THE USE OF PLANT EXTRACTS AND ESSENTIAL OILS AS BIOPESTICIDES

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THE USE OF PLANT EXTRACTS AND ESSENTIAL OILS AS BIOPESTICIDES

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EDITORIAL
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Editorial: The Use of Plant Extracts and Essential Oils as Biopesticides

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Keywords: plant pathogens, pests, plant extracts, essential oils, biopesticides

Editorial on the Research Topic

The Use of Plant Extracts and Essential Oils as Biopesticides

Essential oils (EOs) and plant extracts contain valuable natural products, many of which can be used in pest and disease control safely due to their ability to degrade in nature (Ni et al., 2021; El Khetabi et al., 2022). Despite their qualities, biopesticides represent only 5% of the overall pesticide market (Balog et al., 2017; Kumar et al., 2021; Rakshit et al., 2021). Nevertheless, biopesticides are experiencing rapid growth in recent years with an average annual growth rate of 9–20%, predicted to outpace that of chemical pesticides (Balog et al., 2017; Marrone, 2019; Kumar et al., 2021; Rakshit et al., 2021). Common management practices focus on the application of EOs and plant extract-based biopesticides without deeply understanding their mode of action (Álvarez-Martínez et al., 2021). Recent research focusing on pest management using EOs and plant extracts revealed several mechanisms involved in the insecticidal effects of EOs on targeted organisms (Ni et al., 2021). Moreover, several reports suggest specific strategies that could help in optimizing application of EOs and plant extracts as part of integrated pest management programs (de Oliveira, 2021). Apart from their role in plant development and growth, plant secondary compounds are also essential in plant resistance to biotic and abiotic stressors, and can be involved in metabolic processes that control plant tolerance (Yang et al., 2018; Karimi and Meiners, 2021; Ni et al., 2021). However, EOs and plant extracts are biologically unstable as they are easily destroyed by environmental pH, oxygen, light, and moderate temperatures. EOs exhibit poor aqueous solubility and high volatility in general. Efforts are being made to overcome these challenges. The present Research Topic gathers together studies that focus on the use of plant extracts and essential oils as biopesticides while aiming at the same time to shed light on their mode of action on different targeted key agricultural pest and plant diseases including insects, mites, nematodes, and oomycetes.

Several bioassays of different EOs and plant extracts have been used to demonstrate potential control of different key insect and mite populations. In one study, the strong ixodicidal and antifeedant agents included EOs of *Thymus zygis*, *T. vulgaris*, and *Mentha suaveolens*, which were suggested to be developed as biopesticides to effectively control ticks and insect pests (Valcárcel et al.). Interestingly, synergistic effects were also observed between these EOs (Valcárcel et al.). In a large-scale field trial, the application of aqueous extracts of *Murraya paniculata*, *Cassia tora*, *Amphineuron opulentum*, *Tithonia diversifolia*, and *C. alata* equally reduced the population of the red spider mite, a major tea pest, with a lower impact on natural enemies and increased the yield of tea plants without lethal consequence for the tea plants or consumers (Deka et al.).

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Furthermore, the larvicidal activity of EOs from five piper species including *Piper aduncum*, *P. marginatum*, *P. gaudichaudianum*, *P. crassinervium*, and *P. arboreum* against the yellow fever mosquito *Aedes aegypti* showed up to a 90% lethality at a screening concentration of 100 ppm, making these EOs potential alternatives for control of *A. aegypti* mosquito larvae (Pereira Filho et al.). Despite these effects, EO-based products are often chemically variable, requiring rigorous control of the cultivation process and an understanding of the regulatory aspects of the biosynthesis of these phenylpropanoids (Pereira Filho et al.), especially in developing countries such as sub-Saharan Africa (SSA) (Uyi et al.). The latter review summarized the existing insecticidal activity of invasive plants in SSA including Asteraceae, Solanaceae, Fabaceae, and Euphorbiaceae amongst others. These plants caused 50–100% mortality against various insect pests (Uyi et al.). However, using extracts from these invasive plants as biopesticides in African countries, especially among resource-poor smallholder farmers and locals, remains challenging (Stevenson et al., 2017; Uyi et al.).

Unlike studies on insects and mites, very few bioactivity evaluation bioassays with different EOs and plant extracts have been carried out on controlling different nematode populations. Recently the nematicidal potential of *Citrus sinensis*, *Cymbopogon nardus*, and *Melaleuca alternifolia* has been reported against the cotton root-knot nematode *Meloidogyne incognita* (Kundu et al.). On the other hand, potential botanical products against plant diseases have been extensively studied. For example, volatile organic compounds (VOCs) from soybean plants have been tested against harmful oomycetes, represented by *Phytophthora* (Ge et al.). The VOCs, mainly containing 4-ethylphenol, were simultaneously inoculated with the causal agent of soybean root rot, *P. sojae* and the black shank, *P. nicotiana*. VOCs inhibited the growth of the pathogens by destroying their cell membrane (Ge et al.). These VOCs have potent antifungal activity against other soil-borne phytopathogenic fungi including *Rhizoctonia solani*, *Fusarium graminearum*, and *Gaeumannomyces graminis* var *tritici*, and four forma specialis of *Fusarium oxysporum* (Ge et al.), making it ideal for simultaneously controlling major soilborne diseases.

The identification of active compounds of EOs and plant extracts is crucial to study their mode of action and thus develop effective biopesticide products. Nanotechnology using active biomolecules represents a potential solution to control the release of the active ingredient with less product waste. Biochemical and molecular modes of action of plant extracts and EOs have been recently investigated. *Thymus proximus* EO contains carvacrol, *p*-cymene, and γ-terpinene, representing 85.9% of the total oil, and these major constituents are responsible for both the plant suppressive effect and the insecticidal activity of the EOs (Zhou et al.). Furthermore, Azadirachtin, a tetranortriterpenoid derived from the neem seed of the Indian neem tree, have been reviewed many, many times in the past going back to the early 1980s for its insecticidal activity (Schmutterer, 2002). In this Research Topic, the literature review of Kilani-Morakchi et al. summarized the state of the art on key azadirachtin insecticidal activities and risk assessment. The effect of *T. zygis*, *T. vulgaris*, and *M. suaveolens* was mainly due to the presence of active compounds including piperitenone oxide, carvacrol, piperitenone, and thymol (Valcárcel et al.). The major compounds of the EOs from *Piper* species were identified and included β-asarone, (E)-anethole, (E)-β-caryophyllene, γ-terpinene, *p*-cymene, limonene, α-pinene, and β-pinene and showed larvicidal activity with mortality between 90 and 100% (Pereira Filho et al.). Phytochemical analyses of EOs of *Origanum compactum* tested against *Callosobruchus maculatus* showed that the main components were carvacrol and thymol (38 and 31.5%, respectively) (Aimad et al.). A comprehensive chemoprofiling of nematicidal action of EOs was performed to understand their possible interactions with the target sites of *M. incognita*, suggesting the most prominent monoterpene was l-limonene, with a range of 32–98%. In particular, industrially important *Commiphora myrrha*, *Cymbopogon nardus*, *Artemisia absinthium*, and *Pogostemon cablin* contained a higher amount of furanoeudesm 1,3 diene, geraniol, myrcene, camphor, and patchoulol, respectively. *In silico* analysis suggested a higher binding capacity of geraniol, β-terpineol, citronellal, l-limonene, and γ-terpinene, to the selected target proteins (Kundu et al.). Terpenoids which are present in most essential oils have been reported responsible for their bioactivity (Kundu et al.). Interestingly, several studies pointed out that synergistic interactions among terpenoids in EOs can be important (Tak and Isman, 2015, 2017). Therefore, these biochemical analyses of EOs and plant extracts will open a new door to specifically devise efficient and eco-friendly biopesticides and will help in effectively targeting the plant system (Werrie et al.).

The present Research Topic provides important updates on the roles of EOs and plant extracts in pest and disease management and highlights the chemical compositions responsible for their mode of action. This Research Topic contributes to define potential EO and plant extract candidates, which can be implemented in eco-friendly and sustainable management strategies. Moreover, plants are an important source of biomolecules, which are essential for fighting against economically devastating pests and diseases, and are also listed in this Research Topic. As such, it contributes to advancing the development of sustainable strategies for pest and disease management of food crops. However, there is a big disconnect between academic studies on insecticidal activity of plant metabolites and production of commercial bioinsecticides. Many such compounds are touted as potential biopesticides, but very few meet all the necessary criteria to be produced at scale for commercial use (Isman, 2017, 2020). Finally, besides providing an update on the state of the art of biopesticide research, the current Research Topic offers a perspective on future research needs and priorities. Emerging areas of research related to biopesticides include investigating (i) biopesticides' roles and function in plant metabolism and (ii) novel biopesticide management strategies to address biopesticide waste such as that of nanotechnology. Biopesticides and related products should be evaluated in a more biological and ecological context to further enhance the penetration of biopesticides into plant tissues, thus decreasing the waste and degradation of biopesticides

and contributing to more sustainable integrated pest management systems.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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A Comprehensive *in vitro* and *in silico* Analysis of Nematicidal Action of Essential Oils

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Nematicidal potential of essential oils (EOs) has been widely reported. Terpenoids present in most of the essential oils have been reported responsible for their bioactivity though very less is known about their modes of action. In the present study, an *in vitro* screening of nine Eos, namely, *Citrus sinensis* (OEO), *Myrtus communis* (MTEO), *Eucalyptus citriodora* (CEO), *Melaleuca alternifolia* (TEO), *Acorus calamus* (AEO), *Commiphora myrrha* (MREO), *Cymbopogon nardus* (CNEO), *Artemisia absinthium* (WEO), and *Pogostemon cablin* (PEO) against *Meloidogyne incognita* revealed OEO, CNEO, and TEO as most effective with LC_{50} 39.37, 43.22, and 76.28 μg ml^{-1} respectively. EOs had varying compositions of mono- and sesquiterpenes determined by gas chromatography-mass spectrometry (GC-MS) analysis. The *in silico* molecular interactions screening of major EO constituents and the seven selected target proteins of the nematode indicated highest binding affinity of geraniol-ODR1 (odorant response gene 1) complex (ΔG = -36.9 kcal mol^{-1}), due to extensive H-bonding, hydrophobic and π-alkyl interactions. The relative binding affinity followed the order: geraniol-ODR1 > β-terpineol-ODR1 > citronellal-ODR1 > *l*-limonene-ODR1 > γ-terpinene-ODR1. Taken together, the cumulative *in vitro* and computational bioefficacy analysis related to the chemoprofiles of EOs provides useful leads on harnessing the potential of EOs as bionematicides. The insight on biochemical ligand–target protein interactions described in the present work will be helpful in logical selection of biomolecules and essential oils for development of practically viable bionematicidal products.

Keywords: volatile oils, gas chromatography-mass spectrometry analysis, *Meloidogyne incognita*, molecular docking, odorant response gene 1

INTRODUCTION

Root knot nematodes, pose a major challenge to the global pest management programs due to devastating crop losses caused by these organisms (Sidhu et al., 2017). Among the root knot nematodes, *Meloidogyne incognita* is most abundant in tropical soils, barely sparing any crop family, and the most challenging part is to control its population below economic damage levels

(Collange et al., 2011; de Freitas Silva et al., 2020). Synthetic recommended nematicides like carbofuran, fluopyram owing to their associated detrimental effects on the environment, non-target organisms besides phytotoxicity, necessitate safer approaches for nematode management in cropping systems (Westphal, 2011; Jones et al., 2017). To develop ecofriendly alternatives, a wide spectrum of plant metabolites with nematostatic and nematicidal actions has extensively been reported (Atolani and Fabiyi, 2020).

Phytochemicals have been extensively reported as potential sources of bioactive ingredients for the development of natural nematicides (Oka, 2001). Long-chain hydrocarbons, sulfur compounds, alkenes, furans, acetogenins, phenolics, saponins, etc., have been reported to be effective against various phytoparasitic nematodes (Aissani et al., 2018). Most of the phytochemicals have served as models for the identification of a lead molecule with potential commercial applications (Cantrell et al., 2012). Volatile organic compounds of botanical origin, most commonly found in essential oils, have particularly been recognized as highly effective against *M. incognita* (Aissani et al., 2015; Silva et al., 2018; Pedroso et al., 2019).

Plant essential oils (EOs) are complex mixtures of terpenoids and their oxygenated derivatives, produced by isoprenoid pathways (Tetali, 2019). Only ∼10% of the reported plant species produce EOs (Kalemba and Kunicka, 2003). Stored in secretory glands in epidermic cells, secretory hair, glandular trichomes, EOs play a key role in plant defense against biotic stresses (Bakkali et al., 2008). Known for their bioactive potential against diverse agriculturally important pests, EOs in numerous reports have been mentioned as very effective against *M. incognita* (Caboni et al., 2013). EOs of *Acorus calamus* and *Pogostemon cablin* have been tested for nematicidal activities (Perrett and Whitfield, 1995; Lee et al., 2009). EO from *Eucalyptus citriodora* was found highly toxic to *M. incognita* at 500 $\mu l\ ml^{-1}$ by Pandey et al. (2000). *Citrus sinensis* EO was reported effective against the phytonematodes, *M. incognita*, *Pratylenchus vulnus*, and *Xiphinema index* (Avato et al., 2017). Similarly, EO of *Myrtus communis* was reported to kill 100% of *M. incognita* juveniles at 4,000 $\mu l\ ml^{-1}$ concentration (Ardakani et al., 2013). Another study reported that EO of *Melaleuca alternifolia* was highly active against the larvae of *Anisakis simplex* at a concentration of 7 $\mu l\ mL^{-1}$ (Andrés et al., 2012). The EO of *Cymbopogon nardus* tested against *M. incognita* exhibited moderate effectiveness in a study reported by Sinha et al. (2006). Similarly, the toxicity of *Artemisia absinthium* EO has been documented against *M. incognita* on the tomato plant (Amora et al., 2017). Promising nematicidal action of sesquiterpenes rich in the EO of *Commiphora myrrha* against juveniles of *M. incognita* was reported by Kong et al. (2006) and Ardakani et al. (2013).

A review of literature clearly showed that EO bioactivity evaluation against nematodes largely remained restricted so far to the evaluation of EOs against different plant parasitic nematodes. Emphasis on the correlation of their anti-nemic activity with chemical compositions and mechanism of interaction at molecular level with the possible target sites of action has remained lacking. Therefore, the present study was performed to characterize the chemical composition of the selected EOs, evaluate their bio-efficacy *in vitro* against *M. incognita*, and subject the most effective EOs to *in silico* analysis for a likely mode of action, using molecular docking and modeling approach.

MATERIALS AND METHODS

Essential Oils

Commercially available EOs (99% purity) of different plants, namely, OEO (*Citrus sinensis* (L.) Osbeck; family Rutaceae, orange essential oil), MTEO (*Myrtus communis* L.; family Myrtaceae, myrtle essential oil), CEO (*Eucalyptus citriodora* L.; family Myrtaceae, citriodora essential oil), TEO (*Melaleuca alternifolia* L.; family Myrtaceae, tea tree oil), AEO (*Acorus calamus* L.; family Acoraceae, calamus essential oil), MREO [*Commiphora myrrha* (Nees) Engl; family Burseraceae, myrrh essential oil], CNEO (*Cymbopogon nardus* L. Rendle.; family Poaceae, citronella oil), WEO (*Artemisia absinthium* L.; family Asteraceae, wormwood essential oil), and PEO [*Pogostemon cablin* (Blanco) Benth.; family Lamiaceae, patchouli essential oil] were purchased from CDH Fine Chemicals (New Delhi, India) and Merck® (New Delhi, India) and used without further purification.

Chemicals and Reagents

All the solvents used were of AR grade, purchased from Merck® (New Delhi, India). Surfactants, Atlas G5002 and Triton X-100 were procured from Croda India Company Pvt. Ltd. (Navi Mumbai, India) and Loba Chemie Pvt., Ltd. (Mumbai, India), respectively. For GC-MS analysis, helium (He) gas of high purity (99%) was used.

Gas Chromatography-Mass Spectrometry Analysis

Volatile constituents of EOs were analyzed in GC-MS in a 7890A GC instrument (Agilent Technologies®, United States) equipped with an HP-5MS column (30 m × 0.25 μm;/0.25 μm, Agilent Co., United States) as stationary phase, which was directly connected to a triple axis HED-EM 5975C mass spectrometer (Agilent Co., United States). The injection volume was 1 μl with flow mode in split control. Helium was used as carrier gas at a head pressure of 10 psi, and flow was set at 1 ml min^{-1}. The GC-MS condition was programmed with the oven temperature initially held at 40°C for 1 min, thereafter increased with a gradient of 3°C min^{-1}, until the temperature reached to 120°C and held constant for 2 min. The temperature was raised again with a gradient of 5°C min^{-1} up to 220°C and held constant for 1 min and finally raised to 280°C with an increment of 4°C min^{-1}. The total run time of the analysis was 65 min. The MS acquisition parameters were ion source temperature 180°C, electron ionization 70 eV, full scan mode (50–550 AMU), transfer line temperature 280°C, solvent delay 3 min, and E.M voltage 1,380 V. Compounds were identified by matching their mass spectra and fragmentation pattern using NIST (National Institute of Standards and Technologies) Mass Spectra Library. Further rentention indices (RI) have been calculated following Kovats (1978):

TABLE 1 | Target sequences screened for *in silico* nematicidal activity.

