grown until 90–100% confluent. These were exposed to 0.5 and 1 × MIC_{50} of each compound for 2 min. Cell viability was assessed using an XTT assay after incubation in 5% CO_2 at 37°C for 2 h. Viability was calculated based on unexposed control cells. For cytokine studies OKF6 cells were grown in 12 well tissue culture trays treated with TTO and T-4-ol at 0.5 × MIC_{50} for 2 min, washed then stimulated with zymosan (50 μg/ml). Cells were processed for **(B)** qPCR analysis and **(C)** supernatant was processed for protein analysis of IL-8. Each assay was performed on three independent occasions in triplicate. *$p < 0.05$. Error bars represent the ±standard error of the mean.

T-4-ol and α-terpineol exhibited the greatest and comparable antifungal activity against both planktonic and sessile cells, which has been confirmed from reports elsewhere (Hammer et al., 2003a; Mondello et al., 2006; Terzi et al., 2007). Moreover, time-kill studies showed a rapid and sustained level of activity of both compounds, significantly superior to TTO at 2 × MIC_{90} after 60 min. This superior activity was less evident during the early biofilm inhibition studies (0–2 h), however, at 4 h TTO showed a significant decline in its ability to inhibit biofilm growth. Both T-4-ol and α-terpineol have hydroxyl groups in their chemical structures, making them moderately water-soluble. This allows them to diffuse through water, enter, and destabilize cell membranes, resulting in osmotic shock (Straede et al., 2007). This was evident from analysis of cell membrane integrity using a PI uptake assay and microscopic examination by SEM.

T-4-ol and α-terpineol both showed excellent activity, but the remainder of the studies compared TTO with T-4-ol, primarily because of the high bioavailability of the latter within TTO and its overall antifungal profile (Carson et al., 2006). Cytotoxicity was observed at MIC_{50} levels for both TTO and T-4-ol, whereas at 0.5 × MIC_{50} (concentrations able to inhibit *C. albicans* growth) OKF6 cells remained viable. Previous reports have demonstrated varying levels of cytotoxicity to primary fibroblasts and primary epithelial cells, with a 1 h exposure to 0.03%TTO shown to be toxic (Soderberg et al., 1996). Further reports indicated that a 4 h exposure to 0.28% TTO was required to inhibit the HeLa epithelial cell line (Hayes et al., 1997). In addition, it was shown previously that TTO was highly toxic to monocytes and neutrophils, but after a prolonged 20 h exposure (Hart et al., 2000). The relevance of this length of exposure is difficult to interpret. Collectively, these studies highlight the importance of the cell line being tested and in what context. Given that our primary interests lay in developing a mouthwash to prevent candidal growth as opposed to treating an active infection, a 2 min exposure of sub-inhibitory concentrations was deemed optimal.

These data were used to assess the impact of TTO-based compounds on inflammation, using IL-8 as a biomarker. Both transcriptional and protein analysis showed that IL-8 was regulated by both compounds, with TTO showing protein reduction of approximately 50% 24 h post treatment, whereas T-4-ol only caused a 14% reduction. However, at 4 h post treatment T-4-ol inhibited IL-8 protein production by 53% opposed to 16% for TTO, although neither was significant. The differences observed may be accounted for by the quantitative composition of TTO, which contains approximately 40% T-4-ol. A previous study of LPS stimulated monocytes demonstrated a significant IL-8 suppression by 0.052% T-4-ol after 40 h (Hart et al., 2000). The discordance with our data can be explained through differences in concentration and exposure time. Irrespective, both studies indicate that these molecules have the potential capacity to suppress inflammatory mediators, which are common within the oral cavity of OPC sufferers. Indeed, there is *in vivo* evidence to support this. Several murine studies have shown inhibitory effects on inflammatory processes, including reduced contact hypersensitivity (Brand et al., 2002a), reduced histamine-induced edema (Brand et al., 2002b), and blocking of zymosan-induced inflammation by inhaled TTO (Golab and Skwarlo-Sonta, 2007). In humans,

nickel-induced contact hypersensitivity has been reduced by the topical application of 100% TTO (Pearce et al., 2005). Both TTO (Koh et al., 2002) and T-4-ol (Khalil et al., 2004) have also been shown to reduce histamine-induced weal and flare reaction in human skin. Conversely, other studies have implicated TTO as being pro-inflammatory (de Groot and Weyland, 1992; Rutherford et al., 2007). Given the apparent contradictory reports in the literature it seems prudent to focus on utilizing the most biologically active and abundant compound from TTO, i.e., T-4-ol. This will enable investigators to determine accurately the medicinal benefits of pure T-4-ol and exclude deleterious effects caused by the other terpenes that comprise TTO.

In summary, these studies have added to the body of *in vitro* evidence indicating that TTO and some of its individual components, specifically T-4-ol, exhibit strong antimicrobial efficacy against fungal biofilms. Furthermore, this has also demonstrated a potential biofilm inhibiting activity, suggesting that this agent may be suitable for use in prophylactic oral hygiene products such as mouth rinses and denture cleansers, as well as treatment for established OPC infections. The use of T-4-ol, a single component from TTO, has advantages over the complete essential oil in terms of product safety and consistency. Oral candidosis is a continuing problem for cancer patients, as well as other groups of immunocompromised hosts. In the face of increasing resistance to azoles and other established antifungal drugs, the need for novel preventive and therapeutic agents has never been greater. The weight of laboratory data that has now accumulated and the anecdotal reports of clinical efficacy suggest that clinical trials of TTO components, particularly T-4-ol, would be merited.

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Powerful bacterial killing by buckwheat honeys is concentration-dependent, involves complete DNA degradation and requires hydrogen peroxide

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Exposure of bacterial cells to honey inhibits their growth and may cause cell death. Our previous studies showed a cause-effect relationship between hydroxyl radical generated from honey hydrogen peroxide and growth arrest. Here we explored the role of hydroxyl radicals as inducers of bacterial cells death. The bactericidal effect of ·OH on antibiotic-resistant clinical isolates of MRSA and VRE and standard bacterial strains of *E. coli* and *B. subtiles* was examined using a broth microdilution assay supplemented with 3′-(*p*-aminophenyl) fluorescein (APF) as the ·OH trap, followed by colony enumeration. Bactericidal activities of eight honeys (six varieties of buckwheat, blueberry and manuka honeys) were analyzed. The MBC/MIC ratio ≤ 4 and the killing curves indicated that honeys exhibited powerful, concentration-dependent bactericidal effect. The extent of killing depended on the ratio of honey concentration to bacterial load, indicating that honey dose was critical for its bactericidal efficacy. The killing rate and potency varied between honeys and ranged from over a 6-$\log_{10}$ to 4-$\log_{10}$ CFU/ml reduction of viable cells, equivalent to complete bacterial eradication. The maximal killing was associated with the extensive degradation of bacterial DNA. Honey concentration at which DNA degradation occurred correlated with cell death observed in the concentration-dependent cell-kill on agar plates. There was no quantitative relationship between the ·OH generation by honey and bactericidal effect. At the MBC, where there was no surviving cells and no DNA was visible on agarose gels, the ·OH levels were on average 2–3x lower than at Minimum Inhibitory Concentration (MICs) ($p < 0.0001$). Pre-treatment of honey with catalase, abolished the bactericidal effect. This raised possibilities that either the abrupt killing prevented accumulation of ·OH (dead cells did not generate ·OH) or that DNA degradation and killing is the actual footprint of ·OH action. In conclusion, honeys of buckwheat origin exhibited powerful, concentration-dependent bactericidal effect. The killing and DNA degradation showed a cause-effect relationship. Hydrogen peroxide was an active part of honey killing mechanism.

Keywords: bactericidal action, honey, MBC/MIC ratio, killing curves, MRSA and VRE, hydrogen peroxide, hydroxyl radicals, aminophenyl fluorescein

INTRODUCTION

At present, the exact mechanism of honey action that leads to bacterial cell death is unknown. The early honey research was focused primarily on providing evidence of antibacterial activity by defining the spectrum of susceptible bacteria, by comparing efficacy of different honeys in bacterial growth inhibition and by identifying the activity-related compounds (Allen et al., 1991; Molan, 1992; Cooper et al., 1999, 2000, 2002a,b; Lusby et al., 2002; Wilkinson and Cavanagh, 2005; Brudzynski and Kim, 2011). In due course, it became apparent that there are two main groups of honeys with the respect to the main component involved in bacterial growth inhibition: the group of European and American honeys whose activity was catalase-sensitive and showed substantial correlation with the internal levels of hydrogen peroxide and the group of honeys of *Leptospermum* spp. whose activity was hydrogen peroxide-independent (Molan and Russell, 1988; Allen et al., 1991) but instead correlated well with the levels of internal methylglyoxal (Adams et al., 2008; Mavric et al., 2008). Bacterial cultures exposed to the former group of honeys showed signs of increased oxidative stress that correlated with generation and accumulation of hydroxyl radicals (Brudzynski et al., 2011, 2012; Brudzynski and Lannigan, 2012). Our recent studies documented that hydrogen peroxide was a necessary substrate for ·OH formation via the metal-catalyzed Fenton reaction. ·OH formation and accumulation inhibited bacterial growth in a dose-dependent manner. Addition of transition metal, Cu(II) to this system enhanced honey bacteriostatic action as manifested by a marked decrease of Minimum Inhibitory Concentration

MIC values against both standard and antibiotic-resistant clinical isolates (Brudzynski et al., 2011; Brudzynski and Lannigan, 2012). These studies provided evidence that ·OH generated from honey H_2O_2 occupied a key position in the bacteriostatic mechanism of action.

It was therefore plausible that ·OH radicals may play similar role in the bactericidal effect of honey. ·OH radicals are powerful but short-lived oxidants that indiscriminately target macromolecules located in close vicinity to sites of ·OH formation (Roots and Okada, 1975). In bacterial cells, ·OH radicals were shown to cause protein and lipid peroxidation, and DNA and RNA degradation. The oxidative injury to these macromolecules impaired permeability of cell membranes and cell proliferation, respectively, and ultimately led to the decrease in cell viability and cell death (Imlay and Linn, 1988; Imlay et al., 1988; Cabiscol et al., 2000; Sakihama et al., 2002). Recently, Kohanski et al. (2007, 2010) provided evidence that bactericidal efficacy of different groups of antibiotics was ultimately linked to the overproduction of hydroxyl radicals inside bacterial cell. These results gave support to our hypothesis that ·OH produced from honey's hydrogen peroxide may also underlie the bactericidal action of honey.

The bactericidal effect of antimicrobial drugs is usually characterized by pharmacodynamic parameters. However, the dynamics of bacterial killing by different honeys have not been thoroughly investigated. Honey antibacterial activity is commonly defined in terms of its growth inhibitory activity and usually quantitated using the MIC method. Quite often in literature this activity was equated with honey ability to kill microorganisms. In only a couple of examples have data from bacteriostatic and bactericidal assays been simultaneously analyzed (Blair et al., 2009; Tan et al., 2009; Sherlock et al., 2010). A recurrent finding from the above studies was that the maximal growth inhibitory and bactericidal effects of honeys lied in the narrow concentration range. The MICs for honeys of different botanical origins ranged between 4–16% w/v, averaging around 8% w/v (Willix et al., 1992; Cooper and Molan, 1999; Cooper et al., 1999, 2000; Blair et al., 2009; Brudzynski et al., 2011). Clearly, concentrations of honey active components were critical for the antibacterial effects and this information gave the first indication of a possible mode of bactericidal action.

Recent results from our laboratory have suggested that the oxidative stress evoked by honeys on bacterial cells resulted from the coupling chemistry between polyphenols, H_2O_2 and transition metals. Concentrations of these components were responsible for the suppression of bacterial growth (MIC) as well as for the extent of DNA degradation (Brudzynski et al., 2012). The single and double DNA strand-breaks have been clearly observed after incubation of plasmid DNA with honeys of different botanical origin. DNA degrading potencies of these honeys were closely related to the total phenolic content and redox capacity of polyphenols (Brudzynski et al., 2012). The latter activity play central role in facilitating the polyphenol-mediated Fenton reaction and generation of hydroxyl radicals (Sakihama et al., 2002). Depending on the concentration and in the presence of catalytic amounts of transition metals, flavonoids of certain structure induce free radicals generation (Cao et al., 1997; Fukumoto and Mazza, 2000).

Indeed, polyphenols emerged as important functional honey constituents. The redox capacity of polyphenols enabled them to interact with each other, and with proteins and sugars leading to a formation of high molecular structures called melanoidins (Brudzynski and Miotto, 2011a,b). In the structure-depending way, polyphenols were capable of influencing the levels of antioxidant and antibacterial activities as well as the extent of polymerization of active honey components (Brudzynski and Miotto, 2011a,b,c). As a consequence of their redox activity, the balance between antioxidant/proxidant activities of polyphenols could be changed in the presence of oxygen or hydrogen peroxide and traces of metal ions, and result in the generation of cytotoxic free radicals (Cao et al., 1997; Fukumoto and Mazza, 2000; Sakihama et al., 2002). These in turn, could be responsible for cell injury and DNA damage, mimicking the antibacterial action of phagocytic cells (Gutteridge et al., 1998) or antibiotics (Kohanski et al., 2007, 2010). The concentration of polyphenols and hydrogen peroxide in different honeys may therefore be of critical importance for bacterial cell survival.

The aim of the present study was to (a) demonstrate and compare bactericidal effect of different honeys, (b) establish pharmacodynamic parameters such as killing rates, maximum bactericidal concentration and potency of different honeys, and (c) to explore the role of hydroxyl radical in the killing mechanism of honey.

MATERIALS AND METHODS

HONEYS

Honeys were donated by Canadian beekeepers and included both commercial (pasteurized) and apiary (unprocessed) samples. The list of honeys and their plant origin is given in **Table 1**.

BACTERIAL STRAINS

Standard strains of *Bacillus subtilis* (ATCC 6633) and *Escherichia coli* (ATCC 14948) purchased from Thermo Fisher Scientific Remel Products (Lenexa, KS 66215) were grown in Mueller-Hinton Broth (MHB) (Difco Laboratories) overnight in a shaking water bath at 37°C. Overnight cultures were diluted with broth to the equivalent of the 0.5 McFarland standard.

Clinical isolates, vancomycin-resistant *Enteroccus faecium* (VRE2) and methicillin-resistant *Staphylococcus aureus* (MRSA6) were obtained from the Clinical Microbiology Laboratory of the London Health Science Centre, London Ontario. Strains were subcultured from swabs onto Mueller-Hinton II agar (Difco Laboratories). Isolates were identified to genus and species and their susceptibility to antibiotics was confirmed using an automated system (Vitek^R, Biomérieux^R). The presence of the *mec*A gene, *nuc* genes and *van*A and B genes were determined by polymerase chain reaction. This work was conducted by the Clinical Microbiology Laboratory, London Health Sciences Centre, London, Ontario as described previously (Brudzynski and Lannigan, 2012).

BROTH MICRODILUTION ASSAY

The antibacterial activity of honeys was performed using a broth microdilution assay in sterile, 96-well format as described previously (Brudzynski et al., 2011). Bacterial growth was measured

Table 1 | List of honeys.