Receptor	Amino Acid Seq	Source
Cytochrome c oxidase subunit 1	LVTKSVTHKNIGFIYLFFSFWSGLMGLSLSMLLRMDLMKSGMVIGDGQLYNVILTSHALVMIFFMVMPG LIGGFGNFFFPILINCIDLFLPRVNNMSYWFLPGSLILLMFSLFMDKGSGTGWTLYPPLMI DGQPGRSTDLVIFSLHFSGISSISSGINFLSTCHEMRLEVKTLEIMSLFVWCLIITVFLLVLSLPVLASGITMGLSDRN FNTGFFDSNMGGNILMFQHLFWFFGHPEVYVLIAPAFGLVSMVMVLLSSKKDLYGRK GMILAIMSIGFIGCLVWGHHMFTVGMDHDSRAYFSSATMIIAIPTGMKIFSWMMTLYGSKLNWNYLIL WIMGFIFMFTVGGLSGLILSNAGLDIFLHDTYYVVAHFHYVLSMGAVFGIFLGFFFSYGFMFGLMMNSVLVK SFFYIFFLGVNLTFFPMHFSGLQGQPRKYMSYSSDYLFWQMFASIGS LLSLFSIFLLIYLILESMIIFRLLIFDLFSFSMVSLNVNNYFHTNLDLSMIWLK	NCBI GenBank
AChE	MRKRRRKTTAFSINTSELLRLYFKFSSHSCLTFIFCCFFCLIVYCSSVHGRSSPVALTDVLIQTTLGKIIGFKQK FDGKSVHTFLGVPYAKSPTGSGRFGLPEMIEPWEGEFRADKPARTCFFSRDTMFPDFPGAEMWNPPNDIDEDCLAMNIW VPEHHDGTVLVWIYGGGFYSGSPSLDLYDGRVLAVQERAVVININYRLGPFGFLYFGDD TSVPGNMGLQDQQMALKWIHEHIAHFGGDPRRVTLFGESAGSASAMAHMFADG SYSLFSRIIAQSGSIINNWATKPKASILQISLQLAHHLNCSNGNN STKAMQNIVECIRRVPTSIIQRAGDAVSQSLSLPMDFAFVPIDEDTHFFRGNV FDKLRRKNFKRDVSILVGTVRDEGTYWLPYCLQKNGFGFNHTISPEDHINQALISETDYTKAFDAFLPYFGN SNLVRHALMHAYSHLPTEKQEQRWRDGVARF LGDYFFTCDSIEFADIVSDELYGSVYSFYFTRRSSANPWPQWMGAMHGYEIEYVFGLPLRSPHLYDPSELELEISFSTKIMEF WGHFARTGEPVEFWPKYNRITRKSLVLSEEIATGTSHRIYVDVHGKLCRLLEEAQAVAGITGEQRSRICPDGRATTVNYGQE ISMEDVKEEMQLNRGISGINRIPSIKIYISLIILSLALLRSPEISFLYSSFIFK	NCBI GenBank
Hsp90	MSLIINTFYSNKEIFLRELISNSSDALDKIRYQALTDPAQLETGKDLYIKIVPN KADKTLTIMDTGVGMTKADLVNNLETIAKSGTKAFMEALQAGADISMIGQFGVGFYSAFLVADRVTVTSEHNDDDCHQ WESSAGGSFIIRNCVDPEMTRGTKITLYLKEDQTDYLEERRIREVVKKHSQFIGYPIKLLVEKERDKEISDDEAEDEKKDVK KEEEKEEEKEIKKEEGEDKEGEDEDKDKKDGEKKKKTKKIKEKYTE DEELNKTKPIWTRNPDDITNEEYAEFYKSLSNDWEDHLAVKHLSVEGQLEFR ALLFVPQRAPFDMFENKKQKNAIKLYVRRVFIMENCEELMPEYLNFIKGVV DSEDLPLNISREMLQQSKILKVIRKNLVKKCIELFDEIAEDKDNFKKFYEQFSKNLKLGIHEDSVNRKKLAEYL RYNTSSSGDELVSLKDYVGRMKENQTCIYYITGESKEVVQNSAFVERVKKRGFEVIYMVDPIDE YCIQQLKEFDGKKLVSVTKEGLELPESEEEKKKFEEDKVKF EKLCKVIKDILDKKVQKVSVSNRLVSSPCCIVTGEYGWTANMERIMKAQALRDSSTMG YMASKKNLEINPDHSIIKSLRERIDSDQDDKTAKDLVVLLYETALLTS GFSLEDPQQHASRIYRMVKLGLDITEEDLEGGEQQPCTSGEPVEKIAGAEEDASRMEEVD	NCBI GenBank
ODR1	MMTGQQSTESFLATLAIYNACYGFCLGSSLTSTGSFASDPNNPAFVANLRGKSFQGIKKFLLPK RNFQFKGSFGQVNLTSWPAPLQNLAIYTLPSSGGQYSLIYTAISIPSSSCGT FECFDIQLQTSPNISEDLLWQKQCSNTIPSCIYSGGCSSLVPYFSAGAAIVLVAAAAGIVYTIQRKKRLDVFRVH WRIGRQQFKVIENKQAKGKATGIGQGEGAWSKRRQLHAYALIGTNKAEFIV LRQMKKIYWDKIELHFIFELKKLNHDNLTTFMGICYNDGDKFYVCHSLVERGTLEDYIHDLD FQLDNTFRSAFLRDILKGVKYLHKSSIGYHGMLNLQNVLIDSNWVLKLTNFGIGNLLNRAIRREQLQLIELIPLNTYLT VAPENLIDISYGREYPNGTTIGDIYSMGMVMYHILFRLAPYERTTLSPKEVIDQVRQHNLKPILENTLPEEK PLVDAMEQCWQKNLDLRPRLRQLAQVVSTVFQASQGNLIDQMRRMNEKHALNLEKLV TQRNAELAQAREQTERLLNEMLPPSIAAQLKEHKSV EPRSYDSATVLFCQLVDFSTVLSKFPPDQVIDFLNQVFSTFDTIIRNHDAYKVETTGETYMVAS GVPNENENRHVFEISEVAMEFREVSYTYKSINFP DWKLQLRIGYHCGPIAAGVIGIKAPRYCLFGDTVNFASRMQSNAAPNQIQMSESTALLLMGVSKYKLTKRGIVKVKGKER	WormBase ParaSite Database
ODR3	SCQSEEVREQLSKNKAIEKQLTSDRRAASSIIKLLLLGAGECGKSTVLKQMQILHSNG FTEEEINERKAVVYSNTVTSMAAILKAMDNVLHMPMDDASKERDRNLIFRAIENGEENLPFTDPIAKALQNLWGDKAVK KAYEMRSEYQLNDSAKYFLDSVSRIHEPGYRPTEQDILYSRVATTGVVEVKFIIKGNMEFRVFD VGGQRSERRKWIHCFDNVEAIIFITAISEYDQVLF EDETTNRMIESMQLFSSICNSSWFLNTAMILFLNKKDLFLEKIQRVNITTCF PDYEGSQNYEEAVNFIKMKFAELNQHPDKKTIYMHETCATDTN	WormBase ParaSite Database
Neuropeptide GPCR	MVSSISLNQQINQIEIENCIELNSVLDQFGDWTLRLDVKFFYSLFYAAIFIVGL IGNGFLVGTIRRRMTVANVFLMNLAISDLLLCITALPITPVLAFVKRWIFGLALCKLVPLCQGISVLISSY CLCLIAVDRYRSIVTPLKVPWNIQXAQWLMTLCWTFCIIISSPLFIVQGLQQIVYKNMTFCGEFCTEL NWPTDFRIKLFYGISLLSIQFLIPTLIMTYCYWKILQKVRQDWLVPTNNSIMSLEQQAQTAI RKRRVMYVLILMVLIFMGSWMPLTFVNLLRDIGISFLET QMYFKLLNVXAVAMTSVVSNPLLYFYMSKRXRRALRDDMYWLTNARRQQNQXVGGLLAKF TPSPSIGLLYKKSLERHILQNATAKYNPYRRGTLADPTTLGREKVLQEMHANCFLLVPL MPLCVANQQRLATNQREISNNNNINLNFKRQKHPKFVCEA	NCBI GenBank
CLAVATA3/ESR (CLE)-related protein	MFTNSIKNLIIYLMPLMVTLMLLSVSFVDAGKKPSGPNPGGNN	UNIPROT Database

Meloidogyne incognita acetyl cholinesterase (AChE), M. incognita heat shock protein 90 (Hsp90), M. incognita odorant response gene-1 (ODR1), and M. incognita neuropeptide G-protein coupled receptor (nGPCR).

TABLE 2 | Chemical composition of various EOs as analyzed in GC-MS and content (%)[c] of constituents.

Compounds[a]	RI[b]	OEO	MTEO	CEO	TEO	AEO	MREO	CNEO	WEO	PEO
Monoterpene hydrocarbons										
Thujene	930	nd	0.2 ± 0.1	nd	2.3 ± 0.1	nd	nd	nd	0.1 ± 0.0	nd
o-Cymene	937	nd	nd	nd	nd	nd	nd	nd	5.1 ± 0.2	nd
α-Pinene	939	1.6 ± 0.1	42.3 ± 1.1	0.1 ± 0.0	3.2 ± 0.2	0.2 ± 0.1	0.1 ± 0.0	nd	2.7 ± 0.2	0.2 ± 0.0
Camphene	954	nd	4.2 ± 1.0	1.0 ± 0.1	nd	0.1 ± 0.0	0.3 ± 0.0	nd	nd	nd
t-Ocimene	955	0.1 ± 0.0	nd	nd	nd	nd	nd	nd	nd	nd
Sabinene	975	0.5 ± 0.1	nd	nd	nd	nd	nd	nd	5.6 ± 0.3	nd
β-Pinene	979	nd	1.0 ± 0.2	0.2 ± 0.0	1.3 ± 0.1	nd	0.2 ± 0.0	nd	0.3 ± 0.1	0.3 ± 0.0
β-Myrcene	991	2.2 ± 0.2	nd	nd	2.3 ± 0.1	0.1 ± 0.0	nd	nd	0.2 ± 0.0	nd
Phellandrene	1,003	nd	nd	nd	0.6 ± 0.0	nd	0.1 ± 0.0	nd	nd	nd
p-Cymene	1,013	nd	nd	nd	4.8 ± 0.2	nd	nd	0.1 ± 0.0	0.1 ± 0.0	nd
α-Terpinene	1,017	nd	nd	nd	8.7 ± 0.6	nd	nd	nd	0.9 ± 0.3	0.2 ± 0.0
l-Limonene	1,029	93.2 ± 2.3	nd	nd	nd	nd	nd	5.7 ± 0.5	nd	nd
δ-3-Carene	1,033	0.4 ± 0.1	1.9 ± 0.5	nd	nd	nd	nd	nd	nd	nd
β-Ocimene	1,051	nd	nd	nd	nd	nd	0.2 ± 0.0	nd	0.4 ± 0.0	nd
γ-Terpinene	1,060	nd	nd	nd	17.5 ± 1.1	nd	nd	nd	1.1 ± 0.2	nd
α-Terpinolene	1,089	nd	nd	nd	2.8 ± 0.3	nd	nd	nd	1.1 ± 0.2	nd
Oxygenated monoterpenes										
Fenchone	1,008	nd	nd	2.3 ± 0.3	nd	0.1 ± 0.0	nd	nd	nd	nd
1,8-Cineole	1,035	nd	30.3 ± 1.3	nd	3.1 ± 0.4	nd	nd	nd	10.6 ± 0.5	nd
Limonene oxide	1,087	0.2 ± 0.0	nd	nd	nd	nd	nd	nd	nd	nd
Linalool	1,089	0.4 ± 0.1	7.6 ± 0.9	nd	nd	0.2 ± 0.0	1.7 ± 0.3	0.8 ± 0.2	6.4 ± 0.4	nd
Eucalyptol	1,093	nd	nd	0.2 ± 0.0	nd	nd	nd	nd	nd	nd
p-Menth-8-en-2-ol	1,090	nd	nd	nd	3.2 ± 0.5	nd	nd	nd	nd	nd
t-Thujone	1,102	nd	nd	nd	nd	nd	nd	nd	0.3 ± 0.0	nd
Pulegol	1,116	nd	nd	nd	nd	nd	nd	0.1 ± 0.0	nd	nd
Camphor	1,146	nd	nd	nd	nd	nd	nd	nd	3.8 ± 0.5	nd
Citronellal	1,148	0.1 ± 0.0	nd	81.9 ± 1.1	nd	nd	nd	31.5 ± 1.1	nd	nd
Isopulegol	1,150	nd	nd	nd	nd	nd	nd	1.0 ± 0.1	nd	nd
β-Terpineol	1,163	nd	nd	nd	35.7 ± 1.2	nd	nd	nd	16.2 ± 0.9	nd
Borneol	1,169	nd	nd	nd	nd	0.1 ± 0.0	nd	nd	0.5 ± 0.1	nd
4-Caranol	1,185	nd	nd	nd	nd	nd	nd	nd	0.3 ± 0.0	nd
Decanal	1,202	0.2 ± 0.0	nd	nd	nd	nd	nd	nd	nd	nd
Citronellol	1,226	nd	nd	5.8 ± 0.7	nd	nd	nd	9.6 ± 0.3	nd	nd
Geraniol	1,231	nd	nd	nd	nd	nd	nd	30.6 ± 1.3	0.6 ± 0.1	nd
Citral	1,236	nd	nd	0.1 ± 0.0	nd	nd	nd	nd	nd	nd
Neral	1,240	nd	nd	nd	nd	nd	nd	0.5 ±	nd	nd
Linalyl acetate	1,257	nd	6.6 ± 0.5	nd	nd	nd	nd	nd	nd	nd
Geranial	1,270	nd	nd	nd	nd	nd	nd	0.7 ±	nd	nd
Borneol acetate	1,289	nd	nd	nd	nd	nd	nd	nd	26.6 ± 1.7	nd
Neryl acetate	1,362	nd	nd	nd	nd	nd	nd	2.1 ± 0.2	0.7 ± 0.1	nd
Geranyl acetate	1,383	nd	nd	nd	nd	0.1 ± 0.0	nd	nd	0.2 ± 0.0	nd
Thymol	1,470	nd	nd	nd	nd	nd	nd	nd	0.3 ± 0.0	nd
Sesquiterpene hydrocarbon										
δ-Elemene	1,343	nd	nd	nd	nd	nd	2.1 ± 0.2	nd	2.0 ± 0.3	nd
α-Cubebene	1,348	nd	nd	nd	nd	0.1 ± 0.0	nd	nd	nd	nd
α-Copaene	1,377	nd	nd	nd	0.1 ± 0.0	nd	0.2 ± 0.0	nd	nd	nd
β-Patchoulene	1,382	nd	nd	nd	nd	nd	nd	nd	nd	5.8 ± 0.5
β-Elemene	1,389	nd	nd	nd	nd	nd	4.8 ± 0.7	3.3 ± 0.1	nd	2.6 ± 0.1
α-Gurjunene	1,410	nd	nd	nd	2.1 ± 0.1	nd	nd	nd	nd	0.1 ± 0.0

(Continued)

TABLE 2 | Continued

Compounds[a]	RI[b]	OEO	MTEO	CEO	TEO	AEO	MREO	CNEO	WEO	PEO
β-Caryophyllene	1,419	nd	nd	0.8 ± 0.1	0.3 ± 0.0	2.4 ± 0.3	0.5 ± 0.1	nd	8.2 ± 1.0	6.9 ± 0.5
α-Guaiene	1,430	nd	nd	nd	nd	nd	nd	nd	0.1 ± 0.0	40.6 ± 1.3
α-Bergamotene	1,436	nd	nd	nd	nd	nd	0.4 ± 0.0	nd	nd	nd
Aromadendrene	1,443	nd	nd	nd	1.0 ± 0.1	nd	nd	1.4 ± 0.1	nd	nd
α-Humulene	1,455	nd	1.9 ± 0.3	nd	nd	nd	0.3 ± 0.0	nd	nd	nd
Farnesene	1,457	nd	nd	nd	nd	nd	nd	nd	0.1 ± 0.0	nd
α-Patchoulene	1,460	nd	nd	nd	nd	nd	nd	nd	nd	10.7 ± 0.5
Neoisolongifolene	1,462	nd	nd	nd	nd	nd	nd	nd	nd	1.0 ± 0.1
Alloaromadendrene	1,466	nd	nd	nd	0.7 ± 0.0	nd	nd	nd	nd	4.4 ± 0.2
β-Salinene	1,473	nd	nd	nd	0.3 ± 0.0	nd	nd	nd	nd	nd
γ-Muurolene	1,477	nd	nd	nd	0.4 ± 0.1	1.2 ± 0.2	nd	0.3 ± 0.0	nd	nd
Germacrene D	1,485	nd	nd	nd	nd	nd	1.2 ± 0.1	1.4 ± 0.2	nd	nd
Epi-bicyclophellandrene	1,489	nd	nd	nd	0.4 ± 0.1	nd	nd	nd	nd	nd
Aciphyllene	1,492	nd	nd	nd	nd	nd	nd	nd	nd	2.8 ± 0.5
α-Bulnesene	1,498	nd	nd	nd	nd	nd	nd	nd	nd	14.6 ± 0.9
δ-Cadinene	1,507	nd	nd	nd	1.6 ± 0.2	nd	nd	nd	nd	nd
Curcerene	1,511	nd	nd	nd	nd	nd	23.9 ± 2.0	nd	nd	nd
α-Panasinsene	1,519	nd	nd	nd	0.2 ± 0.0	nd	nd	nd	nd	nd
Sesquiphellandrene	1,523	nd	nd	nd	0.1 ± 0.0	nd	nd	nd	nd	nd
γ-Cadinene	1,526	nd	nd	nd	nd	nd	0.3 ± 0.0	1.7 ± 0.2	nd	nd
α-Bisabolene	1,539	nd	nd	0.1 ± 0.0	nd	nd	nd	nd	nd	nd
α-Calacorene	1,547	nd	nd	nd	nd	nd	0.8 ± 0.1	nd	nd	nd
Oxygenated sesquiterpene										
Methyl eugenol	1,401	nd	nd	nd	nd	0.1 ± 0.0	nd	1.1 ± 0.1	nd	nd
Methyl isoeugenol	1,455	nd	nd	nd	nd	3.1 ± 0.2	nd	nd	nd	nd
Elemol	1,550	nd	nd	nd	nd	nd	nd	3.3 ± 0.5	nd	nd
Spathulenol	1,561	nd	nd	nd	nd	1.7 ± 0.1	nd	nd	nd	nd
Caryophyllene oxide	1,583	nd	nd	0.4 ± 0.0	nd	1.4 ± 0.1	0.4 ± 0.0	nd	0.2 ± 0.0	0.2 ± 0.0
Viridiforol	1,588	nd	nd	nd	0.3 ± 0.1	nd	0.2 ± 0.0	nd	nd	nd
β-Asarone	1,622	nd	nd	nd	nd	85.4 ± 1.1	nd	nd	nd	nd
Cadinol	1,645	nd	nd	nd	nd	nd	0.9 ± 0.1	0.6 ± 0.1	nd	nd
γ-Eudesmol	1,625	nd	nd	nd	nd	nd	0.2 ± 0.1	nd	nd	nd
β-Cudesmol	1,649	nd	nd	nd	0.2 ± 0.0	nd	1.1 ± 0.1	nd	nd	nd
Patchoulol	1,668	nd	nd	nd	nd	nd	nd	nd	nd	6.7 ± 0.3
Elemol acetate	1,674	nd	nd	nd	nd	nd	1.2 ± 0.2	nd	nd	nd
α-Bisabolol	1,678	nd	nd	nd	nd	nd	0.2 ± 0.0	nd	nd	nd
α-Asarone	1,679	nd	nd	nd	nd	1.9 ± 0.3	nd	nd	nd	nd
Farnesol	1,706	nd	nd	0.3 ± 0.0	nd	nd	nd	nd	3.0 ± 0.2	nd
Guaiol acetate	1,721	nd	nd	nd	0.1 ± 0.0	nd	nd	nd	nd	nd
Furanoeudesm-1,3-diene	2,091	nd	nd	nd	nd	nd	41.9 ± 2.4	nd	nd	nd
Others										
2,6-Dimethyl-5-heptenal		nd	nd	0.2 ± 0.0	nd	nd	nd	nd	Nd	nd
4,8-Dimethyl-3,7-non-adienal		nd	nd	0.1 ± 0.0	nd	nd	nd	nd	Nd	nd

[a] *EOs, essential oils; OEO: Citrus sinensis (OEO); Myrtus communis (MTEO), Eucalyptus citriodora (CEO), Melaleuca alternifolia (TEO), Acorus calamus (AEO), Commiphora myrrha (MREO), Cymbopogon nardus (CNEO), Artemisia absinthium (WEO), and Pogostemon cablin (PEO); compounds are listed in order of elution from a HP-5MS capillary column. Identification performed by comparison of mass spectra with the corresponding data in NIST library with respect to total ion chromatogram as well as retention indices, calculated for alkanes C_9 to C_{24} followed by comparison with the Adams (2007) report.*

[b] *Retention indices on the HP-5MS capillary column.*

[c] *Mean value of three replicates calculated from gas chromatography-mass spectrometry (GC-MS) areas, nd, not detected respective data of NIST and Willey (30: 70) libraries in total ion current (TIC) and the literature, as well as retention indices as calculated according to Kovats (1978), for alkanes C 9 to C 24 compared with those reported by Adams.*

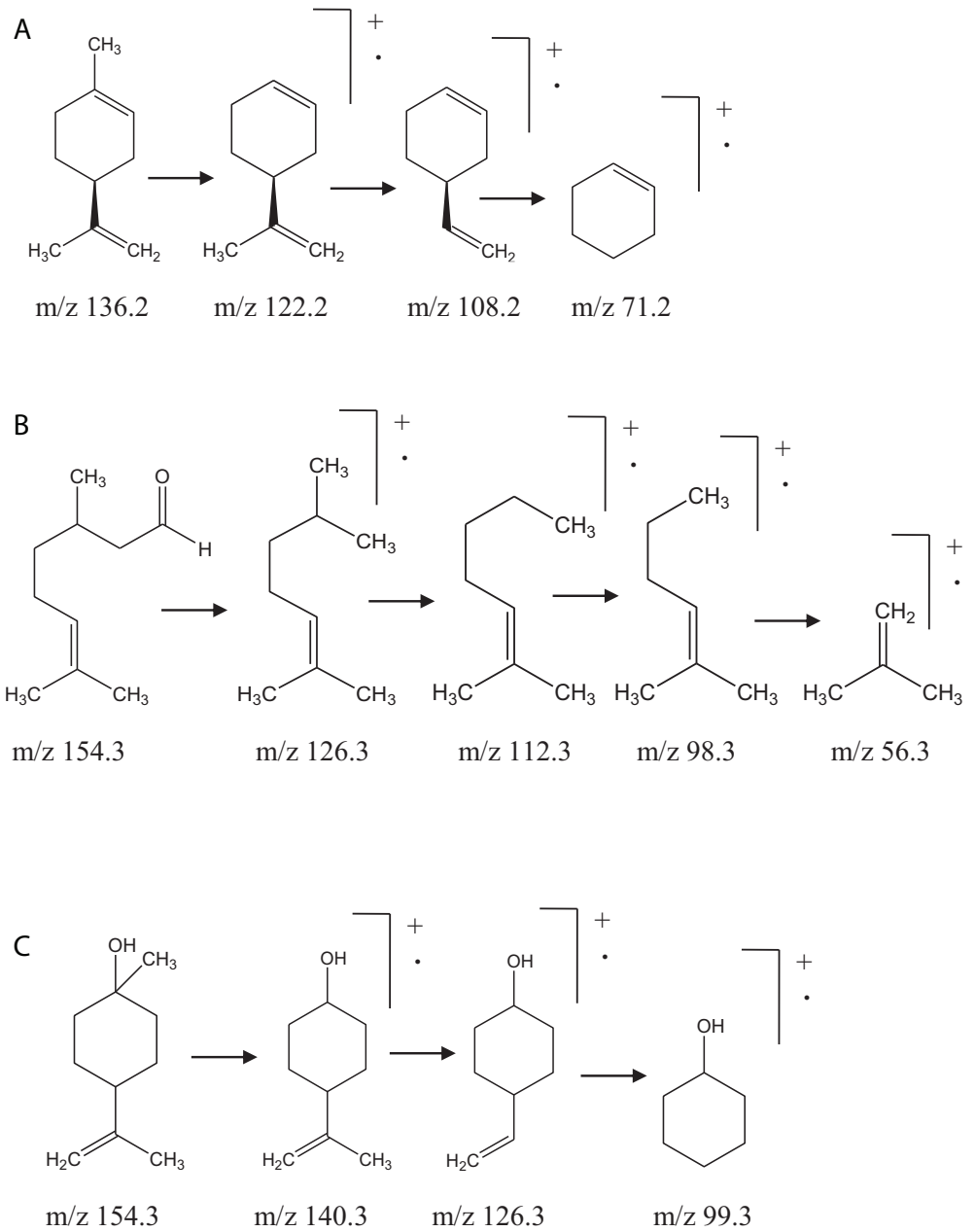

FIGURE 1 | Mass fragmentation pattern of **(A)** *l*-limonene, **(B)** citronellal, **(C)** and β-terpineol.

RI = 100 * n + [log($RT_{compound}$ - v) - log(RT - v)]/[log($RT_{larger\ alkane}$ - v) - log($RT_{smalleralkane}$ - v)]
where n = the number of C in the smaller alkane, $RT_{compound}$ = the retention time of the compound, v = the column void time, $RT_{larger\ alkane}$ = the retention time of the larger alkane, and $RT_{smaller\ alkane}$ = the retention time of the smaller alkane.

Nematicidal Assay

Collection of Nematodes

Nematode culture was maintained on infected tomato plants (var. Pusa Ruby) under greenhouse conditions. Second instar juveniles (J_2s) of *M. incognita* were collected from roots of 21-day-old infected tomato seedlings. Nematode-infested soil was screened through water screening method following Cobb's sieving and decanting technique (Cobb, 1918). Further, nematode egg masses were picked up from the sterilized infected roots of tomato seedlings, transferred to fresh distilled water in Petri plates, and allowed to hatch under ambient condition of 27 ± 1°C for 5 days. The hatched nematode juveniles travel through soft wet tissue placed on the wire of the Petri plate on the surface water (Julio et al., 2017). Nematode J_2s suspensions were combined and counted under light microscope.

TABLE 3 | Chemical composition, number of compounds, group-wise classification of EO constituents.

Classification	OEO	MTEO	CEO	TEO	AEO	MREO	CNEO	WEO	PEO
Total identified composition (%)	98.9	96.0	93.5	93.3	98.2	83.2	95.8	97.7	97.1
Number of identified compounds	10	9	14	26	16	24	19	29	15
Total monoterpene constituents (%)	98.9	94.1	91.6	85.5	0.9	2.6	82.7	84.1	0.7
Total sesquiterpene constituents (%)	–	1.9	1.6	7.8	97.3	80.6	13.1	13.6	96.4
Total hydrocarbons	98.0	51.5	2.2	50.7	4.1	35.4	13.9	28.0	90.2
Total oxygenated compounds	0.9	44.5	91.0	42.6	94.1	47.8	81.9	69.7	6.9

EOs, Essential oils; OEO: Citrus sinensis (OEO), Myrtus communis (MTEO), Eucalyptus citriodora (CEO), Melaleuca alternifolia (TEO), Acorus calamus (AEO), Commiphora myrrha (MREO), Cymbopogon nardus (CNEO), Artemisia absinthium (WEO), and Pogostemon cablin (PEO).

Preparation of Essential Oil Emulsions

Primary stock emulsions (10,000 $\mu g\ ml^{-1}$) of all EOs except PEO were prepared using Atlas G5002 surfactant (2% w/w). In the case of PEO, Triton X-100 (2% w/w) was used. Each primary stock emulsion was diluted serially with surfactant solution to prepare secondary test emulsions of varying strengths (1,000–10 $\mu g\ ml^{-1}$).

Nematicidal Activity

Nematicidal assay was conducted under *in vitro* condition to assess the activity of the EOs against *M. incognita* following a known method with slight modifications (Kundu et al., 2016). Treatments comprised of nine Eos, namely, OEO, MTEO, CEO, TEO, AEO, MREO, CNEO, WEO, and PEO. Aqueous suspension (1 µl) containing 25 J_2s of *M. incognita* was added to each well of multiwell plates (15.6-mm diameter), each containing EO emulsion (2 ml) of a particular test strength (1,000–10 µg ml^{-1}). Surfactant solutions used to dissolve EOs were taken as corresponding negative controls. Each treatment was replicated thrice. Multiwell plates were incubated at 27 ± 1°C and examined using a stereoscopic microscope at 24, 48, and 72-h intervals. The numbers of dead vs alive juveniles in each treatment was recorded. Motionless nematodes with straight bodies were counted. The revival test was done as described by Choi et al. (2007). Briefly, the motionless nematodes were teased with a needle followed by transfer to fresh wells containing deionized water. One drop of sodium hydroxide (1M) solution was added to check any movement. Mortality (%) and corrected mortality (%) of J_2s was calculated considering the mortality of juveniles in negative control.

TABLE 4 | LC_{50} and LC_{90} values ($\mu g\ ml^{-1}$) of EOs against *M. incognita*, calculated for three exposure periods in test solutions.

*EOs	**LC_{50} (µ g ml^{-1})			***LC_{90} (µ g ml^{-1})		
	24 h	48 h	72 h	24 h	48 h	72 h
OEO	353.20	79.35	39.37	921.63	556.96	231.70
MTEO	>1,000	932.65	879.40	>1,000	>1,000	>1,000
CEO	746.48	330.41	124.50	>1,000	>1,000	987.42
TEO	404.13	103.64	76.28	>1,000	963.90	943.17
AEO	524.45	90.11	85.23	>1,000	353.21	310.92
MREO	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000
CNEO	325.41	87.27	43.22	912.57	676.28	278.05
WEO	>1,000	937.52	734.72	>1,000	>1,000	>1,000
PEO	>1,000	387.77	290.87	–	>1,000	>1,000

EOs, essential oils; OEO: Citrus sinensis (OEO), Myrtus communis (MTEO), Eucalyptus Citriodora (CEO), Melaleuca alternifolia (TEO), Acorus calamus (AEO), Commiphora myrrha (MREO), Cymbopogon nardus (CNEO), Artemisia absinthium (WEO), and Pogostemon cablin (PEO). **LC_{50} ($\mu g\ mL^{-1}$): Lethal concentration at 50% mortality of nematodes. *LC_{90} ($\mu g\ mL^{-1}$): Lethal concentration at 90% mortality of nematodes.*

Molecular Docking and Simulation

Based on the results of *in vitro* nematicidal assay and GC-MS analysis, major volatile constituents of OEO, CNEO, and TEO were selected out of nine EOs, for *in silico* ligand target protein interaction analysis.

Selection of Protein

Seven target proteins, namely, cytochrome c oxidase subunit 1, AChE, Hsp90, ODR1, ODR3, neuropeptide GPCR, CLAVATA3/ESR (CLE)-related protein of *M. incognita* were selected as target receptors for the molecular docking studies. Cytochrome c oxidase subunit 1 is involved in the oxidative phosphorylation pathway, which is part of the energy metabolism. AChE regulates synaptic transmission and locomotion processes. The full functional activity of Hsp90 is gained in coordination with other co-chaperones, playing an important role in the folding of newly synthesized proteins, stabilization and refolding of denatured proteins during stress. ODR1 and ODR3 regulate chemosensory functions. Neuropeptide GPCR is associated in the regulation of movement of the parasite toward (or within) its host. CLAVATA3/ESR (CLE)-related protein plays an important role in the differentiation or division of feeding cells (syncytia) induced in plant roots during infection. (Ref. for each).

Protein Preparation

The hypothetical protein sequences were taken from the NCBI and UNIPROT database (**Table 1**). The BLAST servers[1,2] were used to search and annotate the molecular and biological functions of the query sequences. The NCBI Blast tool and the PDB database were together used to identify the templates for modeling the secondary structures of the query sequences. Further homology modeling of the proteins was carried out using Modeller v 9.24.

[1]http://blast.ncbi.nlm.nih.gov

[2]https://parasite.wormbase.org//Tools/Blast

TABLE 5 | *In silico* nematicidal activity of OEO, TEO, and CNEO oil constituents against *M. incognita* ODR1.

NAME	Hbond	Hphob	VwInt	Δ G	Heavy_Atoms	Log_P	LE	Relative Percent (>10%)	Oil Constituent
Geraniol	−7.04	−5.05	−18.7	−36.9	11	2.67	0.80	30.6	CNEO
Linalool	−5.08	−5.43	−21.8	−36.2	11	2.67	0.79	<10.0	
Geranial	−2.51	−4.76	−23.7	−33.1	11	2.88	0.72	<10.0	
β-Terpineol	−5.61	−4.95	−15.8	−32.7	11	2.50	0.71	35.7	TEO
t-Ocimene	0.00	−5.89	−20.9	−28.9	10	3.48	0.69	<10.0	
Neral	−3.61	−4.76	−21.0	−31.0	11	2.88	0.67	<10.0	
p-Menth-8-en-2-ol	−4.95	−5.03	−17.4	−30.4	11	2.36	0.66	<10.0	
Pulegol	−3.80	−5.01	−17.4	−29.9	11	2.50	0.65	<10.0	
2,6-Dimethyl-5-heptenal	−2.58	−4.64	−18.5	−26.8	10	2.57	0.64	<10.0	
Citronellal	−2.66	−4.79	−20.5	−29.3	11	2.96	0.64	31.5	CNEO
Limonene oxide	−1.86	−4.30	−15.6	−28.7	11	2.52	0.62	<10.0	
l-Limonene	0.00	−4.74	−15.5	−25.7	10	3.31	0.61	93.2	OEO
β-Myrcene	0.00	−5.42	−21.5	−24.6	10	3.48	0.59	<10.0	
β-pinene	0.00	−4.49	−14.3	−24.2	10	3.00	0.58	<10.0	
β-Pinene	0.00	−4.36	−13.0	−24.1	10	3.00	0.58	<10.0	
β-Terpinolene	0.00	−5.20	−18.5	−24.0	10	3.45	0.57	<10.0	
γ-Terpinene	0.00	−5.58	−17.0	−24.0	10	3.31	0.57	17.5	TEO
Para-cymene	0.00	−5.46	−17.0	−23.9	10	3.12	0.57	<10.0	
4,8-Dimethyl-3,7-non-adienal	−5.24	−5.02	−14.2	−28.6	12	3.27	0.57	<10.0	
Phellandrene	0.00	−5.43	−16.4	−23.6	10	3.16	0.56	<10.0	
α-Terpinene	0.00	−5.18	−18.5	−23.1	10	3.31	0.55	<10.0	
Decanal	−2.45	−5.13	−19.8	−25.4	11	3.33	0.55	<10.0	
Isopulegol	−1.84	−5.04	−18.0	−24.5	11	2.36	0.53	<10.0	
Sabinene	0.00	−5.11	−17.5	−21.0	10	3.00	0.50	<10.0	
δ-Carene	0.00	−5.10	−17.0	−19.8	10	3.00	0.47	<10.0	
Citronellol	−4.66	−5.27	−18.2	−21.6	11	2.75	0.47	9.6	CNEO
Methyl eugenol	0.00	−5.64	−23.9	−25.4	13	2.43	0.47	<10.0	
α-Thujene	0.00	−5.13	−16.3	−19.2	10	3.00	0.46	<10.0	
Neryl acetate	−1.56	−5.92	−21.4	−26.7	14	3.24	0.45	<10.0	
1,8-Cineol	−1.16	−4.88	−14.3	−20.1	11	2.74	0.44	<10.0	
β-Caryophyllene	0.00	−5.72	−17.1	−26.3	15	4.73	0.42	<10.0	
β-Eudesmol	−6.84	−5.95	−8.4	−27.7	16	3.92	0.41	<10.0	
γ-Cadinene	0.00	−5.60	−19.5	−25.3	15	4.58	0.40	<10.0	
Epibicyclosesquiphellandrene	0.00	−5.66	−17.8	−25.2	15	4.58	0.40	<10.0	
Sesquiphellandrene	0.00	−7.01	−23.9	−24.2	15	4.89	0.39	<10.0	
Germacrene D	0.00	−5.71	−16.6	−23.9	15	4.89	0.38	<10.0	
α-Panasinsene	0.00	−5.45	−15.3	−23.4	15	4.56	0.37	<10.0	
Cadinol	−5.20	−6.03	−10.5	−24.8	16	3.78	0.37	<10.0	
β-Selinene	0.00	−5.88	−18.5	−22.9	15	4.73	0.37	<10.0	
Allo-aromadendrene	0.00	−5.50	−14.2	−21.2	15	4.27	0.34	<10.0	
β-Elemene	0.00	−6.11	−18.0	−21.1	15	4.75	0.34	<10.0	
γ-Muurolene	0.00	−5.99	−18.2	−20.5	15	4.58	0.33	<10.0	
α-Gurjunene	0.00	−5.43	−15.1	−20.3	15	4.42	0.32	<10.0	
α-Copaene	0.00	−5.91	−16.7	−19.2	15	4.27	0.31	<10.0	
Viridiflorol	0.00	−5.54	−13.7	−20.1	16	3.47	0.30	<10.0	
Guaiol acetate	−1.47	−6.28	−18.5	−23.3	19	4.49	0.29	<10.0	
Elemol	−3.74	−6.28	−12.7	−19.7	16	3.94	0.29	<10.0	
δ-Cadinene	0.00	−6.78	−10.1	−16.3	15	4.73	0.26	<10.0	
Aromadendrene	0.00	−5.79	−11.9	−15.3	15	4.27	0.24	<10.0	

TABLE 6 | Binding domains in the ODR1 target receptor.

Domain Name	Position (Independent *E*-value)	Description
1 Guanylate_cyc	549. . .. . 724 (2.5e-49)	PF00211, Adenylate and Guanylate cyclase catalytic domain (Adenylate cyclase-activating G protein-coupled receptor signaling pathway and cyclic nucleotide biosynthetic process)
2 PK_Tyr_Ser _Thr	261. . .. . 479 (1.2e-22)	PF07714, Protein tyrosine and serine/threonine kinase (The catalytic domain found in a number of serine/threonine and tyrosine-protein kinases is represented by this entry)
3 HNOBA	498. . .. . 541 (0.0028)	PF07701, Heme NO binding associated (This domain is predicted to function in both bacteria and animals as a heme-dependent sensor for gaseous ligands, and to transduce various downstream signals)

Ligand and Receptor Preparation

The molecular structures of the chemical constituents, referred hereafter as "ligands," of OEO, CNEO, and TEO were downloaded as.sdf file from PUBCHEM database[3]. The ligand structures were minimized using MM2 forcefield in Chem Draw Ultra 11.0 software [Cambridge Soft Corp., Cambridge, MA, United States (2009)] and used for molecular modeling studies. The ligand molecules were customized for docking using the Dock prep tool of Autodock Vina. Hydrogen molecules were added, and the incomplete side chains were replaced using Dunbrack rotamer library (Dunbrack, 2006). Charges were computed using ANTECHAMBER. AMBER ff14SB and Gasteiger charges were allotted to standard residues and to other residue types, respectively. Similarly, receptor molecules were prepared using the same tools except that the ANTECHAMBER was not employed. All the prepared ligand files were saved in the Mol2 format and the receptor files in the.pdb format.

Molecular Docking Simulation

The customized ligand and receptor molecules were used for docking in ICM Molsoft v. 2.8.ICM software, which performed adaptable ligand docking through global optimization of the energy function (Abagyan et al., 1994). The energy functions incorporated the internal energy of the ligand in view of the ECEPP/3 drive field, and van der Waals, hydrogen-holding, electrostatic and hydrophobic ligand/receptor association terms pre-ascertained on the lattice for computational proficiency (Bursulaya et al., 2003). Flexible ligand docking with the ICM software used Monte Carlo simulations to globally optimize a set of ligand internal coordinates in the space of grid potential maps calculated for the protein pocket (Neves et al., 2012). Discovery Studio v. 4.1 Client was used to study the docked receptor–ligand interactions. The most favored docking conformation interactions of *ODR1* with geraniol, β-terpineol, citronellal, *l*-limonene, and γ-terpinene were analyzed on the basis of docking score, binding affinity, and interacting residues. The active site residues were identified, and depictions of all possible interactions in 3D and 2D poses were prepared using DS Visualiser v. 4.1.

In order to avoid affinity-based selection and optimization of larger ligands, the emphasis was given to compounds that most effectively utilized their atoms. In an attempt to measure the compound effectiveness, Hopkins et al. (2014) suggested an estimation of binding affinity of molecule, in terms of ligand efficiency (LE):

$$\mathrm{LE} = \frac{[-2.303(\mathrm{RT}) \times \log Kd]}{\mathrm{HA}} = \frac{-\Delta G}{\mathrm{HA}}$$

where, ΔG is free-binding energy and HA is the number of ligand non-hydrogen atoms. LE is related to the amount and effectiveness of heavy atoms in a molecule toward complex formation. The average affinity contribution per atom was taken into consideration instead of considering the affinity of the whole compound. This enabled measuring the affinity of the corrected molecules with their size. In drug discovery modules, candidate molecules with LE values $\geq$ 0.3 kcal per mole per heavy atom usually are taken ahead as lead molecule (Hopkins et al., 2014).

Statistical Analysis

The bioassay experiments were done in triplicate. The significance of the differences between variables was tested using one-way ANOVA. The means were compared using Duncan's multiple range test. Statistical significance was determined at $p < 0.05$. Percent mortality data were subjected to probit analysis using Polo Plus software to determine lethal concentrations (LC_{50} and LC_{90}, expressed in $\mu g\ ml^{-1}$).

RESULTS

Essential Oil Composition

The compositions of EOs of OEO, MTEO, CEO, TEO, AEO, MREO, CNEO, WEO, and PEO were determined by comparing their mass spectra with data library, corresponding retention indices, and mass fragmentation patterns. The identified chemical constituents of the oils are listed in **Table 2**. The aromatic profile of most of the EOs showed dominance of one or two major constituents. Individually, *l*-limonene (93.2 ± 2.30%) was found to be most abundant in OEO, along with β-myrcene (2.2 ± 0.2%), α-pinene (1.6 ± 0.1%), sabinene (0.5 ± 0.1%), limonene oxide (0.2 ± 0.0%), and decanal (0.2 ± 0.0%). *l*-Limonene was confirmed based on its fragmentation pattern with characteristic daughter ion peaks of m/z 136.2, 108.2, and 71.2, generated due to sequential loss of methyl and ethyl moieties (**Figure 1**). Interestingly, OEO was found to contain only monoterpenes. The monoterpenic constituents of MTEO were identified as α-pinene (42.3 ± 1.1%), 1,8-cineol (30.3 ± 1.3%),

[3]pubchem.ncbi.nlm.nih.gov

TABLE 7 | Molecular interaction details of flexible ligand docking of major oil constituents (the top 5) with the ODR1 receptor.

Constituent	Bondbetween atoms	Distance	Type ofbonding
Geraniol	:LYS599:HZ2 - Lig:Geraniol:O1	2.24581	Conventional Hydrogen
	:TYR607:HH - Lig:Geraniol:O1	2.07546	Conventional Hydrogen
	Lig:Geraniol:H1 -:ASP589:OD1	2.07777	Conventional Hydrogen
	:TRP220 - Lig:Geraniol:C9	4.53747	Pi-Alkyl Hydrophobic
	:PHE585 - Lig:Geraniol:C10	4.62898	Pi-Alkyl Hydrophobic
	:TYR607 - Lig:Geraniol:C8	5.20422	Pi-Alkyl Hydrophobic
β-Terpineol	Lig:β-Terpineol:H1 -:ASN340:OD1	2.14644	Conventional Hydrogen
	Lig:β-Terpineol:C9 -:ILE389	5.19655	Alkyl Hydrophobic
	:TRP220 - Lig:β-Terpineol	5.04265	Pi-Alkyl Hydrobhobic
	:TRP220 - Lig:β-Terpineol:C9	4.59553	Pi-Alkyl Hydrobhobic
Citronellal	:ASN582:HD22 - Lig:Citronellal:O1	1.86408	Conventional Hydrogen
	:ASN582:HA - Lig:Citronellal:O1	2.5931	Carbon Hydrogen
	Lig:Citronellal:C8 -:LEU338	5.31917	Alkyl Hydrophobic
	Lig:Citronellal:C8 -:ILE389	5.35293	Alkyl Hydrophobic
	Lig:Citronellal:C9 -:ILE389	5.26424	Alkyl Hydrophobic
	:TRP220 - Lig:Citronellal:C9	5.02254	Pi-Alkyl Hydrobhobic
	:PHE585 - Lig:Citronellal:C10	4.31251	Pi-Alkyl Hydrobhobic
/-Limonene	:LEU349 - Lig: /-Limonene	5.48522	Alkyl Hydrophobic
	:PRO385 - Lig: /-Limonene	4.65347	Alkyl Hydrophobic
	Lig: /-Limonene:C10 -:VAL383	4.26576	Alkyl Hydrophobic
	Lig: /-Limonene:C10 -:PRO385	3.65949	Alkyl Hydrophobic
	Lig: /-Limonene:C4 -:LEU349	4.67353	Alkyl Hydrophobic
	Lig: /-Limonene:C4 -:VAL383	4.5661	Alkyl Hydrophobic
	:TRP347 - Lig:Limonene	5.44615	Pi-Alkyl Hydrobhobic
γ-Terpinene	:PRO534 - Lig:γ-Terpinene	4.26992	Alkyl Hydrophobic
	:PRO535 - Lig:γ-Terpinene	5.10312	Alkyl Hydrophobic
	:ALA597 - Lig:γ-Terpinene	3.68262	Alkyl Hydrophobic
	:ALA597 - Lig:γ-Terpinene:C8	4.33181	Alkyl Hydrophobic
	:LYS599 - Lig:γ-Terpinene	5.25154	Alkyl Hydrophobic
	:ALA610 - Lig:γ-Terpinene:C9	4.07633	Alkyl Hydrophobic
	Lig:γ-Terpinene:C10 -:PRO534	4.0165	Alkyl Hydrophobic
	Lig:γ-Terpinene:C10 -:VAL613	4.30711	Alkyl Hydrophobic
	Lig:γ-Terpinene:C10 -:PRO614	4.12284	Alkyl Hydrophobic
	Lig:γ-Terpinene:C8 -:PRO535	4.64633	Alkyl Hydrophobic
	:TYR672 - Lig:γ-Terpinene:C10	5.24394	Pi-Alkyl Hydrobhobic

linalool (7.6 ± 0.9%), and linalyl acetate (6.6 ± 0.5%). GC-MS analysis of TEO showed several peaks corresponding to 27 mono and sesquiterpenoids, comprising 93.3% of the total oil. Monoterpenes (43.5%) and their oxygenated derivatives (42.6%) were found to be the most abundant. Among monoterpenes, β-terpineol (35.7 ± 1.2%) was identified as the major constituent followed by γ-terpinene (17.5 ± 1.1%), α-terpinene (8.7 ± 0.6%), *p*-cymene (4.8 ± 0.2%), α-pinene (3.2 ± 0.2%), *p*-menth-8-en-2-ol (3.2 ± 0.5%), and 1,8-cineol (3.1 ± 0.4%). Besides, α-gurjunene (2.1 ± 0.1%) and δ-cadinene (1.6 ± 0.2%) were identified as the major sesquiterpenes.