Honey	Botanical source	Color (A_{560}–A_{720})	Hydrogen peroxide (mM/L)*
Spl. 15	*Vaccinium corymbosum* Blueberry	0.267	1.75 ± 0.02
Spl. 23	*Fagopyrum esculentum* Buckwheat	0.975	2.12 ± 0.022
Spl. 77	*Fagopyrum esculentum* Buckwheat	1.266	2.70 ± 0.06
Manuka	*Leptospermum scoparium*	0.539	1.04 ± 0.17
H203	*Fagopyrum esculentum* Buckwheat	0.300	0.248 ± 0.020
H204	*Fagopyrum esculentum* Buckwheat	0.320	0.740 ± 0.08
H205	*Fagopyrum esculentum* Buckwheat	0.463	1.17 ± 0.05
H206	*Fagopyrum esculentum* Buckwheat	0.965	1.11 ± 0.02

at A_{595} nm using the Synergy HT multidetection microplate reader (Synergy HT, Bio-Tek Instruments, Winooski, VT, USA). In a single experiment, each honey was tested in triplicate. Each microorganism has been analyzed at least in three independent experiments.

Statistical analysis and dose response curves were obtained using K4 software provided by Synergy HT (Bio-Tek Instruments, Winooski, VT, USA).

The MIC were determined from the growth inhibition profiles curves and represented the lowest concentration of the honeys that inhibited the bacterial growth by 90% as measured by the absorbance at A_{595} nm.

KILLING CURVES AND DETERMINATION OF MBC

After determination of the MIC for each strain in broth microdilution assay, the killing curves were constructed by subculturing the entire contents of each well (100 µl) from microplates that showed no visible growth onto Mueller-Hinton agar (MHA). The killing curves were produced from serially diluted honeys against each bacterial strain. To verify the final cell density of bacteria not exposed to honey, wells containing inoculum only (assay control) were serially 10-fold diluted with sterile water to obtain approximate cell density of 10^4 and 10^2 CFU/ml and then 10-µl and 100-µl aliquot from each dilution was streaked onto agar plates.

The MBC endpoint was the minimum concentration of honey at which at least 99.9% of the initial inoculum was eradicated and at which only one or no colonies could be seen on MHA.

HYDROXY-RADICALS MEASUREMENTS

3′-(*p*-aminophenyl) fluorescein (APF) (Invitrogene, Canada) was used for the detection of hydroxyl radicals produced by honeys. A generation of ·OH radicals was monitored using a broth microdilution assay by adding 10 µM of APF (in 50 mM potassium phosphate buffer, pH 7.4) to each experimental wells (containing bacterial inoculum and honey dilutions) as well as the assay controls (bacterial inoculum). The negative control consisted of experimental wells without APF.

The plates were analyzed for both, bacterial growth using absorbance at A_{595} nm and hydroxyl radical generation using fluorescence excitation and emission wavelengths at 490 and 520 nm, respectively.

BACTERIAL DNA ISOLATION

Overnight *E. coli* cultures were adjusted to 10^7 CFU/ml in M-H broth and incubated in 1:1 ratio with honeys at their 2xMBCs and MBCs by shaking (250 rpm) at 37°C overnight for 18 h. Cells were recovered by centrifugation at 3000 × g (Eppendorf) for 60 s The total genomic DNA was isolated from untreated and from honey-treated cells using two methods: plasmid DNA isolation kit (MiniPrep) to monitor DNA degradation fragments and bacterial genomic DNA isolation kit (Norgen Biotek Corporation, St. Catharines, ON., Canada) according to the manufacturer's instructions.

AGROSE GEL ELECTROPHORESIS

Ten microliter aliquots of isolated DNA were analyzed by agarose gel electrophoresis (1%) in 1X TAE buffer containing ethidium bromide with a Gel System from Bio-Rad Laboratories (Mississauga, Ontario). The DNA molecular weight marker was selected to be the HighRanger 1 kb DNA Ladder, MidRanger 1 kb DNA Ladder and PCRSizer 100 bp DNA Ladder from Norgen Biotek Corporation (St. Catharines, ON., Canada). The gels were run at 85 V for 40 min and then visualized and photographed using the Gel Doc 1000 system and the Quantity One 1-D Analysis software (version 4.6.2 Basic) from Bio-Rad.

STATISTICAL ANALYSIS

The GraphPad Instant software version 3.05 (GraphPad Software Inc.) was used to calculate the means and standard deviation in experiments involving triplicate and multiple replicate analyses of samples. Differences between averages were evaluated by the *t*-test with the significance level of $p \leq 0.05$.

RESULTS

BACTERICIDAL EFFECT OF HONEYS

To examine bactericidal effects of honeys, the isolates of MRSA6 and VRE2 and standard bacteria, *E. coli* and *B. subtilis* were analyzed under the same growth conditions, bacterial density (10^7CFU/ml), incubation times (18 h) and temperature (37°C). The MIC and MBC values were determined using broth microdilution assay with the endpoint at which 90% bacteria were inhibited or killed, respectively. The assay was followed by a colony enumeration in the standard plate count.

Among eight honeys tested, the MIC and MBC values ranged from 3.125% to 25% v/v, indicating a rather narrow

concentration range at which honey was effective. There was no difference in median MIC and MBC values obtained in three separate experiments conducted in triplicate over the period of nine month. As shown in **Table 2**, the MBC/MIC ratio ≤ 4 indicated that the honeys displayed a predominantly bactericidal activity.

BACTERIAL KILLING BY HONEY OCCURRED IN A CONCENTRATION-DEPENDENT MANNER

Dynamics of bacterial killing was investigated as a function of honey concentrations. The changes in the bacterial cell count were assessed at 4×MIC, 2×MIC and the MIC. Killing curves showed a rapid and complete reduction of cell viability at or above the MBCs (**Figure 1**). After honey concentrations fall below MBC levels, the bactericidal effect was abruptly diminished and the bacterial re-growth emerged. Since honeys were bactericidal in the narrow zone of concentrations, it suggested that the killing critically depended on the ratio of honey concentration to bacterial load. It is predicted that with the increased bacterial cell densities, higher concentration of honey would be needed to produce the maximal killing effect.

Bactericidal potency, that is the range of concentrations over which honey produced killing effect, seemed to depend on honey variety. Blueberry honey required the highest concentration (50% v/v) to kill MRSA6 and VRE2 as well as *E. coli* and *B. subtilis*, while buckwheat honeys and manuka did kill bacteria but they differ in their MBC (H23< H77< manuka) (**Table 2**, **Figure 1**). Similarly, buckwheat honeys H77 and manuka showed higher bactericidal potency than buckwheat honey H23 because they evoked the same maximal bactericidal effect (a complete bacterial eradication) at lesser concentrations (12.5% v/v vs 25% v/v, respectively) (**Figure 1**).

Nevertheless, the killing curves demonstrated that honeys at their MBCs caused $> 6 \log_{10}$ CFU/ml reduction of colony counts, equivalent to complete bacterial eradication.

Table 2 | Comparison of bactericidal activity of honeys and bacterial susceptibility.

Honey variety	MRSA6		VRE2		*E. coli*		*B. subtilis*	
	MIC (%, v/v)	MBC (%, v/v)	MIC (%, v/v)	MBC (%, v/v)	MIC (%, v/v)	MBC (%, v/v)	MIC (%, v/v)	MBC (%, v/v)
H15	25%	50%	25%	50%	50%	50%	50%	50%
H23	6.25%	12.5%	12.5%	25%	12.5%	25%	12.5%	12.5%
H77	3.125%	6.25%	12.5%	12.5%	12.5%	25%	12.5%	12.5%
M2	3.125%	6.25%	12.5%	25%	12.5%	12.5%	6.25%	6.25%
H203	6.25%	12.5%	25%	25%	25%	25%	12.5%	12.5%
H204	6.25%	12.5%	25%	25%	12.5%	12.5%	12.5%	12.5%
H205	6.25%	12.5%	25%	25%	12.5%	12.5%	12.5%	12.5%
H206	3.125%	6.25%	6.25%	12.5%	6.25%	6.25%	12.5%	12.5%

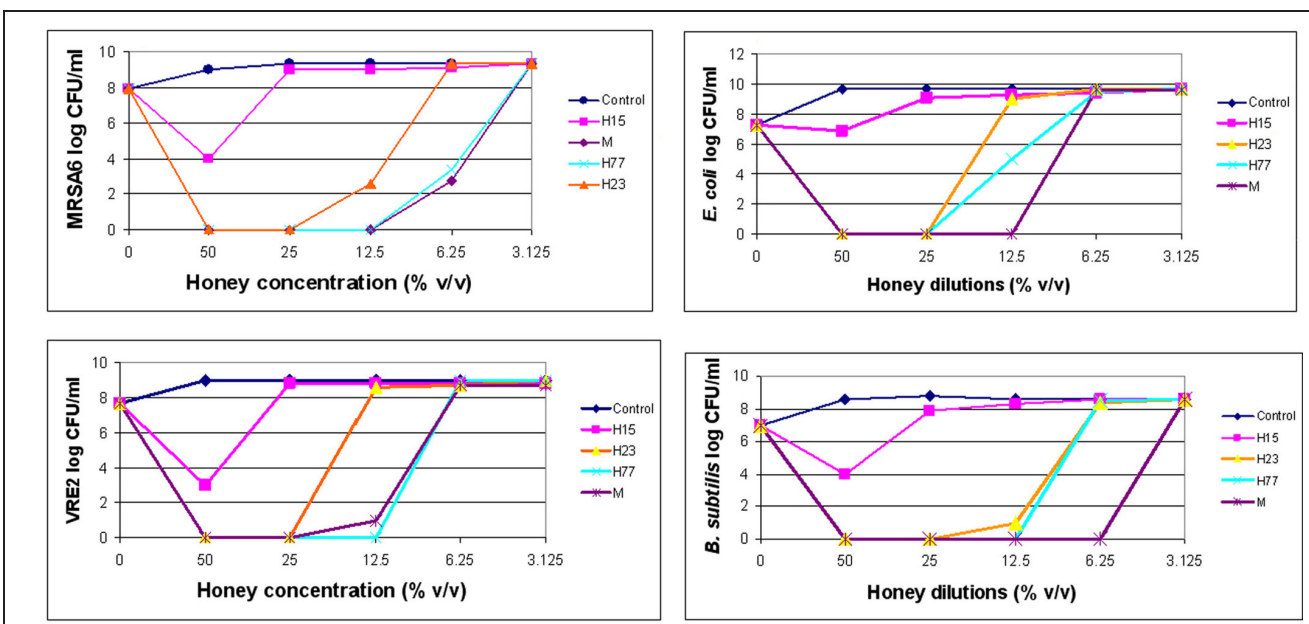

FIGURE 1 | Killing curves of honey for MRSA6, VRE2, *E. coli* and *B. subtilis*. Honey bactericidal effects were evaluated at multiples of MIC. The figure displays concentration-dependent reduction in log CFU/ml and subsequent regrowth of bacteria for each honey.

BACTERIAL KILLING BY HONEYS CORRELATED WITH A COMPLETE DNA DEGRADATION

The observed absence of viable colony counts on agar plates at the MBC endpoints could be indicative of the extensive, catastrophic damage to cell integrity (**Figure 1**). To gather more insight into the mechanism of bacterial cell death after exposure to honey, we investigated a potential causal link between cell killing and DNA damage. DNA degradation and release of DNA fragments was analyzed by agarose gel electrophoresis by employing two methods of DNA extraction, genomic and plasmid DNA isolation methods, respectively.

Since the bacterial killing occurred in the narrow concentration range, we examined whether honey concentrations played analogous role in DNA degradation. *E. coli* and *B. subtilis* cultures were exposed to honey H23 at 4×MIC, 2×MIC and MIC (50%, 25% and 12.5% v/v) and the integrity of their genomic DNA was analyzed on agarose gels. Honeys at their MBC caused extensive double-strand DNA cleavage that led to complete break down of genomic DNA (**Figure 2A**). The appearance of a smudge of small DNA fragments isolated from these cultures using plasmid DNA method captured ongoing DNA degradation (**Figure 2B**). Single strand cuts to the genomic DNA would not yield small fragments and thus would be undetectable using this method. This indicated that DNA degradation involved irreparable double-strand cuts which are lethal for the cell.

In contrast, honey at concentrations beyond the MBC did not degrade DNA. Thus, the complete DNA degradation appeared only at bactericidal concentration. Honey concentration at which DNA degradation occurred correlated well with cell death observed in the concentration-dependent cell-kill on agar plates (**Figure 1**).

These results indicated that bacterial killing and DNA degradation were interdependent events. Thus, there was a cause-and-effect relationship between honey MBCs, cell damage and DNA degradation.

BACTERIAL KILLING BY HONEY REQUIRED HYDROGEN PEROXIDE

Honey hydrogen peroxide played an essential role in the bacterial growth inhibition by being a substrate for hydroxyl radical generation (Brudzynski et al., 2011). Hydroxyl radicals inhibited in a dose-dependent manner the growth of several MRSA and VRE clinical isolates as well as standard *E. coli* and *B. subtilis* (Brudzynski et al., 2012).

To investigate whether H_2O_2 also influenced honey's bactericidal action, honeys were pre-treated with catalase (1000 U/ml honey) prior to the incubation with *E. coli* and MRSA6 and their MIC and MBC values were evaluated using broth microdilution assay followed by agar plating and colony enumeration, respectively. The removal of H_2O_2 reduced bactericidal effect of buckwheat honey H23 as evident by the decrease of the MBC value from 25% v/v to 50% (honey dilutions 4×–2×) against *E. coli* and from 12.5% v/v to 50% v/v against MRSA6 (honey dilutions 8×–2×) (**Figure 3**). The MBC of freshly obtained buckwheat honey H206 was decreased 8-fold from 6.25% v/v to 50% v/v (honey dilution 16×–2×) after H_2O_2 removal. Bactericidal activity of manuka honey and blueberry honey were unaffected by catalase treatment (**Figure 3**). Taken together, these observations imply that bacterial killing by buckwheat honeys required hydrogen peroxide.

DETECTION AND QUANTITATION OF HYDROXYL RADICALS

The combination of 3′-(*p*-aminophenyl) fluorescein (APF) method with microdilution assay and colony enumeration by agar plating allowed us to monitor simultaneously the hydroxyl radical generation (by fluorescence), growth inhibition (by absorbance) and killing rates, respectively. As shown in (**Figure 4**), hydroxyl radical formation was dependent on the honey concentration/dilution. At lethal honey concentrations, ranging from 50% to 12.5% v/v, the levels of ·OH produced by different honeys were significantly lower than ·OH levels produced at non-bactericidal concentrations (12.5–3.125% v/v) (*t*-test, $p < 0.0001$). At maximum killing rate where no visible cells were detected on agar plates, the amount of radicals was 2–3 times lower than at MIC (**Figure 4**). In contrast, at honey concentrations beyond the MBC (12.5–3.125% v/v), the bacterial growth was observed on agar plates and ·OH radicals were clearly detected. These results indicated that the ·OH formation was associated with living cells while the dead cells did not accumulate appreciable levels ·OH.