Analysis of volatiles of CEO and CNEO revealed the presence of various terpenes, representing 93.5% and 95.8% of the total oil composition, respectively. These oils were characterized by the presence of predominant acyclic monoterpene aldehyde and citronellal with its respective contents of 81.9 ± 1.1% and 31.5 ± 1,1%, in two oils. Both CEO and CNEO showed higher content of oxygenated compounds, in which the former attributed an appreciably higher content primarily of 91.3% oxygenated monoterpenes. Similarly, CNEO mainly contained oxygenated terpenoids (81.9%) and hydrocarbons (13.9%). Except citronellal, other constituents of CEO were citronellol (5.8 ± 0.7%) and fenchone (2.3 ± 0.3%), while CNEO contained geraniol (30.6 ± 1.3%), citronellol (9.6 ± 0.3%), *l*-limonene (5.7 ± 0.5%), β-elemene (3.3 ± 0.1%), and neryl acetate (2.1 ± 0.2%).

GC-MS analysis of AEO showed identification of 16 mono and sesquiterpenes, accounting for 98.2% of the total oil. Oxygenated terpenes were the major constituents (94.1%) of the oil with the β-asarone being the highest contributor (85.4 ± 1.1%). Methyl isoeugenol (3.1 ± 0.2%), caryophyllene (2.4 ± 0.3%), α-asarone (1.9 ± 0.3%), spathulenol (1.7 ± 0.1%), caryophyllene oxide (1.4 ± 0.1%), and γ-muurolene (1.2 ± 0.2%) were also detected. Sesquiterpene content was found relatively higher in AEO (3.7%), whereas monoterpene content was meager (0.4%). Volatile composition of MREO showed

abundance of furanoeudesm (41.9%) and curcerene (23.9%), considered as marker components of MREO. Besides these, other sesquiterpenoids such as β-elemene (4.8%), δ-elemene (2.1%), germacrene D (1.2%), elemol acetate (1.2%), and β-cudesmol (1.1%) were also identified.

Total ion chromatogram (TIC) of WEO in GC-MS analysis exhibited characteristic peaks corresponding to 29 mono- and sesquiterpenes, contributing 97.7% of the oil. Oxygenated terpenoids (69.7%) formed the major share of the composition; borneol acetate (26.6 ± 1.7%) and β-terpineol (16.2 ± 0.9%) being the most dominant ones. 1,8-Cineol (10.6 ± 0.5%), linalool (6.4 ± 0.4%), sabinene (5.6 ± 0.3%), *o*-cymene (5.1 ± 0.2%), camphor (3.8 ± 0.5%), and α-pinene (2.7 ± 0.2%) were other important terpenes identified in WEO. Sesquiterpenoids

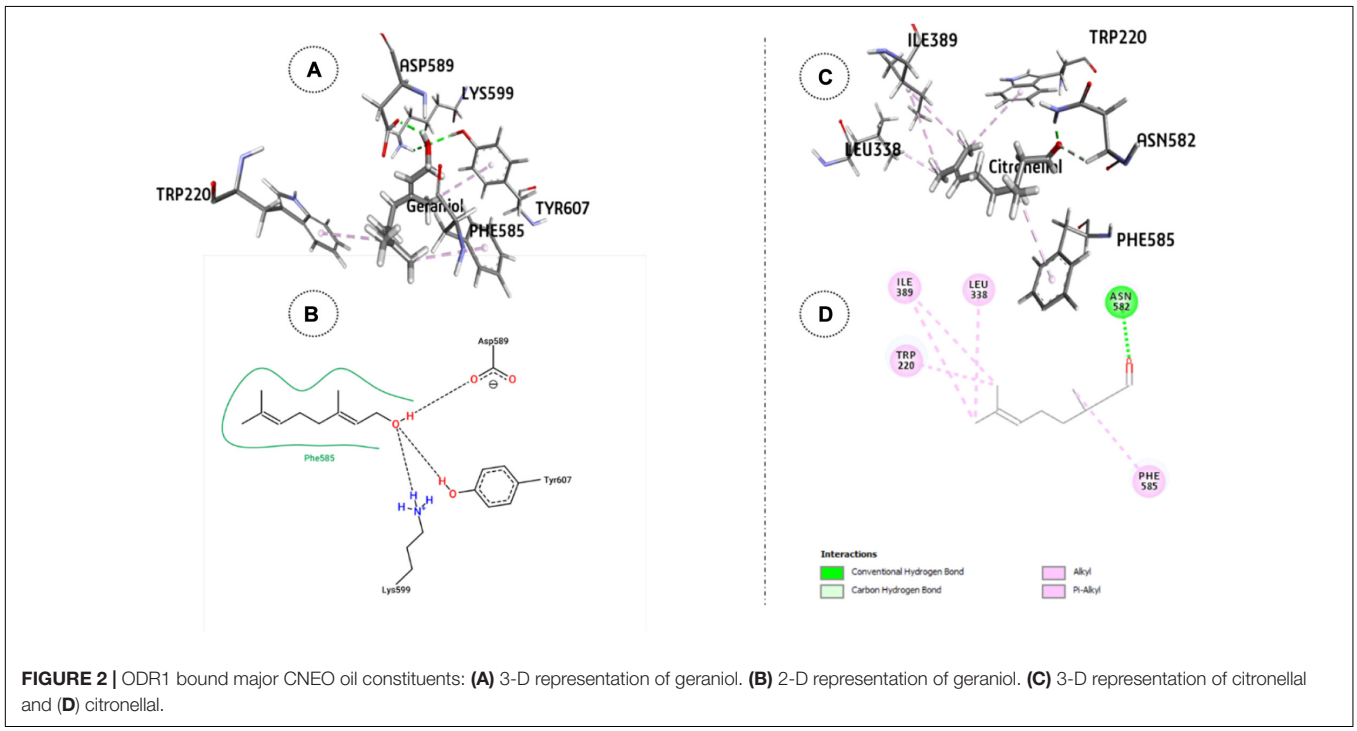

FIGURE 2 | ODR1 bound major CNEO oil constituents: **(A)** 3-D representation of geraniol. **(B)** 2-D representation of geraniol. **(C)** 3-D representation of citronellal and (**D**) citronellal.

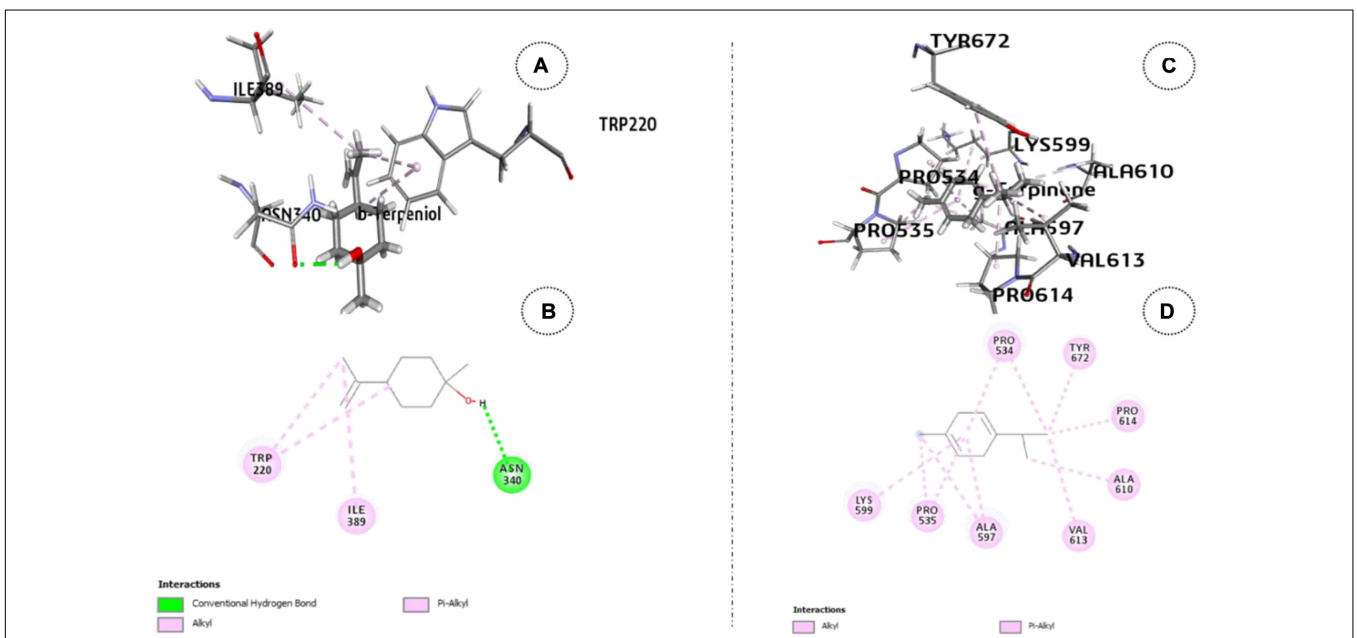

FIGURE 3 | ODR1 bound major TEO oil constituents: **(A)** 3-D representation of β-terpineol. **(B)** 2-D representation of β-terpineol. **(C)** 3-D representation of γ-terpinene and **(D)** 2-D representation of γ-terpinene.

detected in WEO were β-caryophyllene (8.2 ± 1.0%), farnesol (3.0 ± 0.2%), and δ-elemene (2.0 ± 0.3%). Volatile composition of PEO showed various peaks in TIC of GC-MS, representing 15 constituents contributing 97.1% of the oil. Sesquiterpene constituents were highly abundant. Among these, α-guaiene (40.6 ± 1.3%) was the major compound followed by α-bulnesene (14.6 ± 0.9%), α-patchoulene (10.7 ± 0.5%), patchoulol (6.7 ± 0.3%), and β-patchoulene (5.8 ± 0.5%). A comprehensive profile of the chemical composition of the EOs, number of identified compounds, and their group-wise classification is presented in **Table 3**, which described the number of compounds identified along with their content based on functional groups.

Nematicidal Activity of Essential Oils

All the test EOs immobilized more than 50% of juveniles of *M. incognita* at different test concentrations. Antinemic activity of the EOs is depicted in **Table 4**. CNEO exhibited LC_{50} of 325.41, 87.27, and 43.22 μg ml^{-1} concentration after 24, 48, and 72-h exposure, respectively. However, CEO containing a high amount of citronellal showed moderate activity with an LC_{50} of 124.50 μg ml^{-1} after 72 h. OEO rich in *l*-limonene was found to exhibit a comparatively higher nematode toxicity with a lethal concentration LC_{50} of 353.20 μg ml^{-1} within 24 h of J_2 exposure. The nematicidal activity of OEO enhanced with the exposure time, and the highest activity was recorded at an LC_{50} of 79.35 and 39.37 μg ml^{-1} after 48 and 72 h, respectively (**Table 4**).

In this study, TEO and AEO were found effective with an LC_{50} of 76.28 and 85.23 μg ml^{-1} within 72 h, whereas CEO exhibited an LC_{50} of 124.50 μg ml^{-1}. MTEO, however, exerted moderate action with an LC_{50} of 879.40 μg ml^{-1}. The first three Eos, i.e., OEO, CNEO, and TEO, with an LC_{50} (72 h) below 50 μg ml^{-1} except TEO, were subjected to molecular docking analysis, to understand their possible interaction with proteins for nematicidal action.

Molecular Docking Study

Seven receptor proteins (putative target proteins) of *M. incognita* were screened against the biomolecules of OEO, CNEO, and TEO, the three most effective EOs in the present study. Gibb's free energy of binding and other docking parameters of the screened targets are presented in **Table 5**. Bioactivity of OEO, TEO, and CNEO against *M. incognita* J_2s was best explained by the *in silico* inhibition of the odorant response gene 1 (ODR1). The binding pocket of the ODR1 allosteric site is composed of 45 amino acid residues. Screening of the compounds present in the three EOs against the ODR1 gave significantly low binding free energy values ranging from -36.9 to 15.3 kcal mol^{-1}, suggestive of formation of stable protein–ligand complexes.

The relative stability of the docked complexes of 49 ligands (major compounds, >10% present in the OEO, TEO, and CNEO oils) with the ODR1 was computed in terms of ligand efficiency (**Table 5**). It can be seen that the lowest binding energy value for the geraniol–ODR1 complex (-36.9 kcal mol^{-1}), as depicted in **Table 6** may be attributed to the three conventional H-bonds with relatively shorter bond distances (∼2 Å). Additionally, it appeared that the three hydrophobic interactions of π-alkyl type led to further stabilization of the geraniol–ODR1 complex (**Table 7**). Geraniol was bound specifically to the guanylate cyclase catalytic domain of the ODR1 receptor (**Figure 2**).

The citronellal–ODR1 complex with two H bonds (one conventional and one C–H type) and five hydrophobic bonds (three alkyl and two π-alkyl types) (**Figure 2**), exhibited ΔG-29.3 kcal mol^{-1}. In this case, the amino acid residues responsible for the ligand binding interactions belonged to both guanylate cyclase and tyrosine/serine/threonine kinase catalytic domains.

The β-terpineol–ODR1 complex, emerged as the next strongest one with -32.7 kcal mol^{-1} binding energy. One conventional H bond and three hydrophobic bonds (one alkyl type and two π-alkyl types) attributed to complex formation

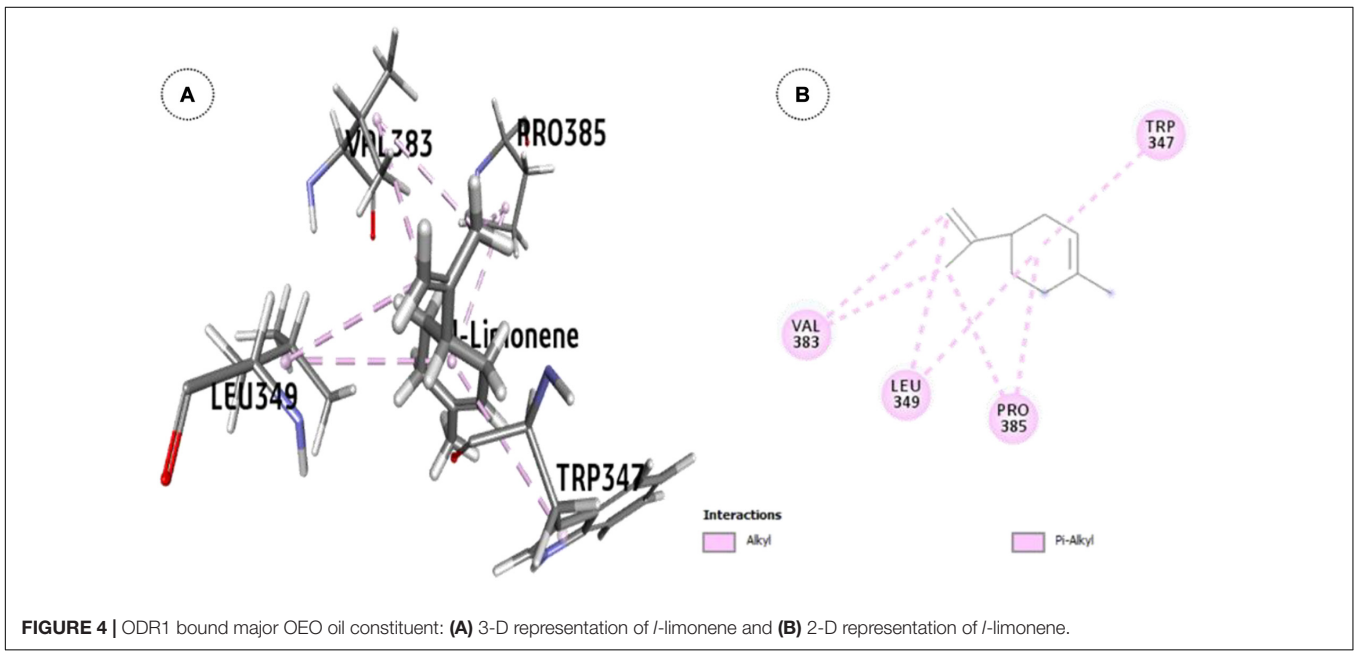

FIGURE 4 | ODR1 bound major OEO oil constituent: **(A)** 3-D representation of *l*-limonene and **(B)** 2-D representation of *l*-limonene.

(**Figure 3**). Here, the specific binding site was tyrosine and serine/threonine kinase catalytic domain.

l-Limonene, the major constituent (93.2% w/w) in OEO showed significant inhibition of the ODR1 gene (ΔG = -25.7 kcal mol^{-1}). The *l*-limonene–ODR1 complex exhibited seven hydrophobic interactions (six alkyl and one π-alkyl type) in between the protein and the ligand (**Figure 4**). Apparently, it was bound to the tyrosine/serine/threonine kinase catalytic domain.

The next major EO constituent showing significantly low free energy of binding was γ-terpinene (ΔG = −24 kcal mol^{-1}, 17.5% in TEO). The γ-terpinene–ODR1 complex showed 10 hydrophobic interactions (nine alkyl and one π-alkyl type). The γ-terpinene molecule bound to the HNOBA and guanylate cyclase catalytic domains.

Based on the observed relative ΔG values of ligand receptor complexes, *l*-limonene ranked fourth (geraniol–ODR1 complex > β-terpineol–ODR1 complex > citronellal–ODR1 complex > *l*-limonene–ODR1 complex > γ-terpinene–ODR1 complex). Inspite of this, the highest observed *in vitro* nematicidal activity of the OEO oil with *l*-limonene could be possible due to the exceptionally high content of *l*-limonene (93.7%). The major constituents in other active EOs was up to about 36% only (**Table 6**).

In order to compare the efficiency of smaller ligands with larger ligands in a non-biased manner, ligand efficiency (LE) calculated for the 49 phytochemical constituents of the three oils varied in the range of 0.8–0.24 kcal mol^{-1} HA^{-1}. Ninety-one percent of the compounds had an LE above the threshold value of 0.3 kcal mol^{-1} HA^{-1}, establishing the discovery of natural leads targeting the ODR1 gene in *M. incognita*. This is the first report on the quantitative binding affinity of the EO constituents toward the ODR1 gene of the root-knot nematode, *M. incognita*, to the best of our information.

DISCUSSION

In the present study, we performed comprehensive chemo-profiling of EOs in order to understand their possible interactions with the target sites of *M. incognita*. The previously investigated reports on OEO suggested the most prominent monoterpene, *l*-limonene, with a range of 32–98% (Zhang et al., 2019; Matuka et al., 2020). Dejam and Farahmand (2017) described MTEO as primarily composed of monoterpenes such as 1,8-cineol, α-pinene, and linalool, which was further confirmed in our study. However, Tunisian MTEO have been reported to be rich in α-pinene (Jamoussi et al., 2005). In our study, α-pinene has been found to be a major component of MTEO. Bioactive terpenic compositions of CEO make it worthy to study on volatile constituents for diverse biological properties. Contrastingly, the oil contains a high amount of citronellal, citronellol, and isopulegol (Singh et al., 2012; Siddique et al., 2013). An earlier report by Madalosso et al. (2017) and Raymond et al. (2017) described the volatile composition of TEO rich in terpinenes, terpinen-4-ol, and methyl eugenol. Our analysis too revealed that TEO comprised of β-terpineol and terpinene. Methyl eugenol, however, was not detected. Our findings on AEO predominantly containing β-asarone have been corroborated by Deepalakshmi et al. (2016). The present study suggested that industrially important MREO, CNEO, WEO, and PEO contained a higher amount of furanoeudesm 1,3 diene, geraniol, myrcene, camphor, and patchoulol, respectively, as reported previously (Buré and Sellier, 2004; Nguyen et al., 2018; Kalaiselvi et al., 2019). Reported variation in chemical profiles of these EOs could be attributed to the plant sources related to locational, seasonal, and climatic factors.

Plant EOs have been described as having great potential in nematode control (Andrés et al., 2012). Oxygenated monoterpenes particularly aldehydes and alcohols have particularly been found effective against *M. incognita* (Echeverrigaray et al., 2010). A similar trend in activity was demonstrated in the case of CNEO comprising an abundance of citronellal and geraniol (Choi et al., 2007). The activity increased both with increasing concentration of EOs and treatment time. Literature also confirmed that EOs containing higher amounts of *l*-limonene usually showed excellent nematicidal potential (Duschatzky et al., 2004). The relative order of nematicidal activity exhibited by the test EOs after a 72-h incubation period, was OEO > CNEO > TEO > AEO > CEO > PEO > WEO > MTEO > MREO.