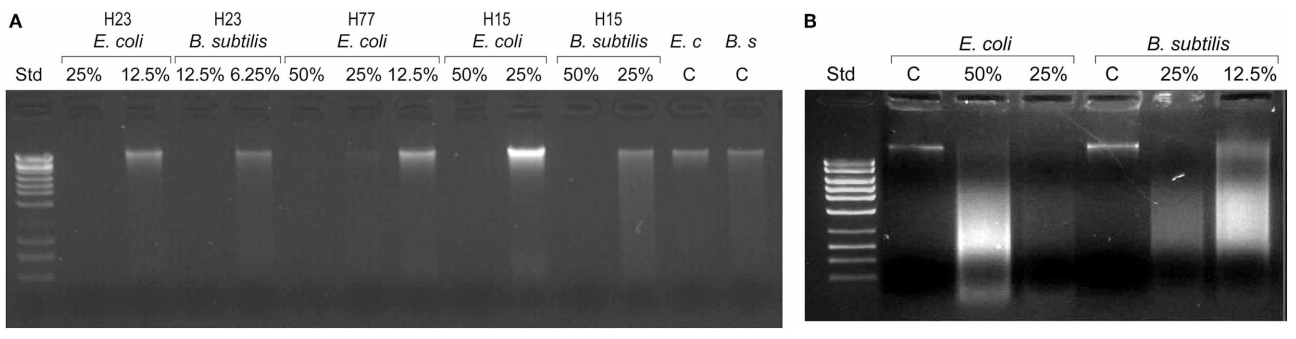

FIGURE 2 | Bacterial DNA degradation by honeys at bactericidal concentrations. Cultures of *E. coli* and *B. subtilis* (10^7 CFU/ml) were treated with honeys at concentrations ranging from 50% v/v to 6.25% v/v, equivalent to multiples of MBC for given honey. DNA degradation was analyzed on 1% agarose gels followed by DNA isolation using bacterial genomic DNA isolation method **(A)** and MiniPrep DNA isolation method **(B)**.

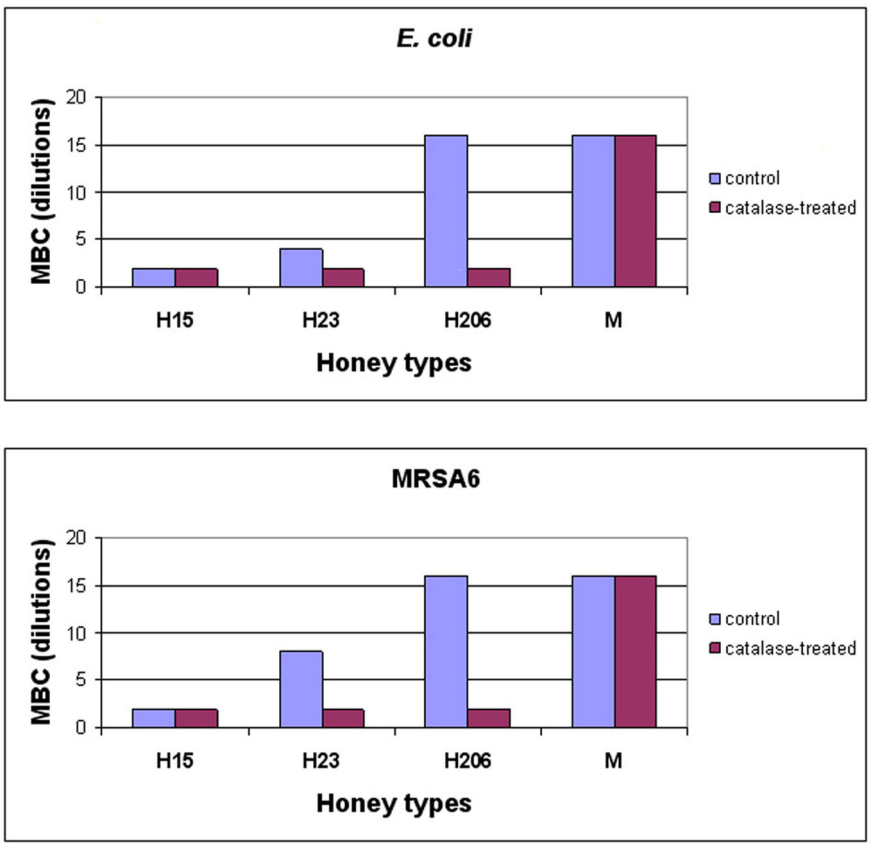

FIGURE 3 | Effect of honey's H_2O_2 on the survival of *E. coli* and MRSA6. Control, untreated and catalase-treated honeys were incubated with *E. coli* and MRSA6 (10^7 CFU/ml) at dilutions ranging from 2× (50 % v/v) to 16× (6.25% v/v). The bacterial survival was analyzed by agar plating and colony enumeration. Values represent averages of three independent determinations where no viable colony counts were found on the agar plates.

Conversion of dilution to honey concentrations (% v/v) is as follows:

Dilutions:	2×	4×	8×	16×	32×
Concentrations:	50%	25%	12.5%	6.25%	3.125%

DISCUSSION

A true therapeutic potential of honey as antibacterial agent depends on honey ability to eradicate infecting pathogens. In this study we have shown that honeys of buckwheat origin exhibited powerful bactericidal effect against standard bacteria as well as against MRSA and VRE. Honey ability to kill bacteria depended on (a) honey concentration, (b) inoculum size, and (c) the presence of H_2O_2. The MBC/MIC ratio ≤ 4 and the killing curves demonstrated that honeys at their MBCs caused > 6 $\log_{10}$ CFU/ml reduction of colony counts, equivalent to complete bacterial eradication. While honey concentration above MIC were lethal (50–6.25% v/v), concentrations at and below MIC caused a rapid bacterial re-growth. This indicates that the narrow concentration range, in which honey was bactericidal, might have a significant impact on therapeutic outcomes. A slight change in the honey concentration or bacterial load would reduce or abolish its bactericidal effect. Therefore, the assessment of concentration-depended killing and the ratio of concentration to bacterial load turn out to be an important indicator predicting bactericidal effect *in vivo*. Often, the infected sites contain bacteria at a higher density than that used in our assays (10^7 CFU/ml). It became evident that honey concentrations should exceed the MBC levels to ensure that the infecting organism is killed.

In this context, potency of honey's bactericidal activity emerged as another factor that influences bactericidal effect. We have shown here that bactericidal potency of honeys depended of honey variety and bacterial susceptibility to honey. The range of concentrations at which honeys exerted the maximal bactericidal effect was the broadest for manuka and buckwheat honey H77 (50–6.25% v/v), while blueberry honey showed bactericidal effect only at the highest concentration tested (50% v/v). Due to their potency, buckwheat honeys and manuka could be diluted up to 12.5% v/v and still maintained their maximal bactericidal efficacy. This leaves a space for an increase in honey dosage if the bacterial load exceeds that used in our experiments (10^7CFU/ml).

No significant differences in susceptibility to honeys were found between antibiotic-resistant clinical isolates of MRSA and VRE and standard *E. coli* and *B. subiltis* bacteria, which suggests that honeys indiscriminately affects broad spectrum of microorganisms. These results are in agreement with previous findings obtained on honeys of different botanical and geographical

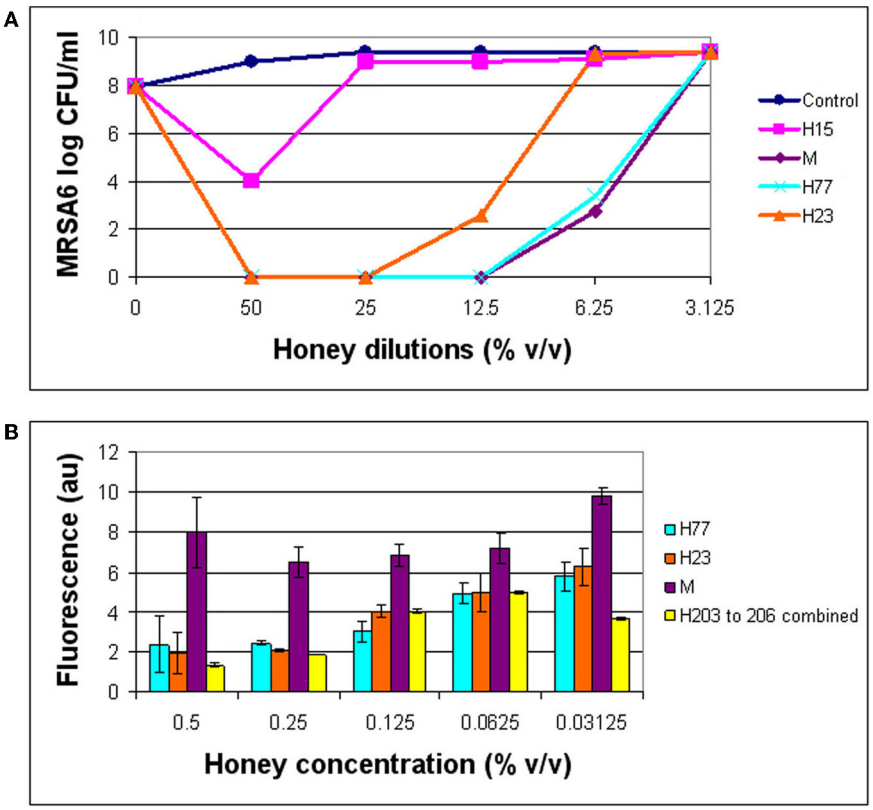

FIGURE 4 | Comparison of bactericidal effect of honeys in relation to the hydroxyl radical levels. The growth inhibition, bactericidal effect of honeys on MRSA6, and hydroxyl radical generation were simultaneously analyzed using broth microdilution assay in the presence of aminophenyl fluorescein (APF) as ·OH trap. **(A)** Honey concentrations at which a complete eradication of MRSA6 was observed. **(B)** Comparison of ·OH radicals formed at bactericidal concentration of honeys (MBC) and beyond MBC.

origins (Cooper et al., 2000, 2002b; Lusby et al., 2005; Blair et al., 2009; Tan et al., 2009; Sherlock et al., 2010).

Honey effectively targeted both rapidly multiplying/dividing bacteria such as MRSA6 as well as those characterized by slower growth such as VRE2. The appropriate honey concentration in each case was required for killing. Honey concentration–dependent killing resembled a class of antibiotics whose activity is concentration dependent: aminoglycosides and quinolones. At low concentration, a primary mode of action of aminoglycosides is the inhibition of protein synthesis through an irreversible binding to the 30S ribosomal subunit. Quinolones, on the other hand, act by promoting cleavage of bacterial DNA in the DNA-DNA gyrase and type IV topoisomerase complexes, thereby inhibiting DNA synthesis and its repair. Recent studies showed however, that both groups of antibiotics have bactericidal activities at high concentrations; aminoglycosides—by creating fissures in the outer cell membrane (Montie and Patamasucon, 1995) and quinolones—by generating irreparable double-strand breaks in DNA resulting in rapid bacterial death (Hooper and Wolfson, 1993).

Whether or not honey action presents such a dual-mode concentration–dependent killing requires further investigations. Nevertheless, some advances have been made in this study toward this goal. Firstly, the causal relationship was observed between the concentration-dependent cell-kill on agar plates and DNA degradation. Honeys at their MBC caused extensive double-strand DNA breaks that led to a complete break down of genomic DNA. In contrast, honey at concentrations beyond the MBC did not degrade DNA. Thus, the complete DNA degradation appeared only at bactericidal concentration. This could suggest a bimodal effect of honey concentration on DNA integrity. Together, these data emphasize that DNA degradation, bactericidal effect and honey concentrations are interrelated. Secondly, we have found that hydrogen peroxide was also involved in the killing mechanism. Removal of H_2O_2 by catalase abolished honey bactericidal action. These results indicate that honey concentration and H_2O_2 both play active part in honey killing mechanism.

Earlier, we have shown that oxidative damage to bacterial cells was conferred by a coupling chemistry between H_2O_2 and honey polyphenols. The extent of oxidative damage depended on H_2O_2 levels and redox capacity of honey polyphenols (Brudzynski et al., 2012). These observations led us to the assumption that hydroxyl radicals generated from H_2O_2 via the polyphenol-mediated, metal-catalyzed Fenton reaction may be responsible for the observed bacterial growth inhibition and DNA degradation. In support of this notion was the fact that supplementation of honey with either Cu(II) or H_2O_2 resulted in a marked increase

in bacteriostatic activity as indicated by over 30-fold decreased in MIC_{90} (from 6.25% v/v to less than 0.78% v/v in a case of Cu(II) supplementation) (Brudzynski and Lannigan, 2012). Subsequently, by including APF as ·OH trap in our broth microdilution assay, we have demonstrated a direct relationship between the ·OH generation and growth inhibition of MRSA and VRE.

However, in this study, we could not conclusively identify ·OH radicals as inducers of bacterial cell death. We did not find the quantitative relationship between ·OH levels and honey bactericidal effect. It could be argued that ·OH radicals were effectively scavenged by honey or bacterial antioxidants and hence not detectable by APF. However, neither honey nor bacterial ·OH scavengers were able to protect bacterial cells against the lethal oxidative damage. Rather, we are tempted to assume that both DNA degradation and the abrupt destruction of bacterial cells represent ·OH footprint, the direct or indirect consequence of fast acting ·OH radicals. The unpaired electron of the hydroxyl radical is highly reactive but short-lived species. Its reaction rates exceed 10^9 M^{-1} sec^{-1} in biological systems (Roots and Okada, 1975). Thus, the action ·OH radical could be only examined indirectly by the detection of the lasting effects of its toxic action. Our data indicated that the generation of ·OH radicals was significantly correlated with living but not dead cells ($p < 0.0001$). The cell death and extensive degradation of genomic DNA may therefore represent this lasting footprint of ·OH radical action. It has to be mentioned here that DNA degradation is a widely accepted marker of oxidative damage caused by ·OH radical action in a variety of model systems (Rozenberg-Arska et al., 1983; Mello Filho and Meneghini, 1984; Imlay and Linn, 1988; Sahu and Gray, 1993; Yang et al., 2006; Keyhani et al., 2007).

Although the role of hydroxyl radicals have been increasingly recognized as the most significant contributor to cell death among reactive oxygen species (Gutteridge et al., 1998; Imlay, 2003; Kohanski et al., 2007, 2010), it is possible that other free radicals generated from the initial Fenton reaction may be the actual effector molecules. Further studies on the role and significance of ·OH radicals in bactericidal activity of honey are needed to reconcile its action with the concentration-dependent killing.

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Enhancement of antimycotic activity of amphotericin B by targeting the oxidative stress response of *Candida* and *Cryptococcus* with natural dihydroxybenzaldehydes

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In addition to the fungal cellular membrane, the cellular antioxidant system can also be a viable target in the antifungal action of amphotericin B (AMB). Co-application of certain redox-potent natural compounds with AMB actually increases efficacy of the drug through chemosensitization. Some redox-potent chemosensitizers and AMB perturb common cellular targets, resulting in synergistic inhibition of fungal growth. Chemosensitizing activities of four redox-potent benzaldehydes were tested against clinical and reference strains of *Candida albicans*, *C. krusei*, *C. tropicalis*, and *Cryptococcus neoformans* in combination with AMB, based on assays outlined by the European Committee on Antimicrobial Susceptibility Testing. Two dihydroxybenzaldehydes (DHBAs), i.e., 2,3-DHBA and 2,5-DHBA, significantly enhanced activity of AMB against most strains, as measured by lower minimum inhibitory concentrations and/or minimum fungicidal concentrations (MFCs). A non-hydroxylated benzaldehyde, *trans*-cinnamaldehyde, showed chemosensitizing activity through lower MFCs, only. Contrastingly, a methoxylated benzaldehyde (3,5-dimethoxybenzaldehyde) had no chemosensitizing activity, as all strains were hypertolerant to this compound. Bioassays using deletion mutants of the model yeast, *Saccharomyces cerevisiae*, indicated DHBAs exerted their chemosensitizing activity by targeting mitochondrial superoxide dismutase. This targeting, in turn, disrupted the ability of the yeast strains to respond to AMB-induced oxidative stress. These *in vitro* results indicate that certain DHBAs are potent chemosensitizing agents to AMB through co-disruption of the oxidative stress response capacity of yeasts. Such redox-potent compounds show promise for enhancing AMB-based antifungal therapy for candidiasis and cryptococcosis.

Keywords: amphotericin B, dihydroxybenzaldehydes, chemosensitization, *Candida*, *Cryptococcus*, antioxidant system, superoxide dismutase

INTRODUCTION

There has been a persistent effort to improve efficacy of conventional antimycotic drugs, especially for treatment of human candidiasis and cryptococcosis. Currently, liposomal amphotericin B (LAMB), AMB lipid complex, etc., are preferred for clinical therapy of these mycoses, in that conventional AMB (e.g., AMB deoxycholate) is hepatotoxic/nephrotoxic (Patel et al., 2011). The lipid-based AMBs are generally recommended for patients who are intolerant to conventional AMB, which is still administered for treatment of mycoses, such as pediatric fungal infections (Allen, 2010 and references therein). However, high doses of LAMBs cause nephrosis and other tissue-damage in murine models of invasive pulmonary aspergillosis (Clemons et al., 2011). Thus, an antifungal therapeutic strategy to reduce side effects of AMB is warranted.

Amphotericin B binds to ergosterol in the fungal plasma membrane, undermining cell membrane integrity and causing ion leakage. However, formation of channels in the fungal membrane is not the sole mode of action of AMB (Palacios et al., 2007). There is ample literature showing AMB induces oxidative damage to both ascomycete and zygomycete fungal cells (Sokol-Anderson et al., 1986; Graybill et al., 1997, and references therein; Okamoto et al., 2004; An et al., 2009; González-Párraga et al., 2011). For example, *Aspergillus terreus*, a causative agent of human invasive aspergillosis, is intrinsically resistant to AMB, compared to other aspergilli. This resistance was thought to result from lower membrane ergosterol, thus offering fewer target sites for AMB (Walsh et al., 2003). However, this resistance was later found to result from

Abbreviations: AMB, amphotericin B; CFU, colony forming unit; Cinn, cinnamaldehyde; DHBA, dihydroxybenzaldehyde; 2,3-DHBA, 2,3-dihydroxybenzaldehyde; 2,5-DHBA, 2,5-dihydroxybenzaldehyde; 3,5-DMBA, 3,5-dimethoxybenzaldehyde; DMSO, dimethyl sulfoxide; EUCAST, European Committee on Antimicrobial Susceptibility Testing; FFCI, fractional fungicidal concentration indices; FICI, fractional inhibitory concentration indices; Glr1, glutathione reductase; Gsh1, γ-glutamylcysteine synthetase; LAMB, liposomal amphotericin B; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration; Mn-SOD, mitochondrial superoxide dismutase; SG, synthetic glucose; Sod, superoxide dismutase; Sod1, cytosolic superoxide dismutase; Sod2, mitochondrial superoxide dismutase; Trx2, thioredoxin; WT, wild type; Ycf1, glutathione *S*-conjugate pump.

higher catalase activity, an enzyme that protects against oxidative stress. This latter finding indicated there is an alternate or additional mode of action of AMB by causing oxidative damage (Blum et al., 2008). This was further confirmed by the finding that superoxide radical-mediated oxidative damage was caused by AMB activity (Okamoto et al., 2004).

Disrupting fungal redox homeostasis and/or the antioxidant system should augment antimycotic activity of AMB. Moreover, the antioxidant system plays an important role in pathogen virulence and defense against host cellular oxidative burst during infection (Washburn et al., 1987; Hamilton and Holdom, 1999; de Dios et al., 2010). Such disruption of the fungal redox homeostasis/antioxidant system could employ redox-potent natural products or their analogs (Jacob, 2006). The natural phenolic 2,3-dihydroxybenzaldehyde (2,3-DHBA) augments antifungal activity of a number of fungicidal agents by interfering with the fungal oxidative stress response system (Kim et al., 2008, 2011). In view that both 2,3-DHBA and AMB stress the fungal antioxidant system, their co-application should result in elevated antifungal activity.

The aim of this study was to test the concept of using benzaldehydes, such as 2,3-DHBA and some of its structural derivatives, as chemosensitizing agents to AMB. As a proof-of-concept, we used clinical strains and species of *Candida* and *Cryptococcus neoformans* for this test. Specifically, we compared the chemosensitizing activity between two hydroxylated DHBAs (2,3- or 2,5-DHBA) and two non-hydroxylated benzaldehydes [non-DHBAs; *trans*-cinnamaldehyde or 3,5-dimethoxybenzaldehyde (3,5-DMBA)]. We reasoned that use of chemosensitizing agents from natural sources could enhance the activity of AMB, while lowering toxic side effects of this drug to human cells.

MATERIALS AND METHODS

FUNGAL STRAINS AND CULTURE CONDITIONS

Candida albicans 90028 and *C. krusei* 6258 were procured from American Type Culture Collection (Manassas, VA, USA). *C. albicans* CAN276, *C. krusei* CAN75, *C. tropicalis* CAN286 and *C. neoformans* CN24 were procured from *Instituto de Higiene e Medicina Tropical/CREM, Universidade nova de Lisboa*, Portugal. *Saccharomyces cerevisiae* wild type (WT) BY4741 (*Mat* a *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) and selected single gene deletion mutants (see text) were procured from Open Biosystems (Huntsville, AL, USA). Yeast strains were cultured on Synthetic Glucose (SG; Yeast nitrogen base without amino acids 0.67%, glucose 2% with appropriate supplements: uracil 0.02 mg mL^{-1}, amino acids 0.03 mg mL^{-1}) or yeast peptone dextrose (YPD; Bacto yeast extract 1%, Bacto peptone 2%, glucose 2%) agar at 30°C for *S. cerevisiae* or 35°C for yeast pathogens (*Candida*, *Cryptococcus*), respectively.

ANTIFUNGAL DRUGS AND COMPOUNDS

Amphotericin B, diamide, 2,3- or 2,5-DHBA, *trans*-cinnamaldehyde, and 3,5-DMBA were procured from Sigma Co. (St. Louis, MO, USA). Each compound was dissolved in dimethyl sulfoxide (DMSO; absolute DMSO amount: <2% in medium) before incorporation into the culture medium. In all tests, control plates (i.e., "No treatment") contained DMSO at levels equivalent to that of cohorts receiving antifungal agents, within the same set of experiments.

SUSCEPTIBILITY TESTING: PLATE (AGAR) BIOASSAY

Petri plate-based yeast dilution bioassays were performed on the WT and antioxidant mutants of *S. cerevisiae* to assess the effects of AMB (0.0, 0.5, 1.0, 1.5, and 2.0 μg mL^{-1}) on the fungal antioxidant system. These assays were performed in duplicate on SG agar following previously described protocols (Kim et al., 2008). Similar dilution bioassays were performed on *Candida* and *Cryptococcus* to assess their differential sensitivity to AMB (0.0, 0.5, 1.0 μg mL^{-1}) or diamide (0.0, 0.2, 0.4, 0.6, 0.8 mM). Cell growth was observed for 3–5 days.

SUSCEPTIBILITY TESTING: MICROTITER (LIQUID) BIOASSAY

To determine changes in antifungal minimum inhibitory concentrations (MICs), i.e., differences/changes in MICs of each compound (AMB, benzaldehydes) alone as compared to when they were combined, triplicate assays were performed using broth microdilution protocols according to methods outlined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; Arendrup et al., 2012; definitive document EDef 7.2.). MIC was defined as the concentration at which no fungal growth was visible. These assays were performed using a range of concentrations of test compounds, as follows: AMB – 0.0, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0 μg mL^{-1}; 2,3-DHBA, 2,5-DHBA, *trans*-cinnamaldehyde, 3,5-DMBA – 0.0, 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 mM.

To measure changes in minimum fungicidal concentrations (MFCs), i.e., differences/changes of MFCs of each compound (AMB, benzaldehydes) alone compared to when they were combined, the entire volume of each microtiter well (200 μL), after determination of MICs, was spread onto individual YPD plates and cultured an additional 48 h (72 h for *C. neoformans*). The lowest concentration of agent showing ≥99.9% fungal death was defined as the MFC, except where noted (see tables). Student's *t*-test for paired data was used to determine significant differences between means of MICs or MFCs of each compound when combined (i.e., chemosensitization) vs alone (i.e., no chemosensitization) for six yeast pathogens (calculation was based on Kirkman, 1996). Compound interactions [for both fractional inhibitory concentration indices (FICI) and fractional fungicidal concentration indices (FFCI)] were calculated based on: FICI or FFCI = (MIC or MFC of compound A in combination with compound B/MIC or MFC of compound A, alone) + (MIC or MFC of compound B in combination with compound A/MIC or MFC of compound B, alone). FICI or FFCI was defined as: "synergistic" (FICI or FFCI ≤0.5) or "indifferent" (FICI or FFCI >0.5–4; Odds, 2003).

RESULTS

We tested the hypothesis that benzaldehydes could act as chemosensitizing agents to AMB against clinical strains and species of *Candida* and *C. neoformans*. First, Petri plate-based yeast dilution bioassays were used to evaluate any relationship between AMB-sensitivity and lower antioxidant capacity. Duplicate assays were performed on SG agar containing AMB (0.0, 0.5, and 1.0 μg mL^{-1}) according to described protocols (Kim et al.,

2008). In this test, *C. albicans* CAN276 was the most sensitive of all strains when exposed up to 1.0 μg mL^{-1} AMB (**Figure 1**). Next, we examined the effect of diamide (0.0, 0.2, 0.4, 0.6, and 0.8 mM) on these strains. Diamide causes stoichiometric oxidative stress by depleting cellular thiols, such as glutathione. CAN276 was also the most sensitive of *Candida* species or strains to diamide (up to 0.8 mM; **Figure 1**). *C. krusei* 6258, *C. krusei* CAN75, and *C. tropicalis* CAN286 grew similar to control (no diamide) cohorts (i.e., no antifungal activity against these strains at the given concentration). *C. albicans* 90028 and *C. neoformans* CN24 showed slight sensitivity to diamide, >100-fold less than CAN276 (**Figure 1**). The high sensitivity of CAN276 to both AMB and diamide indicated a diminished oxidative stress response system increases sensitivity to AMB.

Identification of target(s) of AMB within the yeast antioxidant system was attempted using deletion mutants of the model fungus, *S. cerevisiae*. Petri plate-based cell-dilution bioassays on SG agar with AMB (0.0, 0.5, 1.0, 1.5, and 2.0 μg mL^{-1}; in duplicate) included the WT and four antioxidant mutant strains, as follows: (1) *yap1*Δ [Yap1p is the transcription factor regulating expression of four downstream genes within the oxidative stress response pathway, i.e., *GLR1* (glutathione reductase), *YCF1* (a glutathione *S*-conjugate pump), *TRX2* (thioredoxin), and *GSH1* (γ-glutamylcysteine synthetase; Fernandes et al., 1997; Lee et al., 1999)]; (2) *sod1*Δ (cytosolic superoxide dismutase); (3) *sod2*Δ (mitochondrial superoxide dismutase, Mn-SOD); and (4) *glr1*Δ (glutathione reductase; see *Saccharomyces* Genome Database; www.yeastgenome.org, accessed May 22, 2012). These representative mutants were selected because: (1) they play key roles in maintaining cellular redox homeostasis in both enzymatic (e.g., superoxide radical-scavenging) and non-enzymatic (e.g., glutathione homeostasis) aspects; (2) among 45 *S. cerevisiae* antioxidant/stress response system mutants examined, tolerance to redox-potent benzo analogs relied upon Mn-SOD (*SOD2*) or glutathione reductase (*GLR1*; Kim et al., 2008); and (3) oxidative damage from AMB in *C. albicans* is induced by superoxide (Okamoto et al., 2004). Of the four deletion mutants, only *sod2*Δ was hypersensitive to AMB (up to 2.0 μg mL^{-1}; **Figure 2**). These results showed Mn-SOD plays a relatively greater role in fungal tolerance to AMB-induced toxicity than the other genes represented, similar to that found for treatment by redox-potent 2,3-DHBA (Kim et al., 2008).

The capacity of benzaldehyde analogs (DHBAs and non-DHBAs) to influence antifungal activity of AMB was examined using triplicate checkerboard microdilution bioassays according to the EUCAST (see Materials and Methods). The four benzaldehydes tested were 2,3- and 2,5-DHBAs, *trans*-cinnamaldehyde, and 3,5-DMBA. All four of these compounds targeted Mn-SOD in *S. cerevisiae* (Kim et al., 2008, 2011). In prior studies, 2,3-DHBA and cinnamaldehyde exhibited the highest antifungal activity against *S. cerevisiae* or filamentous fungi, respectively, when treated alone: *S. cerevisiae*- 2,3-DHBA (MIC 0.08 mM) >2,5-DHBA (MIC 1.8 mM) or filamentous fungi-cinnamaldehyde (MIC 0.58 mM) >3,5-DMBA (MIC 1.17 mM; Kim et al., 2008, 2011). In the present study, the DHBAs had the most potent chemosensitizing activity to AMB (see **Tables 1** and **2**).

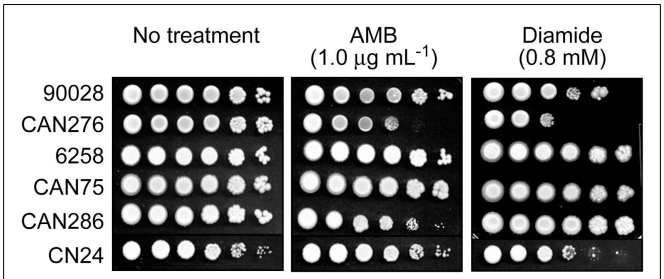

FIGURE 1 | Dilution bioassays showing phenotypic responses of yeast pathogens to amphotericin B (AMB) or diamide. 1 × 10^6 cells were serially diluted 10-fold in SG liquid medium, and were inoculated onto agar plates. Data are representative results shown from 1 μg mL^{-1} (AMB) and 0.8 mM (diamide), respectively.