CONCLUSION

The present study employs analytical and molecular modeling tools to relate the nematicidal activity of potential essential oils and the interactions of their chemical constituents with the target site proteins of the organism. Among the nine essential oils screened against *M. incognita in vitro*, the orange (OEO) and citronella (CNEO) oils were identified in the present work as most effective for immobilization and killing of nematodes. *In silico* analysis suggested a higher binding capacity of geraniol, β-terpineol, citronellal, *l*-limonene, γ-terpinene, to the selected target proteins. Molecular docking-based understanding of the bioactivity of aromatic oils is a novel attempt toward logic-driven selection of natural materials and discovery of biopesticidal leads. The present findings will be further confirmed through wet lab molecular studies and utilized in bionematicide product development.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AS, AdK, AD, and AM conceptualized the study. AdK, AM, AnK, and SD validated the study. AdK, AD, AM, LN, RP, MM, JA, NP, and PS conducted the investigation. AS provided the resources. AdK, AM, AD, and SM wrote and prepared the original

draft. AdK, AM, AD, SS, and RK wrote, reviewed, and edited the manuscript. AS and UR supervised the study. AS acquired the funding. All authors contributed to the article and approved the submitted version.

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Biopesticide Trunk Injection Into Apple Trees: A Proof of Concept for the Systemic Movement of Mint and Cinnamon Essential Oils

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The use of conventional pesticides is debated because of their multiple potential adverse effects on non-target organisms, human health, pest resistance development and environmental contaminations. In this setting, this study focused on developing alternatives, such as trunk-injected essential oil (EO)-based biopesticides. We analysed the ecophysiology of apple trees (*Malus domestica*) following the injection of *Cinnamomum cassia* and *Mentha spicata* nanoemulsions in the tree's vascular system. Targeted and untargeted volatile organic compounds (VOCs) analyses were performed on leaf-contained and leaf-emitted VOCs and analysed through dynamic headspace–gas chromatography–mass spectrometry (DHS-GC-MS) and thermal desorption unit (TDU)-GC-MS. Our results showed that carvone, as a major constituent of the *M. spicata* EO, was contained in the leaves (mean concentrations ranging from 3.39 to 19.7 ng g_{DW}^{-1}) and emitted at a constant rate of approximately 0.2 ng g_{DW}^{-1} h^{-1}. *Trans*-cinnamaldehyde, *C. cassia*'s major component, accumulated in the leaves (mean concentrations of 83.46 and 350.54 ng g_{DW}^{-1}) without being emitted. Furthermore, our results highlighted the increase in various VOCs following EO injection, both in terms of leaf-contained VOCs, such as methyl salicylate, and in terms of leaf-emitted VOCs, such as caryophyllene. Principal component analysis (PCA) highlighted differences in terms of VOC profiles. In addition, an analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) revealed that the VOC profiles were significantly impacted by the treatment. Maximum yields of photosystem II (Fv/Fm) were within the range of 0.80–0.85, indicating that the trees remained healthy throughout the experiment. Our targeted analysis demonstrated the systemic translocation of EOs through the plant's vascular system. The untargeted analysis, on the other hand, highlighted the potential systemic acquired resistance (SAR) induction by these EOs. Lastly, *C. cassia* and *M. spicata* EOs did not appear phytotoxic to the treated trees, as demonstrated through chlorophyll fluorescence measurements. Hence, this work can

be seen as a proof of concept for the use of trunk-injected EOs given the systemic translocation, increased production and release of biogenic VOCs (BVOCs) and absence of phytotoxicity. Further works should focus on the ecological impact of such treatments in orchards, as well as apple quality and production yields.

Keywords: essential oil, biopesticide, *Malus domestica*, trunk-injection, *Cinnamomum cassia*, *Menthas picata*, systemicity

INTRODUCTION

Apple *Malus domestica* Borkh is the most cultivated fruit crop worldwide, reaching a production of 84.7 million tonnes in 2016 and representing a gross product value of US $ 45.8 billion (FAOSTAT). As any other plant, apple trees are subject to abiotic and biotic stresses that cause important economic losses. Apple trees suffer from fungal, viral and bacterial diseases; insects; mites; and nematodes (Kellerhals et al., 2012). The rosy apple aphid, *Dysaphis plantaginea*, and the apple worm, *Cydia pomonella*, are amongst the most serious apple pests (Rousselin et al., 2017), whilst the main diseases are apple scab, powdery mildew, and fire blight caused by the fungi *Venturia inequalis* and *Podosphaera leucotricha* and by the bacteria *Erwinia amylovora*, respectively (Jamar et al., 2010). All these factors can impair production or marketable yields because apples do not fulfil the minimum quality criteria. Currently, the most applied delivery method for pest control is air-blast spray application of pesticides to the tree canopy (Damos et al., 2015). However, pesticide off-target drift can lead to adverse effects on non-target organisms. Over the last 50 years, biodiversity has been reduced by up to 50% in European bird species and by 20–30% in British and German flora (Geiger et al., 2010). Pesticides can cause environmental contamination and risks for human health through excessive residues on the fruit (Damalas and Eleftherohorinos, 2011). Additionally, pests can develop resistance to these pesticides, which usually contain a single active molecule (Alins et al., 2017). Altogether, this suggests that the plant protection product (PPP) mode of application selection is an economic and ecological challenge around the world. As a result of the negative perception of synthetic pesticides, causing negative effects on human health during and after application, and fears of their excessive residues in or on fruit, consumer demand for agricultural products without synthetic pesticide residues from excessive phytosanitary treatments has increased. This is why alternative solutions have been investigated, such as biological pesticides or biopesticides. An abundant body of literature is published each year concerning the prospect of plant essential oils (EOs) as active ingredients in the production of biopesticides (Campos et al., 2019).

The International Organisation for Standardisation (ISO) defines an EO as a "product obtained from vegetable raw material, either by distillation with water or steam, or from the epicarp of citrus fruits by a mechanical process, or by dry distillations." Due to their biological activity, they have long been applied in cosmetics, therapeutics, and food applications (Hüsnü Can Başer and Buchbauer, 2015). The composition of EOs is highly variable and comprises a tremendous diversity of compounds. However, most of them belong to the terpenoids (mono- or sesqui-) or phenylpropanoids class of compounds, both of which have high lipophilicity and volatility, especially at room temperature. The secondary metabolites of EOs originate from methylerythritol phosphate and phenylalanine pathways (Rehman et al., 2016).

Some of the volatile organic compounds (VOCs) contained in EOs play a major role in plant defence mechanisms against bacteria, fungi, viruses, and herbivores (Bakkali et al., 2008). Therefore, much research has been performed to integrate these antibacterial, fungicidal, and insecticidal EOs as alternatives for sustainable agronomic practices, limiting environmental and health hazards. Indeed, due to their rapid degradation and since they are generally recognised as safe (GRAS), they represent an interesting alternative application of most synthetic conventional pesticides (Koul et al., 2008). Two EOs were used in this study:cinnamon EO (*Cinnamomum cassia* J. Presl) and mint EO (*Mentha spicata* L.). They both present well-documented biopesticidal activity (Singh and Pandey, 2018; De Clerck et al., 2020) due to their insecticidal and fungicidal (Muchembled et al., 2018; Lee et al., 2020) properties, which have already led to commercial product development (Isman et al., 2011; Isman, 2020).For example, mint EO has presented an inhibition concentration between 24 and 83 mg L^{-1}on apple scab, depending on the strain (Muchembled et al., 2018). *C. cassia*, on the other hand, possesses a lethal dose 50 of 17.41 µl ml^{-1} on aphid *Myzus persicae* (Ikbal and Pavela, 2019).

Nevertheless, particular attention must be paid to the formulation of EO-based pesticides (Aćimović et al., 2020). A well-studied formulation could, on the one hand, counter the high volatility of EOs and ensure the prolonged release of the active substance and, on the other hand, attenuate potential phytotoxic effects (Moretti et al., 2002; Maes et al., 2019). EOs can impact many plant physiological processes (water status alteration, membrane integrity, respiration, and photosynthesis inhibition) through diverse modes of action, such as reactive oxygen species (ROS) induction and enzymatic or phytohormone regulation (Werrie et al., 2020). In this regards, chlorophyll fluorescence has been proven useful to evaluate plant vitality and response to abiotic stress (Kalaji et al., 2016). The application of EOs in apple tree may lead to phytotoxicity depending on the application method, concentration, and adaptive duration. For example, 7% of flowers were injured for clove oil in a thinning experiment for concentrations as low as 2% (Miller and Tworkoski, 2010). Fruit damages were also reported in postharvest treatment with savory, oregano, and thyme EOs at concentrations of 1–10% for the purpose of controlling *Botrytis cinerea* and *Penicillium expansum* (Lopez-Reyes et al., 2010). Nevertheless, fruit damage was not observed with

thermal fogging treatment of lemongrass and citrus EOs at a concentration of 0.125% to control *B. cinerea* (Mbili et al., 2017). Therefore, the mode of EO application, the formulation and the selection of the active substance must be adapted for specific purposes and carefully evaluated.

Trunk injection is a method of applying chemicals directly to the vascular system of the tree after bark piercing, and the chemicals are then distributed systemically through the xylem tissue. This application method directly targets pests whilst reducing environmental exposure to pesticides and input quantities (Doccola and Wild, 2012; Wise et al., 2014). It has recently been experimented to fight fungi, such as apple scab, *Venturia inaequalis*, and powdery mildew, *Podosphaera leucotricha*, with disease severity reductions of 22–55 and 41.8–73.5% depending on the season and the product considered (potassium phosphites and synthetic fungicides) (Percival and Boyle, 2005; Aćimović et al., 2016b). A similar experiment on insect species [codling moth *Cydia pomonella* (L.), rosy apple aphid *Dysaphis plantaginea* (Passerini) and green apple aphid *Aphis pomi* (Passerini)] reported up to two seasons of control after a single injection of imidacloprid or emamectin benzoate (Wise et al., 2014). Spatial and temporal distributions of imidacloprid in leaves have been investigated (Aćimović et al., 2014), as well as residues to nectar and pollen, which were below the Environmental Protection Agency (EPA) threshold of 25 ng g^{-1} for imidacloprid (Coslor et al., 2019). Although the management of the injection timings may help to keep residue under the toxic limit, systemic resistance inducers have also been explored with the injection of acibenzolar-S-methyl (ASM) to induce systemic acquired resistance (SAR) and to control fire blight (Aćimović et al., 2015).

In the present study, we aimed to determine the distribution of trunk-injected EOs in young apple trees, thus proving their systemic movement by quantifying target VOCs, both within leaves and by aerial emissions. We also determined the impact of injected EOs on tree physiology by monitoring chlorophyll fluorescence and untargeted VOCs.

MATERIALS AND METHODS

Essential Oils

The cinnamon EO (*C. cassia* J. Presl) and mint EO (*M. spicata* L.) used in this study were purchased from Pranarôm (Pranarôm & Herbalgem, Ghislenghien, Belgium). Before formulation of the EOs, the oil composition was analysed by gas chromatography associated with mass spectrometry (GC-MS). These analyses were carried out on a 7890A-5975C GC-MS equipped with an HP-5MS 30 m × 0.25 mm × 0.25 μm capillary silica column (Agilent Technologies Inc., Santa Clara, United States). The operating conditions were the following: helium flow of 1.0 ml min^{-1}; the oven temperature was programmed at 40°C for 2 min, increased to 100°C at a rate of 5°C min^{-1}, increased to 120°C at a rate of 3°C min^{-1}, held for 3 min, increased to 220°C at a rate of 5°C min^{-1}, and finally increased to 310°C at a rate of 15°C min^{-1}. One microliter of a 1 mg ml^{-1} EO solution in hexane (HPLC grade, Merck KGaA, Darmstadt, Germany) was injected in splitless mode. The injector, quadrupole and MS temperatures were 250, 150, and 230°C, respectively. The mass spectrometer (MS) ran in electron impact (EI) mode at an electron energy of 70 eV. Mass spectra were acquired in the range of 30–400 atomic mass units (amu).

Emulsion Formulations

To facilitate injection and diffusion of EOs in the tree vascular tissue, a water-soluble, stable, and homogenous EO emulsion was prepared. To prepare 100 ml of the 0.5% (v/v) EO/water emulsion, 2 ml of Tween 80 (CAS 9005-65-6, Merck KGaA, Darmstadt, Germany) and 20 ml of 100 mM ethylene diaminete traacetic acid (EDTA) (Titriplex III, Merck KGaA, Darmstadt, Germany) solution were added to 15 ml of water under constant agitation at 1,250 rpm. Water was then added to bring the final volume to 100 ml. After 5 min under constant agitation, the solution was then stabilised by high-speed homogenisation for 6 min at 9,500 rpm (Ultra-Turrax T25, IKA WERKE, Staufenim Breisgau, Germany) and by high-pressure homogenisation with eight cycles at 5,000 psi (FMC, Philadelphia, United States). The emulsion stability was checked by analysing the EO particle sizes and distribution in solution with a particle sizer (Beckman Coulter Delsa^{TM} Nano C Particle Analyser, California, United States).

Biological Material

Experiments were performed on 2-years-old apple trees (*M. domestica* Borkh, cv "Jonagold" grafted on M26 rootstock) obtained locally (Serres de Sauvenières, Gembloux, Belgium). The trees were 155 ± 15 cm high and presented a trunk diameter of 2 ± 0.2 cm above the graft union. During the experimental phase, the plants were placed in an environmental chamber with controlled environmental conditions [21 ± 0.5°C, 62 ± 10% relative air humidity and 16:8 h light/dark periods and photosynthetically active radiation (PAR) of 50 μmol m^2 s^{-1}]. Plants were watered every day with 500 ml of water. They developed fully expanded leaves but were free of flowers or fruit.

Trunk Injection System

The trees used in the experiment were drilled right above the grafting union with holes that were 1 mm wide and 1 cm deep. Three trunk injection ports per tree trunk were created and were positioned at an equal distance from each other (each 120° of trunk radius). Each injection port was slanted upward at a 60° angle in relation to the trunk axis (**Figure 1**). Needles (BD vacutainer® safety lock 23G, Becton Dickinson, New Jersey, United States) were inserted into the ports and connected on the other side to drip bags (Baxter®, Baxter International Inc., Deerfield, United States) filled with the solution injected (**Figure 1**). Four different treatments were tested using three biological replicates over a period of 96 h. The first two modalities were treated with EO emulsions (one with cinnamon oil and the other with mint oil), the third was a negative control (emulsion exempt of EOs) and the fourth was a blank treatment (no injection). To avoid cross-contamination, the treatments were delivered separately from each other at different times.

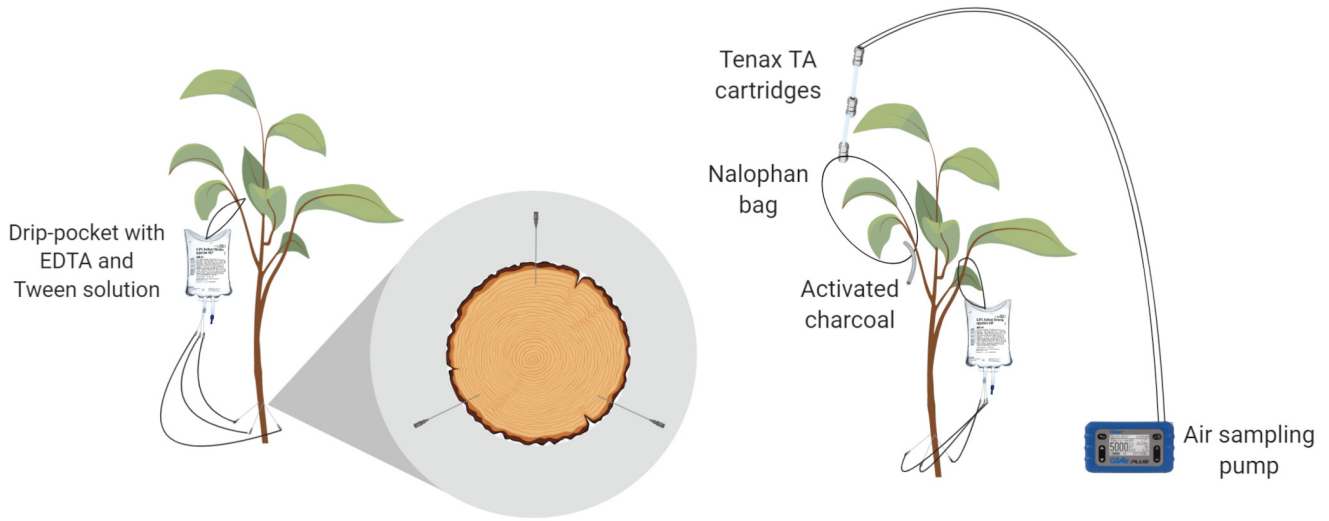

FIGURE 1 | Laboratory trunk-injection device **(left)** and sampling of leaf-emitted volatile organic compounds (VOCs) **(right)**.

Treatments were applied on different trees each time with a chamber ventilating period of 2 days.

Volatile Organic Compound Sampling by Headspace Techniques

Leaf-Contained VOCs

Ten leaves were homogenously sampled at t = 0, 24, 48, 72, and 96 h on each replicate tree. Sampling was performed by cutting the leaves at their base and dipping them into liquid nitrogen before storage at -80°C prior to dynamic headspace–gas chromatography–mass spectrometry (DHS-GC-MS) analysis. A dry weight (DW) measurement was performed at the end of the experiment at 60°C until constant weight to obtain content results in ng g_{DW}^{-1}.

Leaf-Emitted VOCs

The headspace was sampled following the protocol for the volatile collection of aphid-infested leaves from an apple tree (Stewart-Jones and Poppy, 2006). Briefly, two Tenax TA® 60/80 cartridges (Camsco©, Houston, United States) were attached to an inert polyethylene terephthalate (PET) bag (Nalophan®, Odometrics, Arlon, Belgium) enclosing a single branch. The trapping of emitted VOCs was performed by constant air sampling of 50 ml min^{-1} using a Gilian air sampling pump (Sensidyne®, St. Petersburg, United States) attached to the other side of the cartridges (**Figure 1**). Briefly, air enters the bag through the activated charcoal tube, loads in the VOCs and exits the bag through the Tenax TA cartridges, which capture VOCs. The bag and its connected cartridges were set up on each tree (n = 3) at t = 0 h. The cartridges were then replaced at t = 24, 48, 72, and 96 h and stored at −80°C prior to the GC-MS analysis. At the end of the experiment, all leaves enclosed in the bag were sampled and weighed. A DW measurement was also performed on these leaves at 60°C until constant weight to obtain results in ng g_{DW}^{-1} h^{-1}.

VOC Analysis: Sample Preparation and GC-MS Analysis

Leaf-contained VOCs were analysed by DHS-GC-MS. Before dynamic headspace sampling (DHS), the leaves were ground (A11 basic grinder, IKA WERKE, Staufenim Breisgau, Germany) with liquid nitrogen. Then, 1 g of freeze-grinded leaves was put in a 20 ml screw cap vial (Gerstel©, Mülheiman der Ruhr, Germany), and 2 ml of a 20% (w/v) NaCl solution was added to create a salting out effect (Liberto et al., 2020). Afterward, the sealed vial was incubated in the dynamic headspace system at 35°C for 20 min (automated dynamic headspace DHS, Gerstel©, Mülheiman der Ruhr, Germany). The headspace was then dynamically transferred to a Tenax TA cartridge by applying 1,200 ml of nitrogen at a flow of 30 ml min^{-1}. The cartridge was then drypurged at 50 ml min^{-1} for 4 min. The cartridge was then sent to the thermal desorption unit (Thermal Desorption Unit TDU 2, Gerstel©, Mülheiman der Ruhr, Germany) for GC-MS analysis. The thermal desorption parameters used were the same as those described below for leaf-emitted VOCs. Tenax TA® porous polymers, based on 2,6-diphenyl-p-phenylene oxide, are widely used as an adsorbent in purge trap applications and plant headspace analysis due to their high versatility.

Leaf-emitted VOCs were analysed by TDU-GC-MS. Before thermal desorption, 1µl of 0.4 mg ml^{-1} 1-phenyloctane (CAS 2189-60-8, Merck KGaA, Darmstadt, Germany) in hexane was added to the cartridge by a multipurpose sampler (Multi-Purpose Sampler MPS, Gerstel©, Mülheiman der Ruhr, Germany). The addition of 1-phenyloctane as an internal standard (IS) allowed for semiquantification of the VOCs present on the cartridge. The VOCs were then thermally desorbed in the TDU and cryofocused in the cooled injection system (CIS) (Gerstel©, Mülheiman der Ruhr, Germany). The TDU temperature program was 40°C for 1 min and was increased to 280°C at a rate of 100°C min^{-1} and held for 5 min. CIS was mounted

with a baffled glass liner and operated in solvent vent mode, and the temperature program was -60°C for 0.10 min, which was increased to 250°C at a rate of 12°C s^{-1} and held for 2 min following existing protocols (Delory et al., 2016; Durenne et al., 2018).

The analyses were carried out on a 7890A-5975C GC-MS equipped with an HP-5MS 30 m × 0.25 mm × 0.25 µm capillary silica column (Agilent Technologies Inc., Santa Clara, United States). The operating conditions were the following: helium flow of 1 ml min^{-1} and oven temperature 40°C for 2 min, which was increased to 220°C at a rate of 5°C min^{-1} and finally increased to 310°C at a rate of 15°C min^{-1} and held for 3 min. The quadrupole and MS temperatures were 150 and 230°C, respectively. The MS ran in EI mode at an electron energy of 70 eV. Mass spectra were acquired in the range of 30–400 amu.