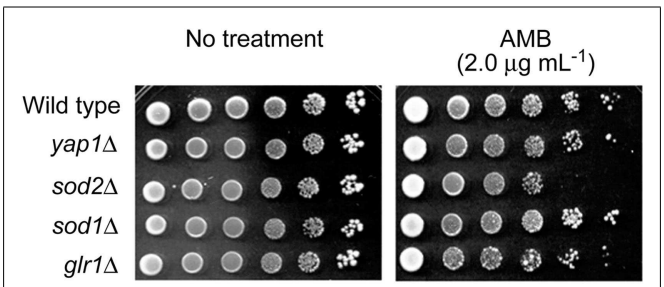

FIGURE 2 | Dilution bioassay showing phenotypic responses of *S. cerevisiae* strains to amphotericin B (AMB). *S. cerevisiae sod2*Δ [mitochondrial superoxide dismutase (Mn-SOD) gene deletion] mutant exhibited increased sensitivity to AMB over that of other deletion mutants (*yap1*Δ, *sod1*Δ, and *glr1*Δ) involving antioxidation responses. Data are representative results shown from 2 μg mL^{-1} (AMB). 1 × 10^6 cells were serially diluted 10-fold in SG liquid medium, and were inoculated onto agar plates.

As an example of DHBA-AMB interactions, the MIC for AMB (MIC_{AMB}), alone, for *C. albicans* 90028 was 2 μg mL^{-1} (**Tables 1** and **2**). However, the MIC_{AMB} was lowered to <1 μg mL^{-1} with either of the DHBAs. MICs of the DHBAs were concomitantly lowered in these co-applications, as well. MFCs were similarly affected, where the MFC of AMB alone (4 μg mL^{-1}) was reduced to <1 μg mL^{-1} by co-treatment with DHBAs. The relatively higher sensitivity of CAN276 than *C. albicans* 90028 to AMB (see **Figure 1**) was also reflected in MFC values; MFC_{AMB} CAN276 = 2 μg mL^{-1}, MFC_{AMB} *C. albicans* 90028 = 4 μg mL^{-1} (**Tables 1** and **2**; See also **Figure 3**). The range of MICs of 2,3-DHBA (0.4–0.8 mM) was lower than that of 2,5-DHBA (1.6–3.2 mM) in all yeasts tested (**Tables 1** and **2**). Thus, the higher to lower antifungal activity of 2,3-DHBA >2,5-DHBA in these yeast pathogens reflected that of *S. cerevisiae* (Kim et al., 2008; see also above).

The non-DHBAs tested were not potent chemosensitizing agents for AMB against the yeasts, as compared with the DHBAs. Interactions of cinnamaldehyde co-applied with AMB, in *C. albicans* 90028, CAN276, *C. krusei* 6258, and *C. neoformans* CN24, were "indifferent," although this co-application showed certain

Table 1 | Chemosensitization of AMB by 2,3-DHBA*.

Strains	Compounds	MIC alone	MIC combined	FICI	MFC alone	MFC combined	FFCI
C. albicans	2,3-DHBA	0.4	0.2	1.0	6.4	0.8	**0.4**
ATCC 90028	AMB	2	1		4	1	
C. albicans	2,3-DHBA	0.4	0.0125	**0.5**	3.2	0.4	0.6
CAN276	AMB	2	1		2	1	
C. krusei	2,3-DHBA	0.8	0.2	0.8	6.4	3.2	1.0
ATCC 6258	AMB	2	1		2	1	
C. krusei	2,3-DHBA	0.8	0.4	1.0	6.4	3.2	0.8
CAN75	AMB	2	1		4	1	
C. tropicalis	2,3-DHBA	0.8	0.2	0.8	3.2	1.6	0.8
CAN286	AMB	2	1		4	1	
C. neoformans	2,3-DHBA	0.8	0.4	1.0	3.2	0.1	**0.5**
CN24	AMB	4	2		4	2	
t-Test	2,3-DHBA		$P < 0.005$			$P < 0.01$	
	AMB		$P < 0.05$			$P < 0.005$	

*MFCs are concentrations where ≥99.9% fungal death was achieved. Synergistic interactions are in **bold** (see Materials and Methods for calculations).*

**2,3-DHBA, 2,3-dihydroxybenzaldehyde (mM); AMB, amphotericin B (μg mL^{-1}); MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration; FICI, fractional inhibitory concentration indices; FFCI, fractional fungicidal concentration indices.*

Table 2 | Chemosensitization of AMB by 2,5-DHBA*.

Strains	Compounds	MIC alone	MIC combined	FICI	MFC alone	MFC combined	FFCI
C. albicans	2,5-DHBA	1.6	0.8	1.0	6.4	3.2	0.8
ATCC 90028	AMB	2	1		4	1	
C. albicans	2,5-DHBA	1.6	0.8	1.0	6.4	3.2	1.0
CAN276	AMB	2	1		2	1	
C. krusei	2,5-DHBA	3.2	3.2	2.0	>6.4[†]	6.4	0.8
ATCC 6258	AMB	2	2		4	1	
C. krusei	2,5-DHBA	3.2	0.0125	**0.5**	>6.4[†]	6.4	1.0
CAN75	AMB	4	2		4	2 (99.7% killing)	
C. tropicalis	2,5-DHBA	3.2	1.6	1.0	>6.4[†]	3.2	0.8
CAN286	AMB	2	1		4	2	
C. neoformans	2,5-DHBA	3.2	1.6	1.0	6.4	3.2	1.0
CN24	AMB	2	1		2	1	
t-Test	2,5-DHBA		$P < 0.05$			$P < 0.01$	
	AMB		$P < 0.05$			$P < 0.005$	

*MFCs are concentrations where ≥99.9% fungal death was achieved, except where noted in the table. Synergistic interactions are in **bold** (see Materials and Methods for calculations).*

**2,5-DHBA, 2,5-dihydroxybenzaldehyde (mM); AMB, amphotericin B (μg mL^{-1}); MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration; FICI, fractional inhibitory concentration indices; FFCI, fractional fungicidal concentration indices.*

[†]Assays were conducted up to the highest concentration of 6.4 mM. For calculation purposes, 12.8 mM (doubling of 6.4 mM) was used.

level of enhanced antifungal activity for MFCs (**Table 3**). Moreover, 3,5-DMBA did not show any antifungal activity in any of the yeast strains, even at the highest concentration tested (6.4 mM), nor any chemosensitization when co-applied with AMB (data not shown). Contrastingly, 3,5-DMBA had potent antifungal activity (average MIC: 1.17 mM) against filamentous fungal pathogens (i.e., species and strains of *Aspergillus*, *Penicillium*; Kim et al., 2011). Perhaps yeast pathogens possess an intrinsic capacity to detoxify 3,5-DMBA.

DISCUSSION

All compounds tested, except for 3,5-DMBA, are known natural volatiles or components of the essential oils of a number of plants, including almond and vanilla. Both 2,3- and 2,5-DHBAs and *trans-cinnamaldehyde* have been shown to have a moderate level (MICs 20–80 μg mL^{-1}) of antibacterial activity (Wong et al., 2008). However, we found that the antifungal activity of these compounds, alone, is not particularly noteworthy.

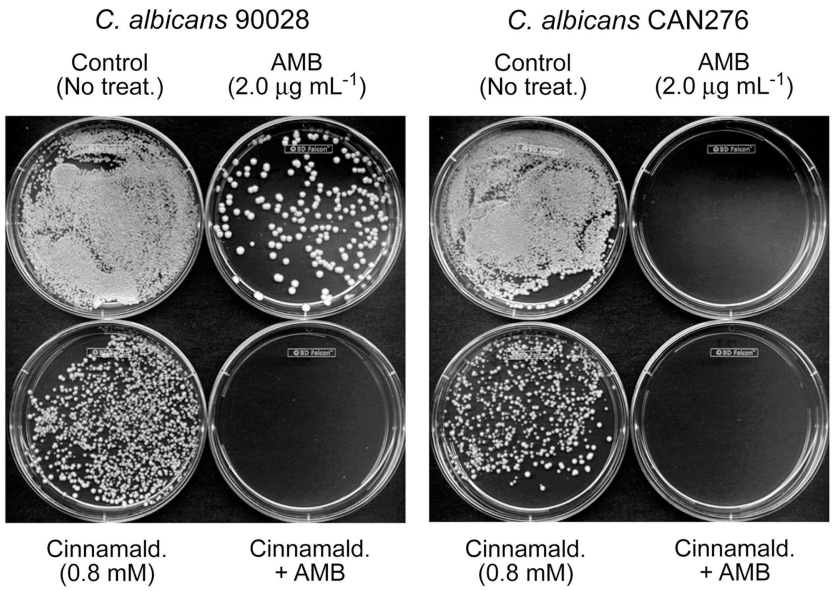

FIGURE 3 | Exemplary plate bioassay to determine minimum fungicidal concentration (MFC) in *C. albicans* 90028 (a reference strain) and CAN276 (a clinical isolate). In *C. albicans* 90028, co-application of AMB (2.0 μg mL^{-1}) and cinnamaldehyde (0.8 mM) completely inhibited colony survival, while survived colonies appeared under the independent treatment of AMB or cinnamaldehyde. Similar assay was performed on *C. albicans* CAN276, where no colonies appeared on 2.0 μg mL^{-1} of AMB, confirming the higher sensitivity of *C. albicans* CAN276 to AMB than *C. albicans* 90028.

Table 3 | Chemosensitization of AMB by cinnamaldehyde*.

Strains	Compounds	MIC alone	MIC combined	FICI	MFC alone	MFC combined	FFCI
C. albicans	Cinn	0.8	0.8	2.0	1.6	0.8	0.8
ATCC 90028	AMB	2	2		4	1	
C. albicans	Cinn	0.8	0.8	2.0	1.6	0.8	1.0
CAN276	AMB	2	2		2	1	
C. krusei	Cinn	0.8	0.8	2.0	1.6	0.8	1.0
ATCC 6258	AMB	4	4		4 (99.8% killing)	2	
C. krusei	Cinn	0.8	0.8	2.0	0.8	0.8	2.0
CAN75	AMB	4	4		4	4	
C. tropicalis	Cinn	1.6	1.6	2.0	1.6	1.6	2.0
CAN286	AMB	2	2		4	4	
C. neoformans	Cinn	0.8	0.8	2.0	0.8	0.4	1.0
CN24	AMB	4	4		4	2 (99.8% killing)	
t-Test	Cinn		*P*-values: not determined			$P < 0.1$	
	AMB		(neutral interaction)			$P < 0.1$	

MFCs are concentrations where ≥99.9% fungal death was achieved, except where noted in the table.

**Cinn, cinnamaldehyde (mM); AMB, amphotericin B (μg mL^{-1}); MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration; FICI, fractional inhibitory concentration indices; FFCI, fractional fungicidal concentration indices.*

However, as previously reported, certain phenolic antioxidants can prolong the activity of AMB against *C. albicans* by stabilizing the multiple double bonds of the polyene moiety. But, the mechanism by which the combination of such phenolics and AMB resulted in a synergistic interaction was unidentified (Beggs et al., 1978). Our results showed the DHBAs also augmented efficacy of AMB, *in vitro*, against yeast pathogens. Co-application of DHBAs with AMB resulted in complete inhibition of fungal growth at lower doses than any of the individual components applied, alone. Based on gene deletion mutant bioassays, it now appears that this synergy between AMB and DHBAs is by targeting at least one common cellular component in the antioxidant system, Mn-SOD. SODs of *C. albicans* are involved in biofilm persistence against miconazole (Bink et al., 2011), further demonstrating the role of fungal SODs in drug resistance. The non-DHBAs tested were poor chemosensitizing agents of AMB against yeast pathogens, indicating hydroxyl (−OH) substituents on the aromatic ring contributed to improved

antifungal/chemosensitizing activity. Of note is benzaldehydes having *ortho*- and *para*-hydroxylation possessed higher antifungal activity than *meta*- or mono-hydroxyl analogs (Kim et al., 2008).

The results of this *in vitro* study demonstrate that chemically targeting the oxidative stress response system of fungi effectively augments antimycotic potency of AMB. DHBAs or their analogs could be developed as potent chemosensitizers to AMB in yeast pathogens. Chemosensitization by using natural compounds could enhance the efficacy of AMB to inhibit fungal growth, and lower the adverse side effects of AMB. Further *in vivo* studies are needed to determine if the activities of chemosensitizers shown in this *in vitro* study can translate to a clinically effective resolution of mycoses.

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Juruin: an antifungal peptide from the venom of the Amazonian Pink Toe spider, *Avicularia juruensis*, which contains the inhibitory cystine knot motif

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The aim of this study was to screen the venom of the theraposid spider *Avicularia juruensis* for the identification of antimicrobial peptides (AMPs) which could be further used as prototypes for drug development. Eleven AMPs, named juruentoxins, with molecular weight ranging from 3.5 to 4.5 kDa, were identified by mass spectrometry after the soluble venom was separated by high performance liquid chromatography. Juruentoxins have a putative inhibitory cystine knot (ICK) motif, generally found in neurotoxins, which are also resistant to proteolysis. One juruentoxin that has 38 amino acid residues and three disulfide bonds were characterized, to which we proposed the name Juruin. Based on liquid growth inhibition assays, it has potent antifungal activity in the micromolar range. Importantly, Juruin lacks haemolytic activity on human erythrocytes at the antimicrobial concentrations. Based on the amino acid sequence, it is highly identical to the insecticidal peptides from the theraposid spiders *Selenocosmia huwena, Chilobrachys jingzhao,* and *Haplopelma schmidti* from China, indicating they belong to a group of conserved toxins which are likely to inhibit voltage-gated ion channels. Juruin is a cationic AMP, and Lys22 and Lys23 show maximum positive charge localization that might be important for receptor recognition. Although it shows marked sequence similarity to neurotoxic peptides, Juruin is a novel exciting molecule with potent antifungal activity, which could be used as a novel template for development of drugs against clinical resistant fungi strains.

Keywords: Juruin, juruentoxins, *Avicularia juruensis*, inhibitory cystine knot motif, Theraphosidae venom, antimicrobial peptides

INTRODUCTION

While combinatorial libraries have been widely used for generating diverse synthetic chemical compounds (Martin et al., 1995; Kirkpatrick et al., 1999), spider venom is a library with naturally selected, biologically active peptides with high target specificity. Therefore, spider toxins have been increasingly used as pharmacological tools and prototypes for drug development. From an evolutionary perspective, spiders belong to a very ancient and diversified group of arthropod with more than 40,700 described species, distributed in approximately 109 families, which makes them the most abundant terrestrial predators (Escoubas and Rash, 2004; Herzig et al., 2011).

The molecular diversity of spider venom is estimated in over 12 million biological active peptides. These toxins show different biologically activities, some of which have evolved into highly selective inhibitors of cell receptors (e.g., voltage-gated K+ ion channels) (Escoubas and Rash, 2004). The mix of several types of cell proteins and toxin peptides may act synergistically against their target, causing the venom noxious effects on its prey (Herzig et al., 2011). Moreover, the structure, function, and pharmacology of specific ion channels have been revealed by the mechanism of action of several spider toxins. Additionally, toxins binding selectivity and neuromodulatory effects could be used in the treatment of neurodegenerative disorders, such as epilepsies, Alzheimer and Parkinson's disease (Estrada et al., 2007; Saez et al., 2010). Advances over the past decades in mass spectrometry and molecular biology methods have allowed the characterization of genes related to such peptide toxins, which shed new light on the molecular diversity and evolution of these living combinatorial libraries (Corzo and Escoubas, 2003; Escoubas and King, 2009).

Spider toxins diversity is mainly based on small sized disulphide-rich peptides, which are suggested to fall into a limited number of structural patterns. Toxic peptides mainly conform to the Inhibitory Cystine Knot (ICK) motif, with a disulfide bond pairing of CI–CIV, CII–CV, CIII–CVI (Escoubas and Rash, 2004). It has been proposed that ICK toxins from spider venom have evolved from β-defensin gene duplications, diversification and further neofunctionalization (Fry et al., 2009). Defensins are among the most widely distributed innate immunity–related antimicrobial peptides (AMPs). In fact, cysteine-knotted (ICK-related) structural dissection revealed a minimal structure with potent antimicrobial activity (Vila-Perelló et al., 2005),

and even highly specific arachnid neurotoxins, which bind to insect voltage-gated ion channels, have demonstrated antimicrobial activity (Redaelli et al., 2010). Convergently, Drosomycin, a β-defensin that acts in the immune response of *Drosophila melanogaster*, inhibits Na^{2+} channels in a manner similar to those of scorpion neurotoxins (Cohen et al., 2009). Therefore, the study of spider venom, a rich source of toxic peptides which exhibit the ICK motif might reveal novel exciting prototypes for antimicrobials design (Escoubas and Rash, 2004).

Accordingly to the ArachnoServer 2.0 <http://www.arachnoserver.org/>, a database of toxic proteins from spiders venom, 916 different peptides from 85 species have been described so far (Herzig et al., 2011). Possible new peptides and toxins have been revealed by the combination of mass spectrometry and transcriptomic analysis, as well as screening the venom for desired properties. However, knowledge about the composition of the venom of many spider species remains very poor (Diego-García et al., 2010).

Here, we started to investigate the venom of the arboreal species *Avicularia juruensis* (**Figure 1**). The *Avicularia* genus comprises 13 species, endemic from regions in Central and South America, with at least three species threatened by habitat loss and illegal trafficking (Bertani and Fukushima, 2009). The Amazonian Pink Toe spider, *Avicularia juruensis* (Mello-Leitão, 1923), is a tarantula considered as an extremely docile species and not toxic to human being. Together with its stunning color and size, tarantulas from the *Avicularia* genus are one of the animals that are most often chosen as exotic pets. Even though the Amazonian Pink Toe spider is widely known, there are no studies available about its venom composition. Hence, the aim of this work was to explore the venom composition from *A. juruensis*, particularly to search novel antimicrobial compounds. This study is to the best of our knowledge the first venom analysis from the Brazilian spider *A. juruensis* (Amazonian Pink Toe), which resulted in the characterization of novel ICK toxins named juruentoxins.

FIGURE 1 | Adult female *Avicularia juruensis* (Theraphosidae, Mygalomorphae). Photo: Ayroza, G.

MATERIALS AND METHODS

BACTERIAL STRAINS

Fungal and bacterial strains were obtained from various sources. *Escherichia coli* SBS363 and *Micrococcus luteus* A270 were from the Pasteur Institut, Paris; *Candida albicans* (MDM8) was from the Department of Microbiology from the University of São Paulo, Brazil; *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, and *S. epidermidis* ATCC 12228 were from the American Type Culture Collection (ATCC). The following human clinical yeast isolates, which can be agents of candidiasis disease, obtained from the Oswaldo Cruz Institute, Brazil, were also used: *Candida krusei* IOC 4559, *C. glabrata* IOC 4565, *C. albicans* IOC 4558, *C. parapsilosis* IOC 4564, *C. tropicalis* IOC 4560, and *C. guilliermondii* IOC 4557. The filamentous fungi *Aspergilus niger* and the entomopathogenic fungus *Beauveria bassiana* were isolated from a mummified spider.

ANIMALS

The spiders (*Avicularia juruensis*, a tarantula of the Theraphosidae family) were kept alive in the biotherium of the Center for Applied Toxinology, of the Butantan Institute (São Paulo, Brazil) (**Figure 1**). These animals were collected under license Permanent Zoological Material no.11024-3-IBAMA and Special Authorization for Access to Genetic Patrimony no.001/2008.

VENOM FRACTIONATION AND JURUIN ISOLATION

In short, adult spiders were electrically stimulated for venom. *A. juruensis* crude venom was resuspended in 0.1% aqueous trifluoroacetic acid containing 10% acetonitrile (CH_3CN), and the insoluble material was removed by centrifugation at 14,000×*g* for 5 min. The supernatant was used directly for HPLC separation. The diluted venom was fractionated using a reverse-phase semipreparative C18 column (Jupiter, 10 × 250 mm) equilibrated in 0.05% trifluoroacetic acid and eluted with a linear gradient from solution A [0.05% (v/v) trifluoroacetic acid in water] to 80% solution B [0.10% (v/v) trifluoroacetic acid in acetonitrile] run for 60 min at a flow rate of 1.5 ml/min. Effluent absorbance was monitored at 225 nm. Fraction with antimicrobial activity (Juruin) was further purified using a distinct gradient from 30 to 40% solution B run for 60 min in the same system. The purity of the peptide was ascertained by a symmetrical peak on the HPLC system, amino acid sequencing, and mass spectrometry analysis.

REDUCTION AND ALKYLATION

Freeze-dried purified protein was dissolved (1 mg/ml) in denaturant buffer [6 M GdmCl (guanidinium chloride), 0.25 M Tris/HCl and 1 mM EDTA, pH 8.5]. To the mixture, 20 µl of 2- mercaptoethanol (Sigma) was added, followed by vortex-mixing and incubating at 37°C for 2 h. After incubation, 100 µl of 4-vinylpyridine was added to the solution, followed by incubation

at room temperature (26°C) for 2 h. It was then subjected to RP-HPLC and the protein was eluted. The reduction and alkylation of the protein were confirmed by checking the mass using MALDI-TOF-MS. The reduced and alkylated protein was fragmented by enzymatic cleavage with trypsin (Boehringer Mannhein). Tryptic peptides were sequenced using tandem mass spectrometry (MS/MS) in a Q-TOF Ultima API (Micromass) spectrometer operating in positive ion mode. The sequence was deposited in UniProt (http://www.uniprot.org/) under accession number B3EWQ0.

MASS SPECTROMETRIC ANALYSIS

The samples containing the peptide fragments (0.5 μl) were spotted onto the sample slide and dried on the bench and crystallized with 0.5 μl of matrix solution [5 mg/ml (w/v) CHCA (α-cyano-4-hydroxycinnamic acid), in 50% acetonitrile and 0.1% TFA] (Sigma). The samples were analyzed on an Ettan MALDI-ToF/Pro spectrometer (Amershan Biosciences) operating in reflectron mode. To determine the amino acid sequence of peptides, the doubly charged ions were subjected to "*de novo*" sequencing in a Q-TOF Ultima API (Micromass) spectrometer operating in positive ion mode. The spectrum was analyzed, and the "y" and "b" fragments were used to elucidate the primary structure of the molecule.

ANTIMICROBIAL ASSAYS

During the purification procedure, the antimicrobial activities of the samples were monitored by liquid growth inhibition assays using the Gram-negative bacteria *Escherichia coli* SBS363 and Gram-positive bacteria *Micrococcus luteus* A270 that were cultured in poor broth nutrient medium (PB: 1.0 g peptone in 100 mL of water containing 86 mM NaCl at pH 7.4; 217 mOsM), whereas yeast strain *Candida albicans* MDM8 was cultured in poor dextrose broth (1/2 PDB: 1.2 g potato dextrose in 100 mL of H_2O at pH 5.0; 79 mOsM) used at half-strength as previsouly described (Hetru and Bulet, 1997; Bulet, 2008). Determination of antimicrobial activity was performed using 5-fold micro titer broth dilution assay in 96-well sterile plates at a final volume of 100 mL. Mid-log phase culture was diluted to a final concentration of 1×10^5 colony forming units/mL. Dried fractions were dissolved in 200 μL of ultrapure water and 20 μL applied in to each well and added to 80 μL of the bacterium/yeast dilution. The fractions were tested in duplicate. 100 μL of sterile water and PB or PDB were used as quality controls. Tetracycline and/or Amphotericin B were also used as controls of growth inhibition. The microtiter plates were incubated for 18 h at 30°C; growth inhibition was determined by measuring absorbance at 595 nm.

MICs DETERMINATION

The minimal inhibitory concentration was determined using the purified peptide against the Gram-negative bacterial strains, the Gram-positive bacterial strains, the fungal strains and the yeast strains, as described above. The peptide was dissolved in sterile water and peptide concentration was measured using the method of Bradford (1976). Determination of minimal inhibitory concentrations (MICs) for Juruin was performed using a five-fold microtiter broth dilution assay of stock solution, and serial dilution in 96-well sterile plates at a final volume of 100 μL where 20 μL of stock solution was applied in to each well at serial dilution two-fold microtiter broth dilution and added to 80 μL of the bacterium/yeast dilution. Microbial growth was measured by monitoring the increase in OD at 595 nm after incubation at 30°C for 18 h (modified from Ehret-Sabatier et al., 1996). Juruin was tested in duplicate. MIC is defined as the minimal concentration of peptide that caused 100% growth inhibitions (Zhu et al., 2007). Juruin was incubated by 18 h with *C. guilliermondii* IOC 4555716, *C. krusei* IOC 4559, *C. glabrata* IOC 45658, *C. tropicalis* IOC 4560, and *Aspergillus niger* in order to identify whether is fungicidal or fungistatic by growth recovery (Baumann et al., 2010), using water, Amphotericin B and Gomesin as controls.

HAEMOLYTIC ACTIVITY

The haemolytic activity of the protein was tested using human erythrocytes. A 2.5% (v/v) suspension of washed erythrocytes in PBS was incubated with Juruin ranging from 0.125 to 10 μM in a 96-well plate for 3 h with intermittent shaking. The absorbance in the supernatant was measured at 415 nm. Haemolysis caused by PBS and 1% (v/v) Triton X-100 were used as 0% and 100% controls, respectively.

HOMOLOGY MODELING

Since Juruin shows more than 60% sequence similarity to U1-theraphotoxin-Ba1a (PDB ID: 2KGH) from *Brachypelma ruhnaui*, with all of the cysteine residues conserved when aligned using Muscle (Edgar, 2004), U1-theraphotoxin-Ba1a structure, determined by NMR (Corzo et al., 2009), was used as a template for homology modeling. SWISS-MODEL (Arnold et al., 2006; Kiefer et al., 2009), an automated protein modeling server (http://www.expasy.ch/swissmod/SWISS-MODEL.html), was used to obtain a preliminary three-dimensional structural model of Juruin. However, three disulfide bonds were missing in the model. Disulfide bonds were incorporated using the Biopolymer module from Insight II software (Accelrys). The model was subjected to energy minimization using the steepest descent method (100 steps) followed by the conjugate gradient method until the RMSD (root mean square deviation) was 0.5 kcal/mol Å (1 kcal ≈4.184 kJ). The resulting structure was checked for bond length and bond angle consistency as well as peptide bond conformation. The quality of the final structure was evaluated using a Ramachandran plot.

RESULTS

PURIFICATION AND PRIMARY STRUCTURE DETERMINATION OF JURUIN

The soluble venom of *A. juruensis* was separated in at least 35 different components by HPLC (**Figure 2**). The fraction eluted at 40.0 min, named Juruin, showed antimicrobial activity against *Candida albicans* MDM8 and was further purified until homogeneity as shown in the inset of **Figure 2A**. The inset graphic shows the elution of a major component (the peptide under study) plus some minor contaminants that were discarded. We have idenfied other three antimicrobial fractions, which eluted at 11.2, 40.6, and 42.0 min (**Figure 2**), respectively. All antimicrobial fractions were analyzed by mass

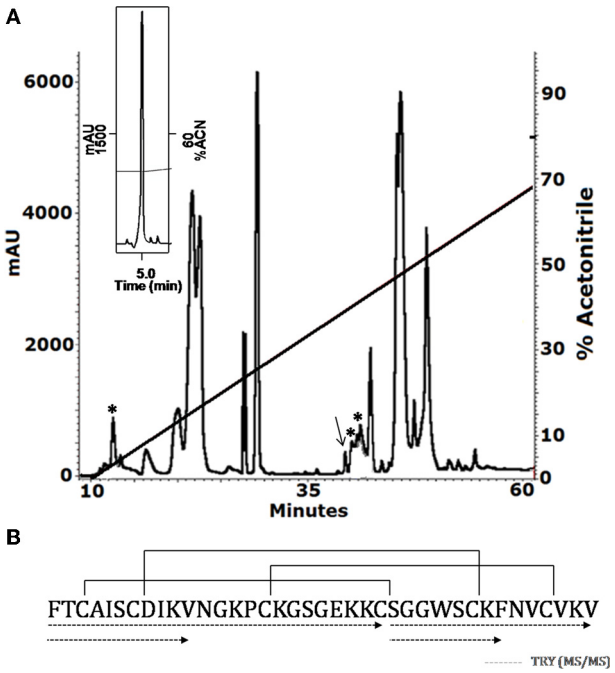

FIGURE 2 | Purification and covalent structure of Juruin. (A) 2.5 mg of soluble venom from *A. juruensis* was separated by HPLC using a C18 reverse-phase column, eluted with a linear gradient from solution A to 80% solution B run for 50 min. The fractions labeled with the asterisk exhibited antimicrobial activity and were eluted at 11.2, 40.6, and 42.0 min, respectively. The fraction labeled with an arrow was eluted at 40.0 min, and was rechromatographed in the same system and run from 30% to 40% solution B in 60 min (inset). The major component is pure Juruin. **(B)** Complete amino acid sequence of Juruin was obtained by mass spectrometry fragmentation of several peptides obtained by enzymatic hydrolysis of Juruin, as indicated by the segments underlined by dotted lines. Solid lines linking the cysteine residues indicate the disulfide bridges in positions Cys^3 to Cys^{24}, Cys^7 to Cys^{30}, and Cys^{15} to Cys^{35}. The N-terminal phenylalanine and the C-terminal amidated valine were determined by mass spectrometry (MS/MS) fragmentation.

spectrometry (**Figure 3**). Fraction eluting at 11.2 min showed two components with molecular weight of 4011.93 and 4033.16. Fraction eluted at 40.6 min showed the presence of six masses: 3506.86, 3599.07, 3629.692, 4004.47, 4252.50, 4319.79. Fraction eluted at 42.0 min showed the presence of more two components: 4252.30, 4319.94. Finally, analysis of Juruin showed the presence of a single component with a molecular weight of 4005.83 (**Figure 3B**). Comparision of molecular masses to previously identified spider toxins suggest these peptides contain an ICK motif (Escoubas and Rash, 2004). Therefore, we proposed the name juruentoxins for the peptides belonging to these group of toxins from *Avicularia juruensis* venom. The componentes with molecular masses: 4005.83, 4011.93, 4033.16, 3506.86, 3599.07, 3629.692, 4004.47, 4252.50, 4319.79, 4252.30, 4319.94 have been named U-theraphotoxin-Aju1a, U-theraphotoxin-Aju2a, U-theraphotoxin-Aju3a, U-theraphotoxin-AjuT4a, U-thera photoxin-AjuT5a, U-theraphotoxin-AjuT6a, U-theraphotoxin-AjuT7a, U-theraphotoxin-Aju8a, U-theraphotoxin-Aju9a, U-theraphotoxin-Aju10a, and U-theraphotoxin-Aju11a, respectively, accordingly to a previous proposed nomenclature (King et al., 2008). Aju1a will be referred with the name Juruin, in reference to the spider *A. juruensis*, as it is the first characterized peptide from this species.