For untargeted analysis, identification was based on comparison of the obtained spectra with the reference mass spectra from the NIST 17, Wiley 275 and pal 600 databases. Moreover, experimental retention indexes (RIs) were calculated using C_7–C_{30} solutions and compared to literature RIs. Technical grade standards were injected to ensure identification (Nea et al., 2019; Tanoh et al., 2020). Semiquantification was performed using the following formula:

$$Compound\ A\ \ concentration = \frac{compound\ A\ area}{IS\ area} * IS\ concentration$$

Detection and quantification of the major compounds of EO were performed in single-ion monitoring (SIM) mode. Based on the characterisation of the selected EOs, calibration curves in TDU-GC-MS using pure standards were established for each major component of the EO: (+)-carvone (CAS 2244-16-8, 99.9% purity, Supelco©, Missouri, United States) for mint and *trans*-cinnamaldehyde (CAS 14371-10-9, ≥99% purity, Merck KGaA, Darmstadt, Germany) for cinnamon oil. The 6-point calibration curves were established by injecting 1µl of the standard solution in hexane (Merck KGaA, Darmstadt, Germany). For (+)-carvone, ions 108 and 93 were selected as qualifiers, and ion 82 was selected as he quantifier. A calibration curve ($y = 0.527x+0.020$, $R^2 = 0.985$) was established in triplicates between 1.50 and 861.05 µg ml^{-1}. For *trans*-cinnamaldehyde, ions 132 and 103 were used as the qualifier, and ion 131 was used as the quantifier. A calibration curve ($y = 0.628x+0.018$, $R^2 = 0.989$) was established in triplicates between 0.623 and 954.50 µg ml^{-1}. The IS 1-phenyloctane was also used at a concentration of 400 µg ml^{-1}.

Chlorophyll Fluorescence Measurements

The potential phytotoxic effect of EOs on the photosynthetic efficiency of plants was evaluated by estimating the maximum quantum efficiency of photosystem II (Fv/Fm) with a fluorimeter (Handy PEA+, Hansatech Instruments Ltd., Norfolk, United Kingdom). For a healthy sample, this ratio is around 0.83 and lowers as plant stress increases, reaching 0.3 at the end of senescence (Bresson et al., 2018). Moreover, the maximum quantum yield of photosystem II has been used to evaluate foliar response after EO application (Synowiec et al., 2015, 2019). Measurements were performed at the same time of day for each time considered (t = 0, 24, 48, 72, and 96 h for each modality tested). Fv/Fm was assessed on three leaves randomly selected on each tree. Before the measurement, the leaves were dark-adapted for 20 min using leafclips. Fv/Fm measurements

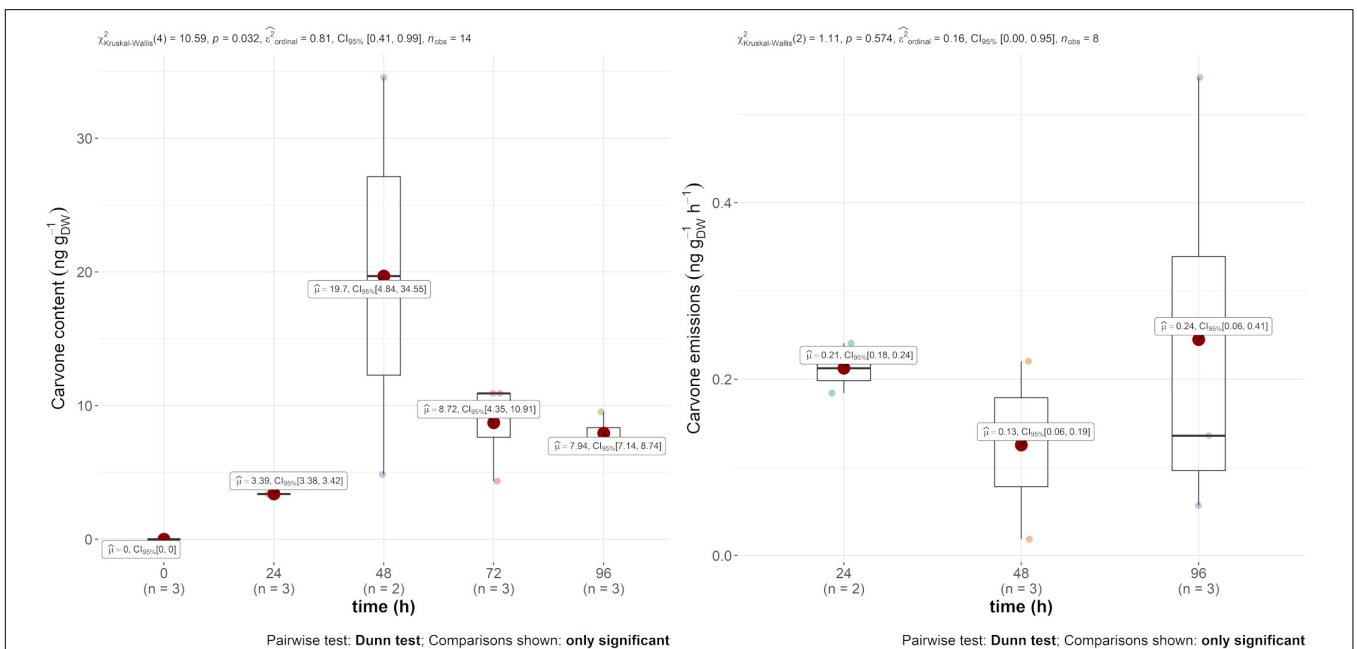

FIGURE 2 | Boxplot of D-carvone contained (in ng g_{DW}^{-1}) in the leaves (left) and in the emissions (ng g_{DW}^{-1} h^{-1}) (right) over time after injection.

were then performed by exposing the leaves to light intensity of 3,000 μmol m^{-2} s^{-1}.

Statistical Analysis

The results from the targeted VOCs were visualised, and detailed nonparametric statistical analysis (Kruskal–Wallis test and Dunn's test) was generated in Rstudio with ggstatplots (Patil, 2018). The untargeted VOC profiles, either contained or emitted, underwent several statistical analyses to understand the impact of the treatments performed on the apple trees. First, one-way analysis of variance (ANOVA) was performed for each VOC present in the profiles to understand which of them were significantly different between treatments using Tukey's *post hoc* test. Descriptive statistics were coupled with principal component analyses (PCA) and heatmaps to visualise treatment effects and which VOCs they impact. All of these analyses were performed with metaboanalyst[1] (Pang et al., 2020). Analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) were performed between the different treatments. PERMANOVA tests the simultaneous response of one or more variables to one or more factors based on a similarity/distance matrix with permutation methods (Anderson, 2017). The ANOSIM and PERMANOVA were calculated in Rstudio (R 3.5.2 software, R Development Core Team, Boston United States) using the VEGAN package. ANOSIM and PERMANOVA were performed to establish if the contained and emitted VOC profiles were significantly impacted by the treatment. For fluorescence measurements, two-way repeated measures ANOVA was performed on the Fv/Fm dataset with treatments and time as a factor, followed by the pairwise *t*-test. A probability cutoff of $\alpha = 0.05$ was applied for tests of significance in all statistical analyses and adjusted with the Bonferroni correction.

[1]www.metaboanalyst.ca

RESULTS

Essential Oil Compositions and Formulations

GC-MS analysis of the EOs demonstrated that *C. cassia* oil was composed of 91.22% *trans*-cinnamaldehyde, and *M. spicata* was mainly composed of carvone (57.78%) and limonene (25.28%). A detailed composition can be found in the **Supplementary Tables 1, 2**. The EO compositions are similar to those reported before (Snoussi et al., 2015; Zhang et al., 2019). A stable nanoemulsion had a mean particle size diameter below 200 nm and a polydispersion index < 0.2.

VOCs Spectra Analysis

Targeted Essential Oil Compounds

Regarding mint EO, the main compound, carvone, was found in both the emission and in the leaves, as displayed in **Figure 2**. The emission rate into the air was constant throughout the experiment at around 0.2 ng g_{DW}^{-1} h^{-1}. The leaf content, however, was more variable within and between 24 and 48 h. Indeed, the carvone content varied between 3.39 and 19.7 ng g_{DW}^{-1}, with a maximum 2 days after injection. However, as this compound was not found in the other treatments of the experiment, it demonstrates the systemic translocation of the trunk-injected mint EO.

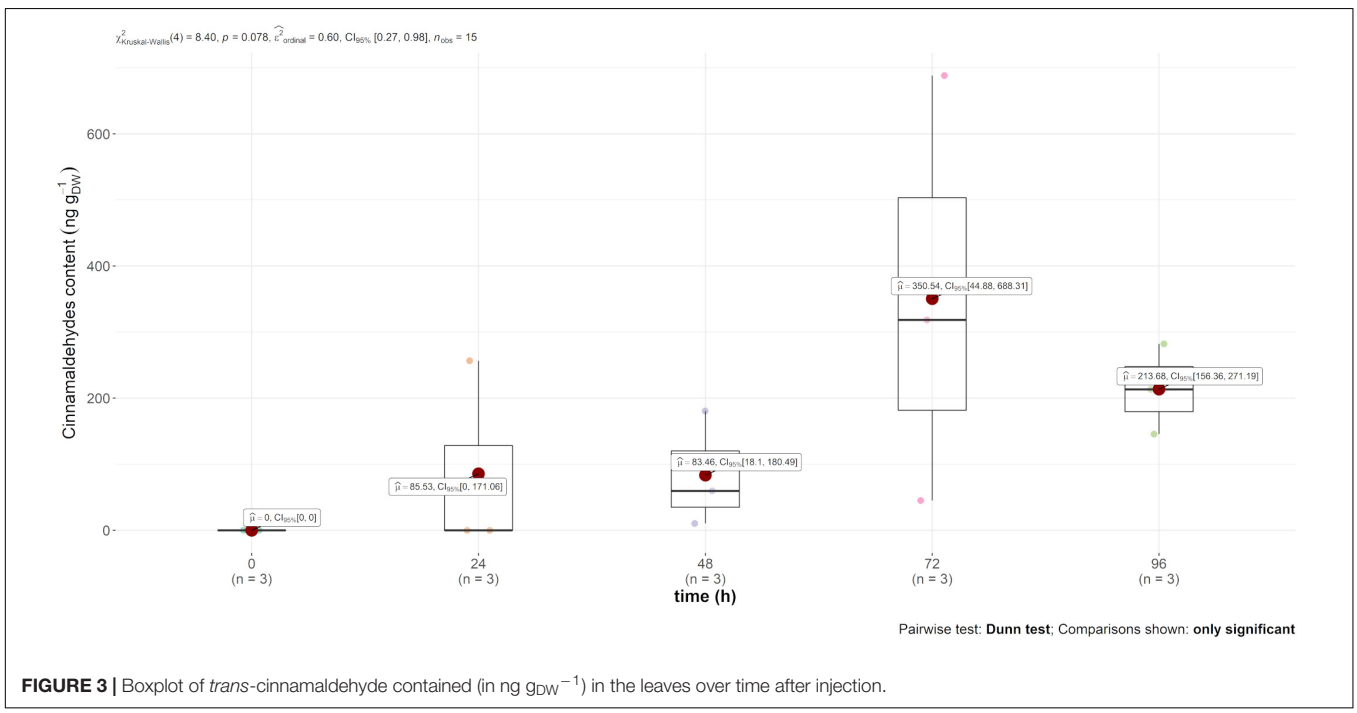

FIGURE 3 | Boxplot of *trans*-cinnamaldehyde contained (in ng g_{DW}^{-1}) in the leaves over time after injection.

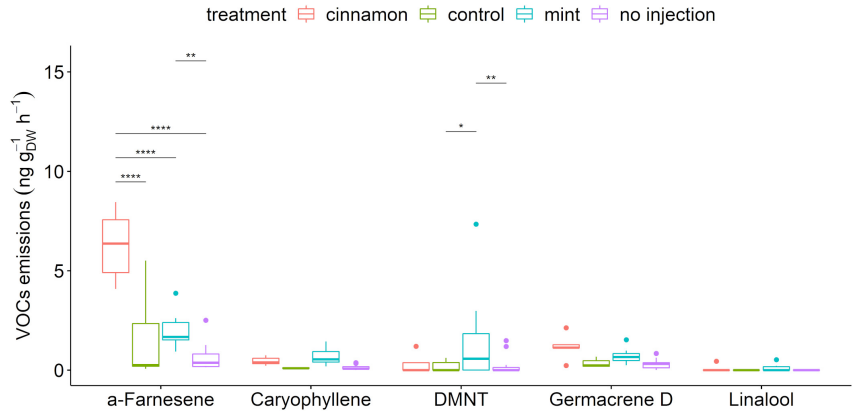

FIGURE 4 | Boxplot of a selection of *Malus domestica* volatile organic compounds (VOCs) emitted (ng g_{DW}^{-1} h^{-1}) from plants injected with essential oils (EOs) and the control. The star symbols above the bars indicate a significant difference between the means ($P < 0.05$).

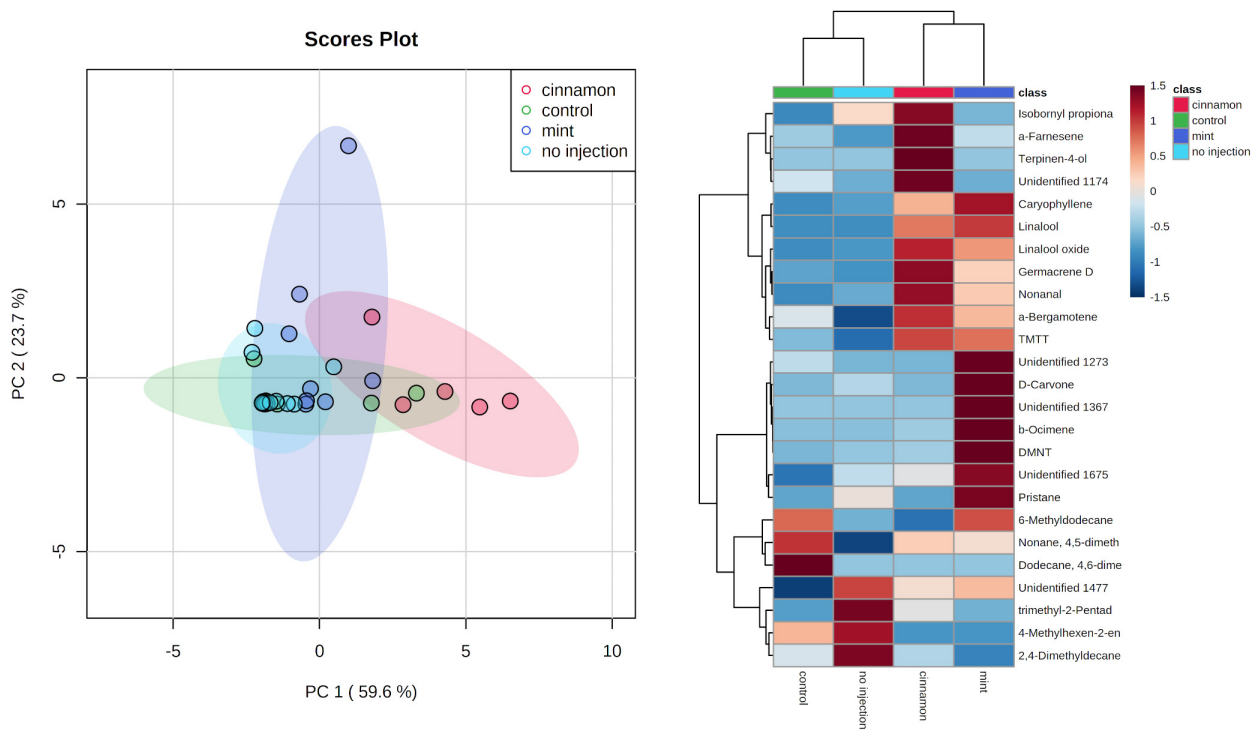

FIGURE 5 | Principal component analysis (PCA) **(left)** and heatmap of the top 25 contributors merged by group **(right)** of *Malus domestica* volatile organic compound (VOC) emissions generated on metaboanalyst after data processing.

Trans-cinnamaldehyde, the main compound of cinnamon EO, was only recovered in the content of the leaf (and not in the air emission). However, this content was much higher in comparison to carvone, i.e., mint EO content, as observed in **Figure 3**, reaching 350 ng g_{DW}^{-1} 72 h after injection.

Untargeted VOCs Emitted (TDU-GC-MS)

A total of 56 compounds were detected in the headspace emissions profiles of *M. domestica* trees belonging to the alkanes, alkenes, alcohols, aldehydes, aliphatic and aromatic esters, furanes, homoterpenes, ketones, monoterpenes, sesquiterpenes, and terpenoids (**Supplementary Table S3**). A selection of biogenic VOCs (BVOCs) that have a major biological role in the environment, such as pest attractant, attraction of pest-killing parasitic wasps, antennal response elicitor, or herbivory-induced plant volatile (Gershenzon and Dudareva, 2007; Hare, 2011; Souza et al., 2017), is presented in **Figure 4**. The apple trees that we injected with both EOs emitted the largest

TABLE 1 | Pairwise permutational multivariate analysis of variance (PERMANOVA) comparisons for volatile organic compounds (VOCs) emissions between treatment.

	No injection	Cinnamon	Control
Cinnamon	0.006**	–	–
Control	0.400	0.013*	–
Mint	0.006**	0.006**	0.253

*The asterisks indicate significant differences: *P ≤0.05, **P ≤ 0.01.*

amounts of caryophyllene, linalool and germacrene D and significantly larger amounts of α-farnesene and (E)-4,8-dimethyl-nonatriene (DMNT).

Multivariate analysis of the emitted VOC profiles performed by PCA captured 83.3% of variance in the first two dimensions (**Figure 5**). VOC profiles of EO-injected trees separated well from the control and no injection treatment. On the other hand, as can be observed in the heatmap (**Figure 5**), some compounds are produced for both oils, such as caryophyllene, germacrene D, bergamotene, (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) and linalool, whereas some of them are specific to a particular oil. Indeed, cinnamon-oil-injected trees emitted more terpinen-4-ol, α-farnesene, and trees injected with mint oil emitted more DMNT and β-ocimene. Amongst the compounds previously mentioned, linalool, germacrene D and terpinen-4-ol are found in mint EO, and caryophyllene is found in cinnamon EO, but as minor compounds at concentrations below 1%.

The VOC emission profiles were significantly impacted by the treatment. ANOSIM revealed significant structural differences for VOC profiles between treatments, with some overlapping (R = 0.281, p = 0.002). On the other hand, PERMANOVA performed on the same data set revealed similar outcomes for comparisons between treatment (F = 3.9517, p = 0.001***). Pairwise PERMANOVA yielded significant differences for multiple comparisons in all cases, except for the no injection-control and mint-control, as shown in **Table 1**.

Untargeted VOCs Contained (DHS-GC-MS)

A total of 67 compounds were detected in the VOCs contained within leaves. These compounds belong to the alcohols, aldehydes, alkadienes, alkanes, aromatic and aliphatic esters, fatty acid esters, homoterpenes, and ketones (**Supplementary Table 4**). Injection of EOs significantly increased methyl salicylate, benzaldehyde, benzeneacetaldehyde, β-ionone, and nonanal (**Figure 6**). Amongst those compounds, only benzaldehyde was found in the cinnamon EO but also as a minor compound below 1%.

VOC profiles for EO-treated trees were much more dispersed in comparison to the control and no injection treatments (**Figure 7**). As for the emitted VOCs, it seems from the heatmap that some compounds increased for both oil treatments, such as decanal, caryophyllene and 1-penten-3-ol. Some increases were specific, such as numerous aldehydes for cinnamon oil (2-heptenal, 2-nonenal, 2,4-hexadienal) and terpenes for mint oil (α-terpineol, eucalyptol, β-homocyclocitral). With regard to the EO composition, only α-terpineol was found in trace amounts within the mint EO at 0.25%.

ANOSIM revealed significant differences for VOC profiles between treatments, with some overlapping between group (R = 0.2712, p = 0.001). PERMANOVA analysis of the same dataset revealed similar outcomes for comparisons between treatments (F = 7.3673, p = 0.001***). Finally, pairwise PERMANOVA revealed significant pairwise differences between all treatments, except for the control–no injection and cinnamon–mint, as shown in **Table 2**.

Chlorophyll Fluorescence

Maximum yields of photosystem II (Fv/Fm) over time are presented in **Figure 8**. Chlorophyll fluorescence showed that most values were located between 0.80 and 0.85, implying that the trees maintained good ecophysiological performances throughout the experiment (**Figure 8**). Two-way repeated measure ANOVA revealed a significant impact of factors (treatment: F = 4.759, p = 0.003, ges = 0.082; day: F = 4.782,

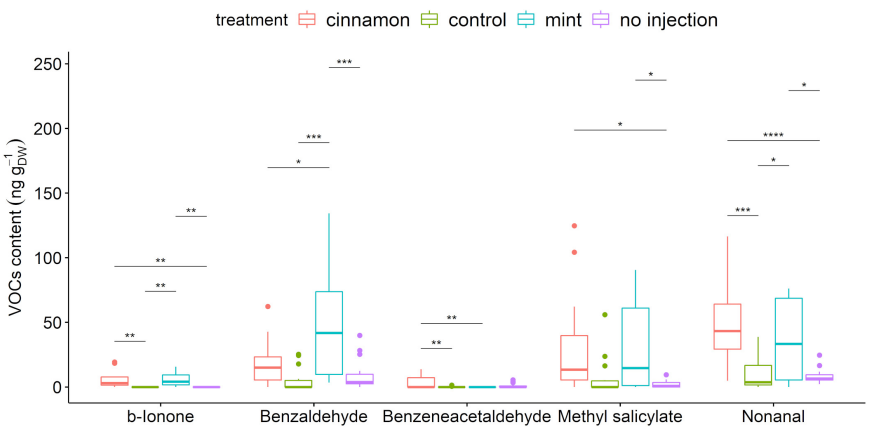

FIGURE 6 | Boxplot of a selection of *Malus domestica* volatile organic compounds (VOCs)content (ng g_{DW}^{-1}) from plants injected with mint and cinnamon essential oils (EOs) and the control. The asterisk symbols above the bars indicate a significant difference between the means (P < 0.05).

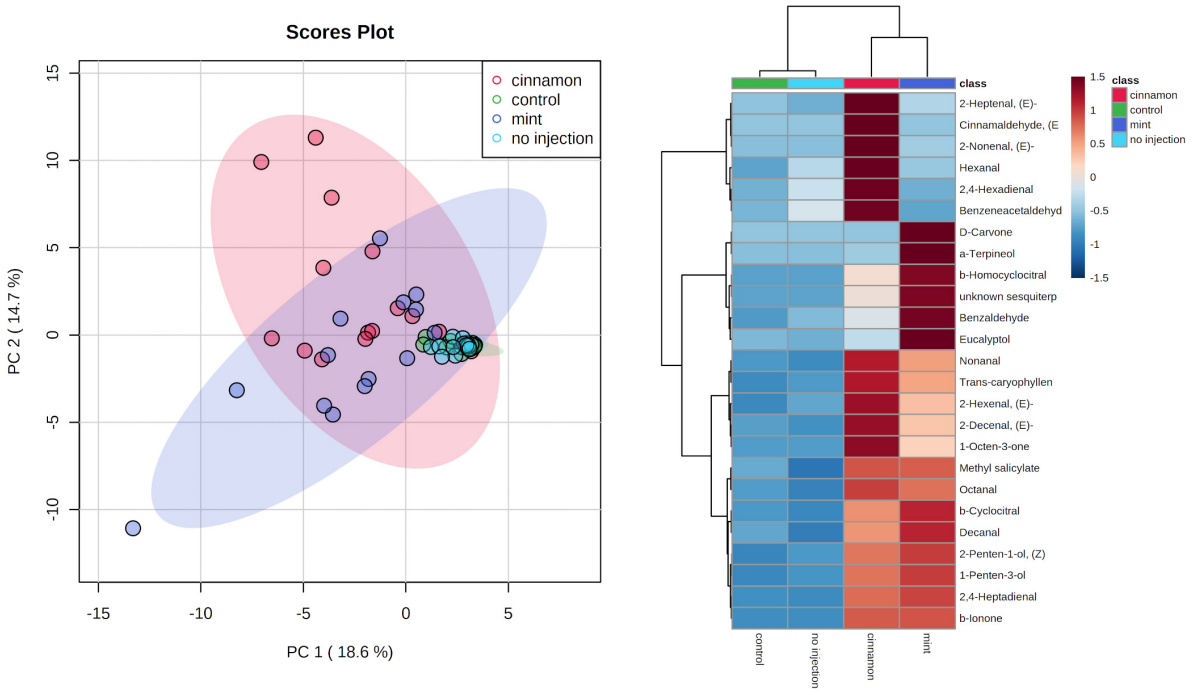

FIGURE 7 | Principal component analysis (PCA) (left) and heatmap of top 25 contributors merged by group (right) of *Malus domestica* volatile organic compounds (VOCs) contained generated on metaboanalyst software after data centring processing.

TABLE 2 | Pairwise permutational multivariate analysis of variance (PERMANOVA) comparisons for volatile organic compounds (VOCs) contained between treatments.

	No injection	Cinnamon	Control
Cinnamon	0.006**	–	–
Control	0.585	0.006**	–
Mint	0.016*	0.151	0.022*

*The asterisks indicate significant differences: *P ≤ 0.05, **P ≤ 0.01.*

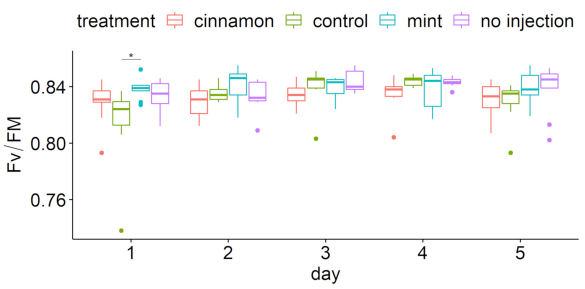

FIGURE 8 | Maximum quantum yield of photosystem II (Fv/Fm) boxplot per treatment during time after injection. The star symbols above the bars indicate a significant difference between the means ($P < 0.05$).

$p = 0.001$, ges = 0.107) without interaction ($F = 1.59$, $p = 0.099$, ges = 0.107). Pairwise comparison demonstrated significant differences only at day 1 between treatment (**Figure 8**) and only for the control between 24 and 48 h and 24 and 96 h.

DISCUSSION

Taken altogether, our results demonstrate, for the first time, the systemic translocation of trunk-injected EOs in apple plants. Carvone increased in the leaf content and was emitted at a constant rate, and *trans*-cinnamaldehyde content increased in the leaves but was not found in detectable amounts in the air emissions. The strong spatial heterogeneity combined with the relatively small sampling may also contribute to the variability of the results. However, it appears that the EO translocation within apple tree tissues and its diffusion in ambient air must be conditioned by its own physicochemical properties. Amongst those properties, vapour pressure, organic carbon–water partitioning coefficient (Ko/c), and the octanol water partition coefficient (Ko/w) may explain the differences observed between carvone and *trans*-cinnamaldehyde (Doccola and Wild, 2012; Montecchio, 2013; Aćimović, 2014). Out of these two, *trans*-cinnamaldehyde was the molecule with the smallest vapour pressure of 15.3 and 3.853 Pa at 25°C for carvone and *trans*-cinnamaldehyde, respectively (European Chemical Agency, 2020). This molecule, following Henry's law, has a smaller tendency to volatilise and hence accumulates in the leaves. Moreover, from a histological point of view, they diffuse slowly through aqueous phases in the mesophyll, lipid bilayer membranes and internal airspace (in the substomatal cavity) before release through the stomata (Calfapietra et al., 2013). This diffusion is conditioned following the compounds' octanol water partition coefficients (Ko/w), which are 2.7 for carvone and 2.1 for *trans*-cinnamaldehyde. It is worth

mentioning that other phenomena could concurrently take place, such as the potential transformation or degradation of these xenobiotic compounds by the apple plants. Diverse mechanisms such as reduction/oxidation, esterification or conjugation with carbohydrates (glycosylation) or glutathione (glutathionylation) were demonstrated *in planta* for numerous GLVs and terpenes (Matsui et al., 2012; Rivas et al., 2013) and by diverse microorganisms (Asakawa et al., 2018). This was specifically demonstrated for *Arabidopsis* aldehydeoxidase 4 (AAO4) extracted from *Arabidopsis thaliana* developing seeds that could convert *trans*-cinnamaldehyde *in vitro* (Ibdah et al., 2009).

In addition to the established systemic circulation of carvone and *trans*-cinnamaldehyde, it is most interesting to look at the modification of other VOCs in the emission profiles that can strongly impact trophic interaction within ecosystems. BVOC emissions can mediate herbivore interactions (Trowbridge and Stoy, 2013). Within the framework of this discussion, one should bear in mind that numerous factors can influence apple tree VOC emissions, including meteorological (Vallat et al., 2005), circadian (Giacomuzzi et al., 2017), physiological (Zeng et al., 2017), and phonological (Casado et al., 2006), as well as interactions with herbivores (Suckling et al., 2012) or fungi (Souleyre et al., 2019). However, systemic release of induced volatiles also occurs in plants in the case of insect feeding to recruit natural enemies. The homoterpenes DMNT and TMTT, the monoterpenes ocimene and linalool and these squiterpenes farnesene and caryophyllene are a shared response to herbivores in diverse plant systems (Paré and Tumlinson, 1999; Holopainen and Gershenzon, 2010). Therefore, modification of emitted VOCs, such as those observed in our work, may alter trophic interactions with regard to chemical ecology. Moreover, germacrene-D, α-farnesene and methyl salicylate may have resulted from SAR activation by the injected EOs, since SAR has been detected after trunk injection of SAR activators (Aćimović et al., 2015). Indeed, monoterpenes have been acknowledged to support SAR amongst different plants (Riedlmeier et al., 2017). The elicitation of resistance in young apple trees by acibenzolar-S-methyl was observed to specifically increase the production of the compounds that were effective against rosy apple aphids and *Erwinia amylovora* (Aćimović et al., 2015; Warneys et al., 2018). Moreover, *Cinnamomum zeylanicum* oil and *trans*-cinnamaldehyde were proven to be efficient against Alternaria brown spot in tangerine by direct effects and resistance induction (Perina et al., 2019). A prospective molecular tool such as quantitative real-time PCR to detect changes in expression levels of genes involved in plant defence mechanisms may prove useful to challenge this hypothesis (Dugé De Bernonville et al., 2014; Aćimović et al., 2015). The plant defence responses include other mechanisms, such as cell wall fortification, antimicrobial compounds such as pathogenesis related (PR)protein productions, phytoalexins or reactive oxygen species (ROS) (Marolleau et al., 2017). Phytoalexins include diverse plant secondary metabolites biosynthesised in response to pathogens and certain abiotic stresses. In the subtribe Malinae of the Rosaceae family, the phytoalexins biphenyl and dibenzofuranare are produced upon pathogen attack (Chizzali and Beerhues, 2012) and after elicitor-treated cell cultures (Saini et al., 2019; Teotia et al., 2019). The production of phytoalexins following treatment with EOs could also be an interesting prospect in order to determine and clarify the defence induction potential of these compounds as well as their potential impact on pathogens.

The results presented in this work clearly exposed the possibility that EO application could trigger different physiological processes within plants, leading to other BVOC emissions. Some compound production seems to be shared for both EOs, whereas some seem to be specifically induced by each EO. These results support the hypothesis of different modes of action for each EO and further demonstrated the plant's reaction to these EO injections. The differences between the two EO profiles may result from their specific interactions with the plant and, more precisely, with the plasma membrane. Recently, molecular techniques of dynamic interaction were applied to study the interaction between a biomimetic membrane with monoterpene (citronellal and citronellol) and with cinnamaldehyde (phenylpropanoids). Briefly, the *in silico* insertion model predicted different behaviours between the two classes (monoterpenes and phenylpropanoids), which are the stable interactions with plant lipids for monoterpene, whilst *trans*-cinnamaldehydes had no stable interaction with the membrane. These predictions were confirmed using *in vitro* biophysical assays (Lins et al., 2019).

Regarding the contained VOC profiles, green leaf volatiles (GLVs) generated by the lipoxygenase (LOX) pathway such as 2-hexenal constitute the major compounds. Due to the extraction protocol, this profile may not be interpreted as a potential pool for VOC emissions in the environment, as *de novo* synthesis could have occurred during incubation and trapping after grinding, especially for GLV. DHS is the most widely used sampling approach in the plant field because of its flexibility (sampled volume, trapping approaches and materials) (Bicchi et al., 2008). A high concentration factor was applied for the trace components under study. However, the analysis of these contained profiles may prove useful to further establish the metabolomic impact of EOs injection into apple trees. The presence of greater amounts of other aldehydes, such as nonanal, and the plant volatile hormone methyl salicylate reinforces the previously formulated hypothesis of resistance induction (Wenig et al., 2019). Other compounds emerged from the degradation of carotenoids, namely β-ionone and homocyclocitral (Dudareva et al., 2013).

Our work did not express foliar phytotoxicity. Chlorophyll fluorescence is a non-destructive and sensitive method that is widely used in eco-physiological studies to assess abiotic stress in plants. Indeed, perturbation in plant metabolism may decrease photosystem II (PSII) performance. However, local toxicity at the injection site cannot be excluded, as well as the mechanical damage that occurs due to the injection procedure (Doccola, 2012; Aćimović et al., 2016a). Furthermore, the specific mode of action of carvone can lead to microtubule depolarisation within cells. Lastly, unspecific generation of ROS has been frequently observed after EO application (Kaur et al., 2010; Sunohara et al., 2015; Dahiya et al., 2020). Carotenoids are amongst the first non-enzymatic antioxidants acting to protect photosystem II from photo-inhibition and ROS (Pospíšil, 2012). Therefore, the higher

content of its degradation product in the leaf may be explained by such a phenomenon. Physiological disorder in phytohormones or ROS balances that may result in chronic and long-term toxicity from EO applications should be addressed before concluding a lack of harmful effects of the treatments.

In terms of agricultural application, trunk injection and EO applications are rarely used (Aćimović et al., 2020). This work was established as a proof of concept that the combination of both may be a suitable strategy to develop the biopesticidal potential of EOs whilst avoiding most of their drawbacks. However, we must highlight that more works in terms of reproducibility of results over different years, with other apple varieties, rootstock and efficiency on diverse pests are needed to establish the agronomic potential of such treatment. The absence of impact on apple quality or yield and on tree growth through long-term phytotoxicity should be established as well. Field trials should be performed to establish efficacy as a biopesticide and the lack of harmful effect to beneficial insects.

Plant VOCs are a promising tool, as they have numerous applications in agriculture, such as parasitoid attractant or through defence induction or priming, growth regulators and abiotic stress protectants (Brilli et al., 2019). Moreover, the use of natural substances that elicit systemic resistance has been proven to be a suitable strategy for pathogen management in orchards (Lateur, 2002). The possibility of combining EOs due to their biopesticidal properties with a new mode of application—trunk injection—was hereby demonstrated. Furthermore, the variations in the emitted and contained VOCs clearly demonstrate that young apple trees react to EO injection and that this reaction may be explored to design sustainable agricultural practices.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

P-YW and CB: conceptualisation, methodology, formal analysis, and writing—original draft preparation. P-YW, CB, M-LF, GL, and TH: validation and writing—review and editing. M-LF: supervision. M-LF, GL, and TH: project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.650132/full#supplementary-material

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Acaricidal and Insect Antifeedant Effects of Essential Oils From Selected Aromatic Plants and Their Main Components

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This work has demonstrated the ixodicidal and insect antifeedant effects of essential oils from 14 experimentally cultivated aromatic plants. The strong ixodicidal and antifeedant oils corresponded to *Thymus zygis*, *Thymus vulgaris*, *Satureja montana*, *Oreganum virens*, and *Mentha suaveolens*. The moderately active oils were from *Lavandula angustifolia*, *Mentha piperita*, *Mentha spicata*, *Artemisa herba-alba*, and *Rosmarinus officinalis.* The most effective larvicidal and antifeedant compounds were piperitenone oxide, carvacrol, piperitenone, and thymol, explaining the effects of the most active essential oils. The rest of the tested compounds were not ixodicidal or antifeedant. Therefore, the activity of moderately active oils cannot be explained by their main components (linalyl acetate, linalool, menthone, menthol, limonene, camphor, 1,8-cineole, *p*-cymene, α-pìnene, and carvone), suggesting synergistic effects. Considering the ixodicidal and antifeedant effects of these extracts, the plants have been ranked in relation to *Thymus vulgare*, a commercial biopesticide ingredient, for their potential as botanical pesticides. *T. zygis*, *S. montana*, and *M. suaveolens* ranked over *T. vulgaris* as ixodicidal agents and *S. montana* as insecticidal. Therefore, we propose the plant populations of *S. montana*, *T. zygis*, and *M. suaveolens* tested here for further development as biopesticide ingredients.

Keywords: aromatic plant, essential oil, ixodicidal, antifeedant, *Hyalomma lusitanicum*, *Spodoptera littoralis*, *Myzus persicae*, *Rhopaslosiphum padi*

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INTRODUCTION

Food safety and environmental concerns related to the use of pesticides have resulted in more restricted regulatory frameworks worldwide, reducing the number of commercial products available for crop protection and other pest management sectors including the control of vectors of human and livestock diseases. Therefore, new safer and effective insecticides are needed. Botanical pesticides are emerging as a solution to meet part of the demand (Isman, 2020a). Essential oils (EOs) that are composed of volatile secondary metabolites, mostly terpenes (Bakkali et al., 2008), are among the most important extracts acting as botanical insecticides (Regnault-Roger et al., 2012; Pavela and Benelli, 2016), and some are being commercialized as commercial pesticide ingredients (Isman, 2020b).

Arthropods, including economically important disease vectors and insect pests, are an important target of the biological effects of EOs (Ntalli et al., 2019; Isman, 2020a,b). Tick-borne diseases are a serious health and economic problem, responsible for over 100,000 cases of human diseases worldwide (de la Fuente et al., 2008) and billions of dollars in losses to the livestock industry (Lotfi and Karima, 2020). Additionally, ticks are in expansion due to climate change (Abbas et al., 2018). For example, *Hyalomma* ticks, vectors of the Crimean-Congo hemorrhagic fever virus, have spread from their original distribution (African and Mediterranean environments) to other European countries, becoming an increasing public health concern (Chitimia-Dobler et al., 2019; Hansford et al., 2019; Buczek et al., 2020; Grandi et al., 2020). For many years, tick control has been carried out with synthetic acaricides, leading to the appearance of resistance (reviewed by Abbas et al., 2014) and being harmful to the environment. Therefore, new effective and safer tick control agents are needed. In this context, EOs have been reported as being toxic and/or repellent to ticks (Benelli et al., 2016, 2017a; Benelli and Pavela, 2018; Salman et al., 2020).

Crop yield damages caused by pest infestations and pesticide use are significant (Oerke, 2006; Gregory et al., 2009) and increasing with global warming. Adaptation measures to increased pest damage related to global warming may involve greater use of pesticides with detrimental effects on health, environmental damage, and increased pesticide resistance (Deutsch et al., 2018). Some important crop pests include the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), a highly polyphagous insect labeled as an A2 quarantine pest by the OEPP/EPPO (2015) due to its host range (Alford, 2007) and distribution (Centre for Agricultural Bioscience International, 2020a). The green peach aphid, *Myzus persicae* Sulzer (Hemiptera: Aphididae), is the most economically important aphid crop pest worldwide (van Emden and Harrington, 2017) due to its distribution (Centre for Agricultural Bioscience International, 2020b), host range (Blackman and Eastop, 2000), mechanisms of plant damage, life cycle, and its ability to evolve resistance to insecticides (Bass et al., 2014). The bird cherry-oat aphid, *Rhopalosiphum padi* L., is a global pest of cereals (van Emden and Harrington, 2017) and a vector of yellow dwarf viruses that cause significant crop losses in cereals (Finlay and Luck, 2011). Many EOs are good insecticidal candidates because of their direct effects, biodegradability, and their low level of toxicity to mammals (Isman, 2020a,b).

The commercial production of a botanical insecticide depends on the sustainable production of plant biomass for extraction. Therefore, the domestication and cultivation of aromatic and medicinal plants (AMPs) for the production of EOs contributes to species conservation and provides sustainability of the production and lower variations in active ingredients. For example, a selected chemotype of wormwood, *Artemisia absinthium* (Asteraceae), that lacks the toxic terpene β-thujone but produces other novel terpenoids that are toxic and antifeedant to a range of pest insects has been domesticated for cultivation and registered as a new plant variety (Gonzalez-Coloma et al., 2017).

TABLE 1 | List of the plant species used and their origin (experimental field locations in Aragón, Spain, and UTM coordinates).

Plant species	Origin
Artemisia dracunculus L.	Ejea de los Caballeros (42°7′45″ N, 1°8′15″ W)
Artemisia herba-alba Asso.	Villafranca (41°34′28″ N, 0°39′01″ W)
Hyssopus officinalis L.	Teruel (40°20′37″ N, 1°06′26″ W)
Lavandula angustifolia L.	Ejea de los Caballeros (42°7′45″ N, 1°8′15″ W)
Mentha piperita L.	La Alfranca (41°36′22″ N, 0°45′22″ O) La Alfranca (41°36′22″ N, 0°45′22″ O)
Mentha spicata L.	
Mentha suaveolens Ehrh.	Ejea de los Caballeros (42°7′45″ N, 1°8′15″ W)
Origanum vulgare subsp.*virens* Hoffmanns and Link	Fabara (41°10′ N, 0°10′ E)
Rosmarinus officinalis L.	Villafranca (41°34′28″ N, 0°39′01″ W)
Satureja montana L.	Ejea de los Caballeros (42°7′45″ N, 1°8′15″ W)
Tanacetum vulgare L.	Ejea de los Caballeros (42°7′45″ N, 1°8′15″ W)
Thymus mastichina L.	Moncayo-Trasobares (41°39′49.43″ N, 1°37′48.11″ W)
Thymus vulgaris L.	Villarroya (41°27′49″ N, 1°47′01″ W)
Thymus zygis Loefl. ex L.	Aguarón (41°20′20″ N, 1°16′11″ W)

As part of an ongoing project on the domestication and valorization of selected AMPs, plant species belonging to the genera *Artemisia*, *Hyssopus*, *Lavandula*, *Mentha*, *Origanum*, *Rosmarinus*, *Satureja*, *Tanacetum*, and *Thymus* have been experimentally cultivated at a small scale. These genera include species traditionally used in medicinal, food, and flavor applications due to their contents in bioactive EOs (Fathiazad and Hamedeyazdan, 2011; Chishti et al., 2013; Kumar and Tyagi, 2013; Tepe and Cilkiz, 2016; Aprotosoaie et al., 2017; Singh and Pandey, 2018; Borges et al., 2019; Li et al., 2019; Isman, 2020a,b).