We determined the amino acid sequence of Juruin by MS/MS fragmentation (**Figure 4**). Three main fragments were obtained after enzymatic hydrolysis with trypsin, as indicated by the dotted line under the sequence shown in **Figure 2**. The fragment corresponding to the positions Phe^1 to Lys^{10} was sequenced (**Figure 4A**) and was further aligned with several peptides obtained by enzymatic hydrolysis, after their mass fragmentation (MS/MS) as indicated under the sequence of **Figure 2**. The second fragment, corresponding to the positions between Phe^1 to Lys^{23} was sequenced (**Figure 4B**), positioned and correctly aligned with the previous subpeptide. The last segment, a subpeptide corresponding to positions Cys^{24} to Lys^{37}, was sequenced (**Figure 4C**) and positioned correctly into the sequence as derived from the results of overlapping sequences obtained by mass fragmentation, as indicated. This sequence was also confirmed through the examination of another peptide, consisting of residues between Cys^{24} and Lys^{31} (data not shown). The fact that the peptide from position Phe^1 to Lys^{37} presented a calculated molecular weight of 3907.59 Da indicates there is a missing residue at C-terminus. Considering a mass difference of 98.2 Da between the calculated mass to the mass observed by MALDI-TOF/MS suggest a valine C-amidated. Confirmation of the sequence came from the results of amino acid sequence comparison of Juruin against 91 known ICK containing peptides deposited at ArachnoServer 2.0 (Herzig et al., 2011). Whitin the Toxin-20 Family (Pfam ID: PF08089), the identities fall higher than 70% against any of the toxins considered. This high sequence similarity suggested that Juruin contains a highly conserved scaffold whitin spider toxins. In several toxins the scaffold compromises a C-terminal amidated valine (Liang, 2004). Therefore, these results suggest that Juruin has a amidated valine at the C-terminus. Additionally, sequence allignement with 26 toxins demonstrates equivalent folding of the disulfide bridges for Juruin, with disulfide pairing made between cysteines that occupy the same relative position (**Figure 5**). The identification of disulfide bridges are as follows: one between Cys^3 and Cys^{24}, the other between Cys^7 and Cys^{30} and the third disulfide pairing formed by Cys^{16} to Cys^{35}. The theoretically expected and the experimentally found molecular weights are similar (MW calculated: 4005.74, MW observed: 4005.83; for reduced Juruin, MW calculated: 4008.2, MW observed: 4007.8).

ANTIMICROBIAL ACTIVITY

Since many ICK toxins are reported to have antimicrobial activity, we tested native Juruin for antimicrobial activity by liquid growth inhibition assays for target pathogens, and compared with Amphotericin B and Gomesin (Silva jr. et al., 2000). Juruin showed high antimicrobial activity against all yeast and filamentous fungi tested, except for *Beauveria bassiana*. The MIC of Juruin against *C. albicans* was 2.5–5 μM. The most sensitive strains were *Candida* spp. Additionally, the filamentous fungi tested *Aspergilus niger* was highly sensitive to Juruin

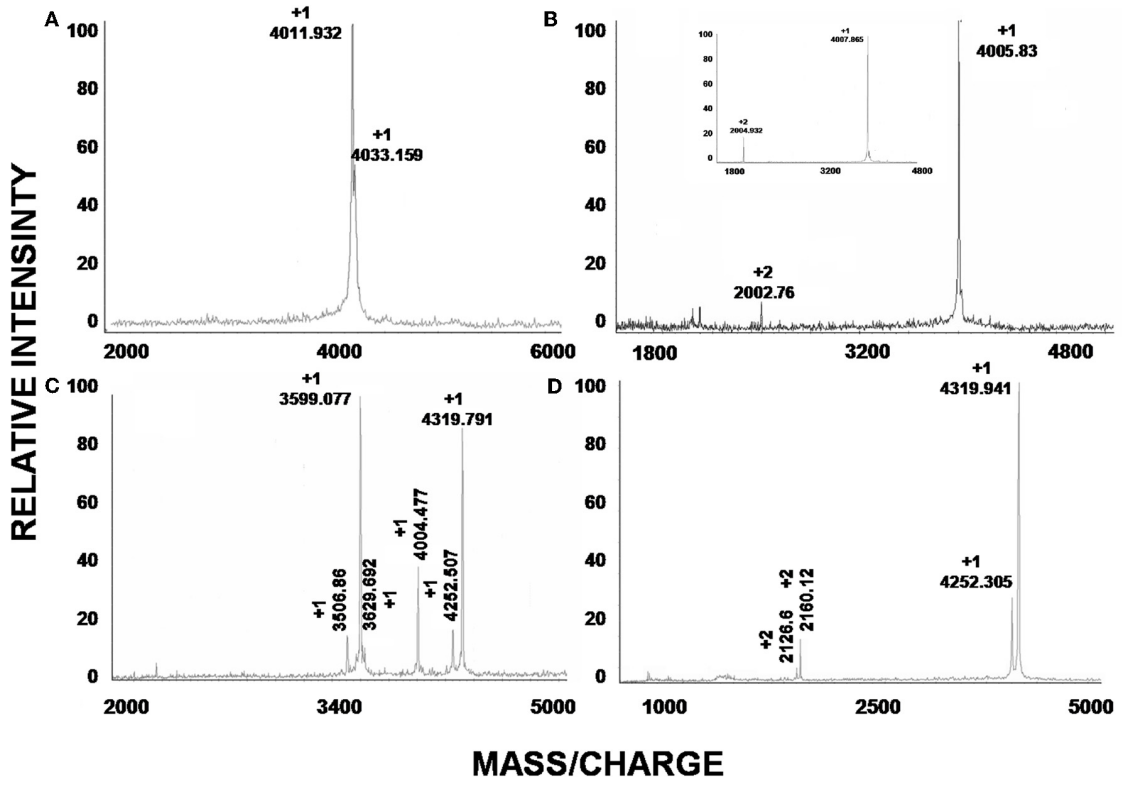

FIGURE 3 | Mass spectroscopic analysis of peptides. (A–D) are the mass spectra of the peaks obtained by HPLC with retention times 11.2, 40.0, 40.6, and 42.0 min, respectively. **(B)** Correspond to the observed mass of native Juruin (4005.83) and reduced Juruin (inset).

(MIC: 10 μM). However, Juruin did not show any antibacterial effect on the three Gram-positive strains tested, *M. luteus, S. epidermidis*, and *S. aureus*, or on the Gram-negative strains *E. coli* and *P. aeruginosa*, even at a concentration as high as 100 μM. Juruin showed similar antifungal activity to other host defense cysteine-rich peptide, Gomesin. However, when compared to Amphotericin B, the MICs are usually six fold lower than Juruin (**Table 1**). When *C. albicans* and *C. tropicalis* were incubated in the presence of Juruin at 20 μM for 16 h, a full growth recovery was not observed, hinting at fungicidal rather than fungistatic activity.

HAEMOLYTIC ACTIVITY

To investigate whether Juruin has any effect on mammalian membranes at the antimicrobial concentration range, its haemolytic effect was tested. After incubating human erythrocytes with the protein up to 10 μM concentrations, no haemoglobin release was observed, indicating that Juruin does not cause lysis of erythrocyte membrane within these concentrations (results not shown).

STRUCTURE-FUNCTION RELATIONSHIP STUDIES

Disulfide bridges are required for the highly compact, stabilized folding of many cysteine-rich proteins and their biological function, such as the antimicrobial properties of β-defensins (Yenugu et al., 2003). Due to the lack of material, we could not determine the importance of disulfide bonds and folding for the antifungal properties of Juruin. Instead, a three-dimensional model of Juruin was built using the known structure of U1-theraphotoxin-Ba1a (PDB ID: 2KGH), from *Brachypelma ruhnaui* (Corzo et al., 2009), since it shows 60% identity, with all of the cysteine residues conserved. The homology model of Juruin is shown in **Figure 6**. The final structure had φ and ψ angles within the allowed region of the Ramachandran map and all the peptide bonds were *trans*. A total of 15 residues (39.47%) were in the fully allowed region, 11 residues (28.95%) were in the additionally allowed region, nine residues (23.7%) were in the generously allowed region and only three residues (7.9%) were in the outside region. Juruin model shows similarity to the structure of U1-theraphotoxin-Ba1a, consisting of an ICK motif with three cross-linked disulfide bonds. The structure consists of three antiparallel β-sheet at residues 14–16, 27–31, and 34–37. The remaining part of the molecule is loop-structured (**Figures 6A–C**). The analysis of the electrostatic potential of the molecule reveals that the charge distribution is distinct, in which the middle segment of the molecule harbors maximum surface positive charge (**Figure 6F**), which may be essential for antimicrobial activity (Yin et al., 2012). This region, between Cys^{16} to Cys^{24} of Juruin, comprises three positively charged residues out of nine residues (CKGSGEKKC).

DISCUSSIONS

The findings described in this study provide novel information for the development of antimicrobial drugs. We have described the isolation and the complete covalent structure determination

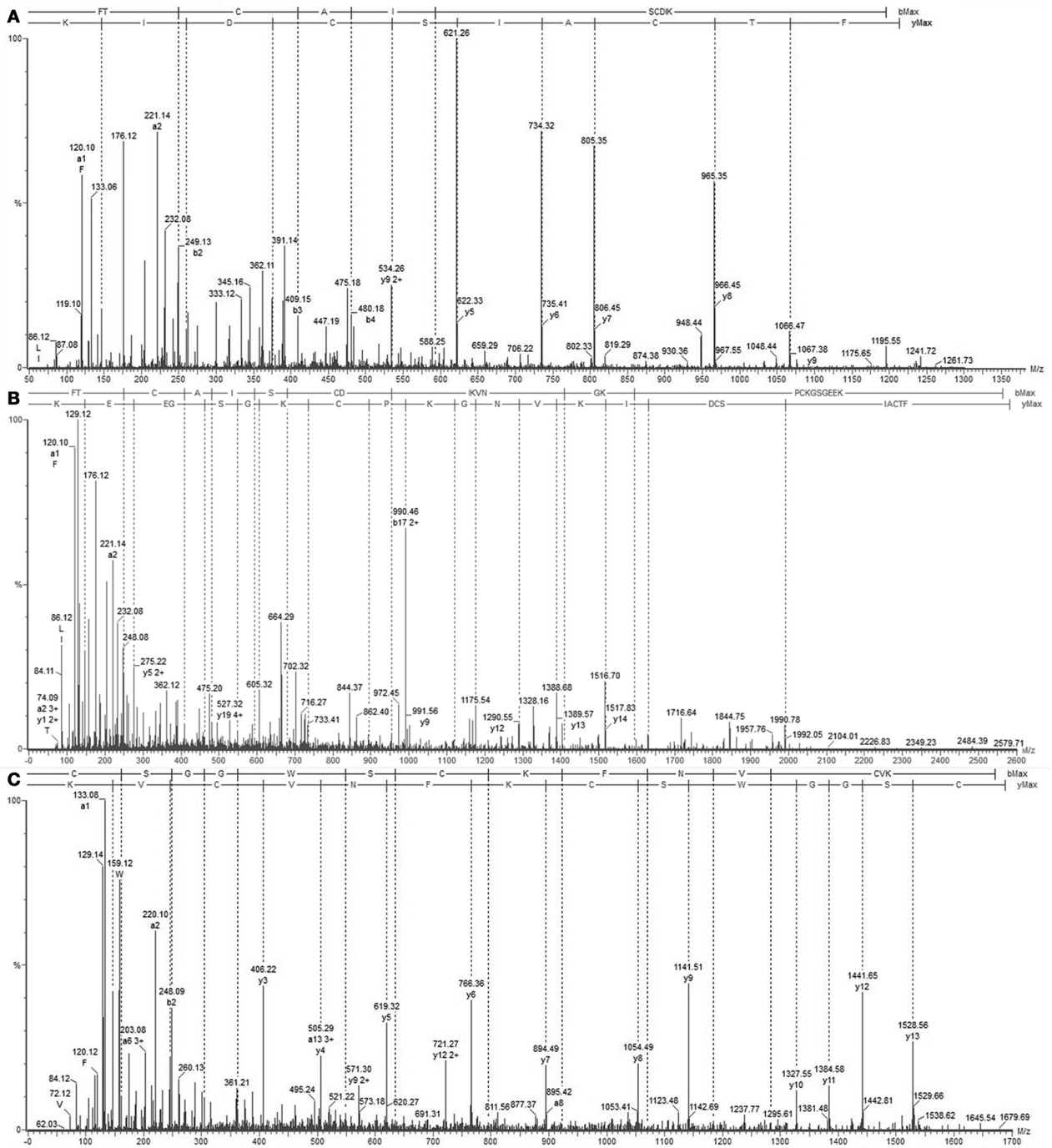

FIGURE 4 | Mass spectrometry analysis of Juruin peptides. **(A)** Collision-induced dissociation spectrum from mass/charge (*m*/*z*) 1211.3 generated by trypsin digestion after analysis by LC/MS, showing the dominant fragment KIDCS with a *m/z* of 621.26, which corresponds to an N-terminal segment. **(B)** Collision-induced dissociation spectrum from *m*/*z* 2579.8, showing the b and y ion series that corresponds to the partial sequencing of the tryptic peptide between residues Phe^{1} to Lys^{30}, which allowed the assignment of four cysteines, Cys^{3}, Cys^{7}, Cys^{24}, and Cys^{30}, as well as the lysine rich region Lys^{22}-Lys^{23}. **(C)** MS/MS spectrum from the precursor ion at *m*/*z* 1679.69 which corresponds to the C-terminus of Juruin, lacking the amidated valine at the end.

of Juruin. To our knowledge, this is the first peptide isolated from *A. juruensis*, a mygalomorph spider which belongs to the Theraphosidae family (**Figure 1**). When we analyzed the structure of Juruin in the context of what is known for the other spider toxins (Escoubas and Rash, 2004; Kuhn-Nentwig et al., 2004; Liang, 2004), it is clear that its structure is highly conserved. However, we could identify six novel residues within a highly conserved scaffold from an arboreal spider toxin (**Figure 5C**).

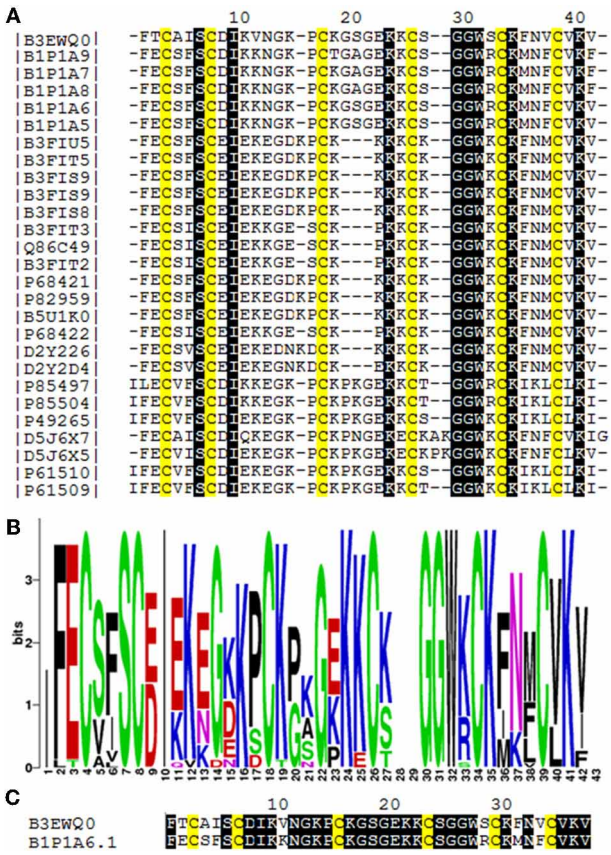

FIGURE 5 | Amino acid sequence comparision of Juruin (Aju1a) to other ICK-containing toxins. (A) Multiple sequence alignement of Juruin to selected toxins. Amino acid sequences of toxins were retrieved from public databases and aligned with Muscle (Egdar, 2004). Cysteines are in yellow. To minimize confusion, all sequences are referred to by their UniProt accession numbers (http://www.uniprot.org/). Juruin (B3EWQ0), U3-theraphotoxin-Cj1a (B1P1A5), U3-theraphotoxin-Cj1a (B1P1A6), U3-theraphotoxin-Cj1b (B1P1A8), U3-theraphotoxin-Cj1b (B1P1A7), U3-theraphotoxin-Cj1c (B1P1A9) from *Chilobrachys jingzhao*. U1-theraphotoxin-Ba1b (P85504), U1-theraphotoxin-Ba1a (P85497) from *Brachypelma ruhnaui*. U1-theraphotoxin-Bs1a (P49265) from *Brachypelma smithi*. U1-theraphotoxin-Asp1b (P61510), U1-theraphotoxin-Asp1a (P61509) from *Aphonopelma californicum*. Putative mature sequence toxin-like RFEC (D5J6X7), putative mature peptide toxin-like LFEC (D5J6X5) from *Pelinobius muticus*. U1-theraphotoxin-Lp1b (P61506) from *Lasiodora parahybana*. Hainantoxin-II-17 (D2Y2D4), Hainantoxin-II-15 (D2Y226) from *Haplopelma hainanum*. Huwentoxin-7 (P68421), U1-theraphotoxin-Hh1a (P82959), HWTX-VIII (B5U1K0), HWTX-II (B3FIU5), HWTX-VIIIa (B3FIT5), HWTX-IIb (B3FIS9), HWTX-IIa (B3FIS8), U1-theraphotoxin-Hh1f (P68422), Huwentoxin-2a (Q86C49), HWTX-VIIb (B3FIT3), HWTX-VIIa (B3FIT2) from *Haplopelma schimidti*. **(B)** Amino acid occurrence within selected toxins. **(C)** Structural similarity of Juruin to that of U3-theraphotoxin-Cj1a, from *Chilobrachys jingzhao,* to which Juruin shares 80% sequence similiarity. Conserved residues are shown in black boxes.

We suggest that these residues might have been positively selected during evolution, which can cause an increase in the binding affinity to its target receptor.

Firstly, we have screened the venom of *A. juruensis,* monitoring the antimicrobial activity using liquid growth inhibition assays. Out of more than 40 fractions, we have identified only four fractions which exhibited toxicity to tested microorganisms (**Figure 2**). Mass spectrometric analysis revealed the masses of 11 compounds: 4005.83, 4011.93, 4033.16, 3506.86, 3599.07, 3629.692, 4004.47, 4252.50, 4319.79, 4252.30, 4319.94 (**Figure 3**), which have been named U-theraphotoxin-Aju1a, U-theraphotoxin-Aju2a, U-thera photoxin-Aju3a, U-theraphotoxin-AjuT4a, U-theraphotoxin-AjuT5a, U-theraphotoxin-AjuT6a, U-theraphotoxin-AjuT7a, U-theraphotoxin-Aju8a, U-theraphotoxin-Aju9a, U-thera photoxin-Aju10a, and U-theraphotoxin-Aju11a, respectively, accordingly to a previous proposed nomenclature (King et al., 2008). Aju1a will be referred with the name Juruin, in reference to the spider *A. juruensis,* as it is the first characterized peptide from this species.

When compared to other known toxins from spiders, these compounds shows similarity to ICK-containing peptides, with molecular masses ranging from 3.0 kDa to 7.5 kDa (Escoubas and Rash, 2004). ICK-containing peptides in spider venom seem to have evolved from β-defensins, while they have gained novel functions during evolutionary recruitment events (Fry et al., 2009). Therefore, even highly specific neurotoxins have been shown to exhibit a high antimicrobial activity (Kuhn-Nentwig, 2003), and also antimicrobial compounds at spider venoms act in synergism to neurotoxins (Kuhn-Nentwig et al., 2004). In this work, we investigated the antimicrobial properties from the compound with 4,005.83 Da, named Juruin, which has been purified to homogeneity (**Figure 2**). Further research will focus on those other compounds.

Juruin is a 38-residue peptide with three disulphide bridges conformed in an ICK motif, and a valine amidated at the C-terminus, similarly to previous identified huwentoxins from *Selenocosmia* (*Ornithoctonus*) *huwena* (Liang, 2004). The disulfide bridges and C-terminal amidation certainly contributes to the stability of the peptide to proteases within the venom or when it is released for defense or against a prey (Silva jr. et al., 2000). Juruin is a highly cationic AMP, with seven positively charged residues (seven Lys) with a calculated pI of 9.08. Our structural model and sequence allignment suggest that the six cysteine residues in Juruin form three disulfide bridges linking Cys^{3}-Cys^{24} (CI-CIV), Cys^{7}-Cys^{30} (CII-CV), and Cys^{16}-Cys^{35} (CIII-CVI), such disulphide array is identical for all ICK-containing toxins from spiders (Escoubas and Rash, 2004). Interestingly, in Juruin an equal number of residues is observed between disulfide bridges as it is observed among other ICK-containing toxins (**Figure 5A**). Sequence comparison between Juruin to U3-theraphotoxin-Cj1a, from *Chilobrachys jingzhao,* to which Juruin shares 80% sequence similarity, reveals the difference of only six residues (**Figure 5C**). It has more than 70% of sequence similarity to Toxin-20 family (Pfam ID: PF08089) of peptides, from the spiders *Ornithoctonus huwena* (Liang, 2004; Yuan et al., 2007; Jiang et al., 2010), *Chilobrachys jingzhao* (Liao et al., 2007; Chen et al., 2008) and *Haplopelma hainanum* from China (Pan and Yu, 2010; Tang et al., 2010), which are neurotoxins with broad biological activities, including: voltage-gated ion channels inhibition, bioinsecticidal activity and inhibition of trypsin (Liang, 2004). While the three residues between Cys^{3}-Cys^{7} (Ala^{4}, Ile^{5}, Ser^{6}) have already been identified in the putative mature sequence toxin-like RFEC

Table 1 | Antimicrobial activities of cysteine-rich antimicrobial peptides against selected microorganisms.

MIC [μM (μg/mL)]	Juruin	Gomesin	Amphotericin B
GRAM-POSITIVE BACTERIA			
Micrococcus luteus A270	ND	0.4 (0.88)	ND
Staphylococcus aureus ATCC 29213	ND	1.2 (2.6)	ND
Staphylococcus epidermidis ATCC12228	ND	NT	ND
GRAM-NEGATIVE BACTERIA			
Escherichia coli SBS363	ND	0.4 (0.88)	ND
Escherichia coli ATCC 25922	ND	0.9 (1.9)	ND
Pseudomonas aeruginosa ATCC 27853	ND	5 (11)	ND
FILAMENTOUS FUNGI			
Beauveria bassiana	ND	NT	0.07-0.15 (0.06-0.13)
Aspergilus niger	5–10 (20–40)	1.2 (2.6)	0.01–0.03 (0.01–0.03)
YEAST			
Candida albicans MDM8	2.5–5 (10–20)	1.25–2.5 (2.7–5.5)	0.01–0.03 (0.01–0.03)
Candida krusei IOC 4559	2.5–5 (10–20)	2.5–5 (5.5–11)	0.07–0.15 (0.06–0.13)
Candida glabrata IOC 45658	2.5–5 (10–20)	>10 (22)	ND
Candida albicans IOC 45588	2.5–5 (10–20)	5–10 (11–22)	ND
Candida parapsilosis IOC 456416	2.5–5 (10–20)	2.5–5 (5.5–11)	0.07–0.15 (0.06–0.13)
Candida tropicalis IOC 45608	2.5–5 (10–20)	0.3–0.6 (0.6–1.2)	0.07–0.15 (0.06–0.13)
Candida guilliermondii IOC 455716	2.5–5 (10–20)	2.5–5 (5.5–11)	0.07–0.15 (0.06–0.13)

The activity of Juruin was compared to Gomesin (Silva jr. et al., 2000) and Amphotericin B. MIC values (μM) refer to the minimal inhibitory concentration required toachieve 100% growth inhibition. ND, not detected in the ranges assayed; NT, not tested.

(Uniprot ID: D5J6X7) from *Pelinobius muticus*, Thr^{2}, Val^{11}, Ser^{29}, Phe^{32}, and Val^{34} are novel residues within this toxin scaffold. When compared to more than 91 toxins, none of them presents those residues at these positions. It is likely that these amino acids residues are important to bioactivity of Juruin, being positively selected during evolution, instead of having appeared from neutral mutations. Surprisingly, Juruin is the unique Toxin-20 Family belonging peptide which has a Thr^{2} at the N-terminal region. In contrast to all species producing Toxin-20 family proteins, which are burrowing tarantulas, *A. juruensis* is an arboreal spider from the Amazonian rainforest also known as bird eating tarantula, because it often prey small vertebrates such as birds, small lizards, and tree frogs. Therefore, higher toxicity against vertebrates as well as unusual activities in the venom of an arboreal species could have appeared from divergent sequence patterns and positive Darwinian selection. The Brazilian insular arboreal pitviper *Bothorps insularis* (Cogo et al., 1998) show variation on the snake venom composition as well as divergent sequence patterns which might be the result of a significant dietary habit change and positive Darwinian selection causing an increase in venom toxicity. Therefore, Juruin should be included in further phylogenetic analyses with other ICK containing toxic peptides.

Juruin is effective against the majority of the fungi and yeast strains tested, with MICs between 2.5–5 μM for all of them, except for *Aspergilus niger* which showed MIC between 5–10 μM. Although cysteine-rich AMPs play an important role on spider immune system and often show a broad spectrum of activity against pathogens (Silva jr. et al., 2000), Juruin, a highly knotted cysteine-rich AMP, didn't show antibacterial activity against Gram-positive and Gram-negative bacteria tested. Also, antifungal activity against *B. bassiana* could not be observed. Juruin has marked activity against a variety of yeast at a rather low concentration, the most resistant strain being the yeast *C. glabrata* (**Table 1**). Interestingly, Juruin has similar MICs to that of Gomesin, a potent host

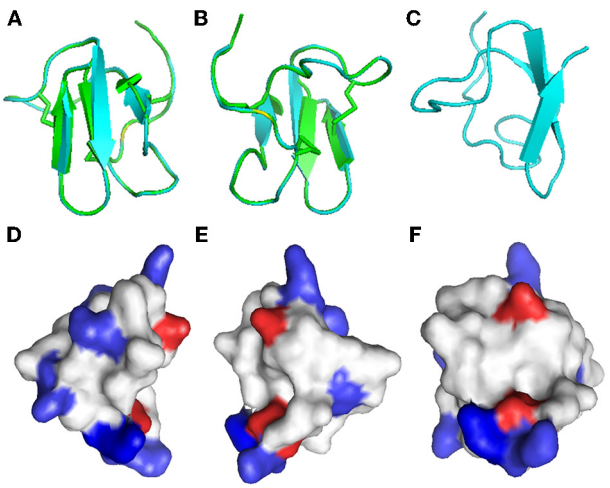

FIGURE 6 | Homology modeling of Juruin. Comparison of Juruin and U1-theraphotoxin-Ba1a (PDB ID: 2KGH), from *Brachypelma ruhnaui*, structures. **(A,B)** Juruin (blue) and U1-theraphotoxin-Ba1a (green) ribbon structures were superimposed over the backbone atoms. **(C)** Ribbon structure of Juruin in a different view related by ∼90° rotation. **(D,E)** Molecular surface of Juruin highlighted to show electrostatic potential, surfaces with positive, negative, and neutral electrostatic potentials are drawn in blue, red, and white, respectively. **(F)** The Lys^{22}-Lys^{23} segment shows maximum positive charge localization represented by the intense blue color. Model pairs show the sides of the protein rotated by ∼180°.

defense peptide previously identified by our group (Silva jr. et al., 2000). Amidated Gomesin has a slightly more pronounced active antimicrobial effect when compared to that of non-amidated form. The lack of disulphide pattern in Gomesin after reduction/alkylation produce a decrease in antimicrobial activity. Similarly, disulphide bridge pattern and post-translational modification might be related to antimicrobial activity and a putative neurotoxic effect of Juruin. However, Amphotericin B is effective even in six-fold lower concentrations (μM) than that of Juruin. On the other hand, Juruin is effective against Amphotericin B-resistant strains, *C. albicans* IOC 45588 and the clinically important *C. glabrata* (Krogh-Madsen et al., 2006; Khan et al., 2008).

We tested the toxicity to human erythrocytes only within the antimicrobial ranges. Juruin do not show haemolytic activity even at the higher concentration tested 10 μM. This data suggests that the mode of action of Juruin is not by disrupting cell membranes. Moreover, the presence of a large number of positively charged amino acids in host defense peptides contributes to a higher specificity of the peptide to a higher electronegative charged targets, such as prokaryotic cells (Silva jr. et al., 2000), nucleic acids or intracellular proteins (Nguyen et al., 2011). Therefore, the positively charged residues (**Figure 6**) might be involved in target receptor recognition and selectivity against pathogens and preys.

The specific antimicrobial activity of Juruin against fungi and yeast gives novel evidences for the origin of arachnid toxins from antifungal β-defensins (Zhu et al., 2005). After one or several recruitment events, defensins sequence duplication and wide divergence driven by positive Darwinian selection might have expanded this class of molecules into new functional groups (Fry et al., 2009), probably including ICK-containing toxins. The evidence of positive Darwinian selection in the ICK fold within spider toxins suggests that adaptive amino acid changes in a conserved scaffold are a major force driving new functional emergence. Therefore, divergent sequence patterns should be used for peptide-based drug design (Zhu et al., 2005, 2011). Hence, the identification of novel residues within a highly conserved scaffold offers a potential to investigate the divergent evolution of *A. juruensis* toxins, and Juruin is a natural template for development of novel therapeutical drugs.

In summary, we have isolated, purified and characterized a new ICK-containing AMP, named Juruin (Aju1a). The remarkable similarity to other toxins with other interesting bioactivities such as ion channels modulations (Liang, 2004) and antiparasitic activity (Pimentel et al., 2006), the highly conserved primary structure of the toxin, along with its selectivity, potent fungicidal activity, and the lack of haemolytic activity against human erythrocytes together with a putative resistance against proteases, makes Juruin pharmacologically interesting and valuable for the design of novel efficient drugs against fungal diseases. Thus, Juruin unique sequence should be investigated as a novel prototype for drug development.

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