In this work, essential oils from selected species of aromatic and medicinal plants cultivated experimentally (**Table 1**) have been evaluated against arthropods of importance in public health and animal and crop production: the tick (*Hyalomma lusitanicum*) and three insect pests (*S. littoralis*, *M. persicae*, and *R. padi*). *Thymus vulgaris* has been included in this study as a reference to compare the rest of the selected species because it is one of the most important aromatic plants grown worldwide (Southern and Central Europe, Southeast Asia, North America, and Africa), and it is an ingredient of botanical insecticides because of its thymol content (Pavela, 2016). Additionally, the composition of the most active EOs has been analyzed and the ixodicidal and insecticidal activities of their main components (**Figure 1**) tested.

MATERIALS AND METHODS

Plant Material

Fourteen plant species belonging to the families Asteraceae and Lamiaceae (**Table 1**) were selected for the study. The plants

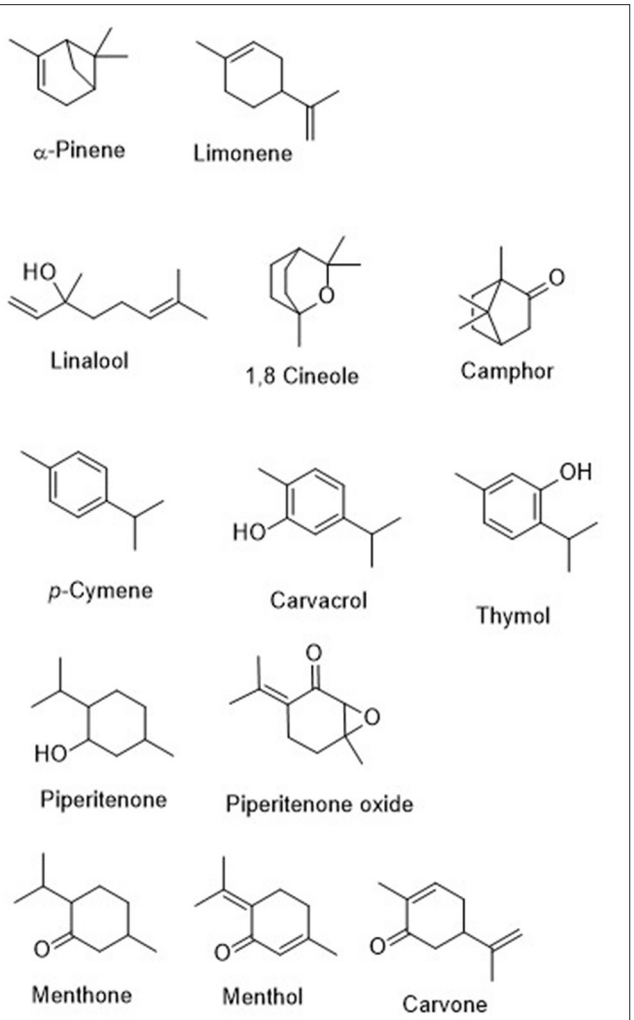

FIGURE 1 | Chemical structures of the essentail oils main components.

come from Spanish native flora and have been experimentally cultivated in several locations in Aragon (Spain) as described (Burillo, 2003; Burillo et al., 2017; Navarro-Rocha et al., 2020).

Aerial parts of these plants were collected at the flowering stage. EOs were obtained in the laboratory by Clevenger hydrodistillation (European Pharmacopoeia, 1975).

Essential Oil Analysis

The essential oils were analyzed by gas chromatography–mass spectrometry (GC-MS) using a Shimadzu GC-2010 gas chromatograph coupled to a Shimadzu GCMS-QP2010 Ultra mass detector (electron ionization, 70 eV) and equipped with a 30-m × 0.25-mm i.d. capillary column (0.25 μm film thickness) Teknokroma TRB-5 (95%) dimethyl–(5%) diphenylpolisiloxane. The working conditions were as follows: split ratio, 20:1; injector temperature, 300°C; temperature of the transfer line connected to the mass spectrometer, 250°C; initial column temperature, 70°C; then heated to 290°C at 6°C/min. The relative amounts of the individual components were calculated based on the peak area without using a correction factor. Electron ionization mass spectra, retention data, and the calculated linear retention indices (LRIs) were used to assess the identity of the compounds by comparing them with those of standards or those found in the Wiley 229 Mass Spectral Database.

Ixodicidal Activity

Hyalomma lusitanicum engorged females were collected from red deer in Ciudad Real (Central Spain) and maintained under laboratory conditions [22–24°C and 80% relative humidity (RH)] until oviposition and egg hatching.

Tick bioassays were performed according to Ruiz-Vásquez et al. (2017). Briefly, 50 μl of the test solution was added to 25 mg of powdered cellulose at different concentrations (initial concentration of 40 or 20 μg/mg for EOs or pure compounds, respectively) and the solvent was evaporated. The ticks and cellulose were then placed in laboratory glass tubes and carefully mixed by rotating the glass several times to ensure full tick–cellulose contact. After mixing, the tubes were kept under laboratory conditions for 24 h. For each test, three replicates with 20 active older than 6 weeks larvae. To validate the tests, three replicates of negative (cellulose, 25 mg) and positive (thymol, 20 μg/mg) controls were also used.

Ticks were considered dead when they could not move from one place to another. Dead ticks were counted after 24 h of contact with the treated cellulose at the laboratory conditions described using a binocular magnifying glass. The larvicidal activity data are presented as percent mortality corrected according to Schneider–Orelli's formula (Püntener, 1981). Effective lethal doses (LC_{50} and LC_{90}) were calculated by Probit analysis (1:2 serial dilutions to cover a range of activities between 100 and <50% mortality with a minimum of three doses) (STATGRAPHICS Centurion XVI, version 16.1.02).

Insect Antifeedant Activity

Spodoptera littoralis, *M. persicae*, and *R. padi* colonies are maintained at ICA-CSIC, reared on artificial diet, bell pepper (*Capsicum annuum*) and barley (*Hordeum vulgare*) plants, respectively, and kept at 22 ± 1°C and >70% RH, with a photoperiod of 16:8 h (L/D) in a custom-made walk-in growth chamber.

The bioassays were conducted as described (Navarro-Rocha et al., 2018). The upper surfaces of the *C. annuum* and *H. vulgare* leaf disks or fragments (1.0 cm^2) were treated with 10 μl of the test substance. The EOs and products were tested at an initial dose of 10 or 5 μg/μl (100 or 50 μg/cm^2), respectively. Five to seven Petri dishes or 20 ventilated plastic boxes (2 × 2 cm) with two sixth-instar *S. littoralis* larvae (≥24 h after molting) or 10 apterous aphid adults (24–48 h old) each were allowed to feed in a growth chamber (until 75% larval consumption of the control disks or 24 h for aphids, environmental conditions as above). Each experiment was repeated twice. Feeding inhibition or aphid settling was calculated by measuring the disk surface consumption (digitalized with https://imagej.nih.gov/ij/) (Rueden et al., 2017) or by counting the number of aphids on each leaf fragment. Feeding/settling inhibition (%FI or %SI) was calculated as $\%FI/SI = [1 - (T/C) \times 100]$, where

TABLE 2 | Larvicidal effects of the selected essential oils on *Hyalomma lusitanicum*.

Essential oil	*Hyalomma lusitanicum*		
	% Mortality[a] (40 μg/mg)	LD_{50} (CL)[b]	LD_{90} (CL)[b]
Artemisia dracunculus	30.20 ± 11.52	>40	>40
Artemisia herba-alba	100	20–40	20–40
Hyssopus officinalis	0	>40	>40
Lavandula angustifolia	100	16.06 (14.72–17.18)	19.71 (18.55–21.18)
Mentha piperita	100	22.96 (21.06–26.16)	30.34 (26.9–37.64)
Mentha suaveolens	100	4.54 (4.18–4.92)	6.12 (5.64–6.92)
Mentha spicata	100	23.58 (21.46–26.14)	33.86 (30.58–38.84)
Origanum vulgare subsp.*virens*	100	6.38 (5.82–7.00)	8.96 (8.18–10.10)
Rosmarinus officinalis	100	∼10	∼12
Satureja montana	100	4.68 (4.14–5.24)	8.33 (7.54–9.38)
Tanacetum vulgare	23.37 ± 6.74	>40	>40
Thymus mastichina	47.69 ± 20.50	>40	>40
Thymus vulgaris	100	5.52 (4.42–6.36)	9.52 (8.46–11.36)
Thymus zygis	100	2.44 (2.18–2.74)	3.88 (3.48–4.48)

[a] *Values (in percent) are the means of three replicates corrected according to Schneider–Orelli's formula (Püntener, 1981).*
[b] *Lethal doses (upper–lower 95% confidence limits) calculated to give 50% (LD_{50}) or 90% (LD_{90}) mortality by Probit analysis.*

T and *C* represent feeding/settling on the treated and control leaf disks, respectively. The antifeedant effects (%FI/SI) were analyzed for significance by the non-parametric Wilcoxon paired signed-rank test comparing the consumption/settling between the treatment and control leaf disks. Extracts and compounds with an SI >70% were further tested in a dose–response experiment (1:2 serial dilutions to cover a range of activities between 100 and <50% feeding inhibition with a minimum of three doses) to calculate their relative potency (EC_{50}, the effective dose to give a 50% settling reduction) from the linear regression analysis (%FI/SI on Log-dose, STATGRAPHICS Centurion XVI, version 16.1.02).

RESULTS

Ixodicidal Effects

Most of the EOs tested (75%) gave significant ixodicidal activity against *H. lusitanicum* larvae (**Table 2**), which can be grouped into four categories as follows:

(1) Strong ixodicidal effects (LC_{50} < 10 μg/mg): *Thymus zygis* (four doses tested, 100–46% mortality), followed by *Mentha suaveolens* (five doses tested, 100–50% mortality), *Satureja montana* (seven doses tested, 100–18% mortality), *T. vulgaris* (four doses tested, 100–5% mortality), and *Origanum vulgare* subsp. *virens* (six doses tested, 100–10% mortality).
(2) Moderate ixodicidal effects (LC_{50} < 16–28 μg/mg): *Mentha piperita* (three doses, 100–2% mortality), *Mentha spicata* (three doses, 100–9% mortality), *Lavandula angustifolia* (two doses, 92–2% mortality), and *Rosmarinus officinalis* (two doses, 100–20% mortality).
(3) Moderate–low ixodicidal effects (LC_{50} < 20–40 μg/mg): *Artemisia herba-alba*, only toxic at the highest dose tested (40 μg/mg, 100% mortality).
(4) No ixodicidal effects (LC_{50} > 40 μg/mg): *Artemisia dranunculus*, *Hyssopus officinalis*, *Tanacetum vulgare*, and *Thymus mastichina*.

Antifeedant Effects

Table 3 shows the insect antifeedant effects of the tested EOs. Overall, the herbivorous insects were less affected by these EOs than the tick (37 and 31% EOs effective against *S. littoralis* and aphids, respectively).

Spodoptera littoralis feeding was strongly affected by *S. montana* (four doses, %FI = 90–5, EC_{50} = 39 μg/cm^2), followed *by M. piperita*, *M. spicata*, *T. vulgaris*, *T. zygis*, and *T. vulgare* (%FI = 70–80).

Mentha persicae and *R. padi* were strongly affected by *T. vulgaris* (four doses, %SI = 81–10 and 84–7, EC_{50} = 29 and 49 μg/cm^2, respectively) and *S. montana* (four doses, %SI = 90–5, EC_{50} = 29 μg/cm^2). *M. suaveolens* (three doses, %SI = 92–20), *O. vulgare* subsp. *virens* (three doses, %SI = 78–10), and *T. zygis* (three doses, %SI = 89–15) showed moderate effects on *M. persicae* (EC_{50} = 35, 34, and 45 μg/cm^2, respectively). *T. zygis*, *T. vulgare*, and *O. vulgare* subsp. *virens* had low effects on *R. padi* (%SI = 65–70).

Plant Species Ranking

Considering the ixodicidal and antifeedant effects of the tested EOs, the plants have been ranked in relation to *T. vulgaris* (**Table 4**) for their potential as botanical pesticide ingredients. The ranking index has been established as [*T. vulgaris* EC_{50} value/ranked species EC_{50} value] for each test with significant effects (see **Tables 2**, **3**).

Overall, considering the sum of all the indices, *S. montana* and *T. zygis* ranked over *T. vulgaris* (value >4). However, *T. zygis*, *S. montana*, and *M. suaveolens* ranked over *T. vulgaris* as

TABLE 3 | Insect antifeedant effects of the selected essential oils.

Essential oil	*Spodoptera littoralis*	*Myzus persicae*	*Rhopalosiphum padi*
	%FI[a]	%SI[b]	
	EC_{50} (CL)[c]		
Artemisia dracunculus	53.4 ± 11	21.8 ± 6	42.9 ± 7
	∼100	>100	>100
Artemisia herba-alba	30.2 ± 10	59.4 ± 7	31.5 ± 7
	>100	>100	>100
Hyssopus officinalis	40.1 ± 3	41.2 ± 8	26.6 ± 7
	>100	>100	>100
Lavandula angustifolia	54.8 ± 11	31.0 ± 8	46.5 ± 6
	∼100	>100	>100
Mentha piperita	74.6 ± 8*	38.5 ± 10	33.2 ± 8
	>70	>100	>100
Mentha spicata	72.84 ± 12*	56.7 ± 8	17.8 ± 5
	>70	∼100	>100
Mentha suaveolens	71.1 ± 14*	92.1 ± 3*	48.5 ± 7
	>70	35.0 (31–39)	>100
Origanum vulgare subsp. *virens*	37.7 ± 11	78.0 ± 7*	67.2 ± 8*
	>100	33.7 (23–50)	>70
Rosmarinus officinalis	35.5 ± 11	51.4 ± 6	19.9 ± 5
	>100	∼100	>100
Satureja montana	94.3 ± 1*	93.5 ± 2*	90.1 ± 3*
	39.5 (13–63)	28.9 (22–34)	29.2 (20–38)
Tanacetum vulgare	68.2 ± 10*	51.8 ± 7	68.1 ± 6*
	>70	∼100	>70
Thymus mastichina	38.7 ± 10	33.5 ± 9	8.7 ± 5
	>100	∼100	>100
Thymus vulgaris	74.9 ± 12*	80.7 ± 6*	83.9 ± 5*
	>70	29.0 (10–35)	49.0 (40–50)
Thymus zygis	72.3 ± 15*	89.3 ± 5*	70.2 ± 8*
	>70	45.0 (40–50)	>70

[a] *Percent feeding (FI) inhibition at a dose of 100 μg/cm². Values are the means of five to seven replicates per dose.*
Values with asterisk are significantly different according to Wilcoxon paired rank test ($P < 0.05$).
[b] *Percent setting (SI) inhibition at a dose of 100 μg/cm². Values are the means of 20 replicates per dose.*
[c] *EC_{50} (95% lower–upper confidence limits), concentration needed to produce 50% feeding/setting inhibition.*

ixodicidal agents (>1), and only *S. montana* ranked better than *T. vulgaris* against insects (**Table 4**).

Essential Oil Composition

Table 5 shows the main components (% abundance >10) of the active EOs. The oils can be grouped according to their main components as follows: camphor/1,8-cineole (+*p*-cymene and *A. herba-alba*; +α-pìnene and *R. officinalis*); carvacrol (*S. montana*); carvone/1,8-cineole (*M. spicata*); *p*-cymene/carvacrol/linalool (*O. vulgare* subsp. *virens*); linalyl acetate/linalool (*L. angustifolia*); menthone/menthol/limonene (*M. piperita*); piperitenone oxide/piperitenone (*M. suaveolens*); and thymol (*T. zygis*) (+*p*-cymene and *T. vulgaris*).

Ixodicidal and Antifeedant Effects of EOs' Main Components

Table 6 shows the ixodicidal effects of the selected individual components. Piperitenone oxide was the strongest acaricidal compound (LD_{50-90} = 0.9–1.1 μg/mg), followed by carvacrol (LD_{50-90} = 1.4–1.7 μg/mg), piperitenone (LD_{50-90} = 1.8–2.2 μg/mg), and thymol (LD_{50-90} = 2.9–6.2 μg/mg).

The antifeedant effects of the individual EO components are shown in **Table 7**. Piperitenone was the most effective antifeedant against *S. littoralis* (EC_{50} = 1.4 μg/cm²), followed by piperitenone oxide (EC_{50} = 5 μg/cm²), thymol (EC_{50} = 21 μg/cm²), and α-pinene with moderate-low effects (EC_{50} = ∼37 μg/cm²). *M. persicae* strongly responded to thymol (EC_{50} = 7.6 μg/cm²) and piperitenone oxide (EC_{50} = 8.6 μg/cm²), followed by carvacrol (EC_{50} = 15 μg/cm²) and menthone (EC_{50} = ∼34 μg/cm²). *R. padi* was the least sensitive insect species and responded to carvacrol (EC_{50} = 15 μg/cm²), thymol (EC_{50} = 19 μg/cm²), and piperitenone oxide (EC_{50} = ∼25 μg/cm²).

DISCUSSION

This work has demonstrated the ixodicidal and insect antifeedant effects of EOs from experimentally cultivated AMPs. Furthermore, more EOs were ixodicidals than insect antifeedants, probably because of their different feeding ecologies (blood sucking vs. herbivores). Ticks are obligate hematophagous ectoparasites (Basu and Charles, 2017) and therefore have not evolved adaptations to plant secondary metabolites. On the other hand, insect herbivores have coevolved with plants and their chemical defenses/secondary metabolites (Maron et al., 2019). These differences in feeding adaptations could explain the selective toxicity of EOs toward the ticks observed here.

The EOs grouped as strong ixodicidal agents corresponded to *T. zygis*, *T. vulgaris*, *S. montana*, *M. suaveolens*, and *Origanum virens*. Similarly, the EOs grouped as strong antifeedants corresponded to *S. montana*, *T. zygis*, and *T. vulgaris*, followed by *O. virens* and *M. suaveolens*.

Thymus vulgaris EO, an ingredient of botanical pesticides (Pavela, 2016), has been included in this work as a reference for further species selection. In this work, the EO from *T. vulgaris* (thymol/*p*-cymene) was the third most ixodicidal and the second most antifeedant against the insect species tested. The most common *T. vulgaris* chemotypes are thymol/carvacrol (György et al., 2020), which have reported ixodicidal effects including repellency against nymphs of *Ixodes ricinus* and adults of *Dermacentor reticulatus* (Štefanidesová et al., 2017; Goode et al., 2018), but not on its larvicidal effects against *H. lusitanicum*. EO from *T. vulgaris* has also been described as being insecticidal against several insect species, including *S. littoralis*, *M. persicae* (toxicity; Pavela, 2012; Ikbal and Pavela, 2019), and *R. padi* (antifeedant; Grul'ová et al., 2017). The EO from *T. zygis* (thymol) was the most effective ixodicidal agent tested here, with insect antifeedant effects similar to *T. vulgaris*. Previous

TABLE 4 | Rank index [calculated as EC_{50} of *Thymus vulgaris* essential oil (EO)/EC_{50} of ranked species' EO] of the bioactive EO-producing plant species tested for further selection.

Essential oil	*Hyalomma lusitanicum*	*Spodoptera littoralis*	*Myzus persicae*	*Rhopaslosiphum padi*	Total index
Thymus vulgaris	1	1	1	1	4
Satureja montana	1.17	1.67	1	2.43	6.27
Thymus zygis	2.25	0.95	0.64	0.69	4.53
Mentha suaveolens	1.22	0.94	0.83		2.99
Origanum vulgare subsp. *virens*	0.86		0.86	0.66	2.05
Mentha spicata	0.23	1			1.23
Mentha piperita	0.23	1			1.23
Rosmarinus officinalis	0.55				0.55
Lavandula angustifolia	0.34				0.34

TABLE 5 | Main components of the active essential oils.

Plant species	Compound (% abundance)
Artemisa herba-alba	Camphor (19), 1,8-cineole (12), *p*-cymene (8), borneol (1)
Lavandula angustifolia	Linalyl acetate (30), linalool (30), geranyl acetate (7), terpineol (4), *c*-linalool oxide (3), *t*-linalool oxide (3), caryophyllene oxide (3), neryl acetate (2)
Mentha piperita	Menthone (41), menthol (31), limonene (13)
Mentha spicata	Carvone (79),1,8-cineole (12), menthol (2)
Mentha suaveolens	Piperitenone oxide (37), piperitenone (21), limonene (7), D-germacrone (7), *t*-caryophyllene (6)
Origanum vulgare subsp. *virens*	*p*-Cymene (30), carvacrol (17), linalool (14), α-terpinene (3), myrcene (2), β-caryophyllene (2)
Rosmarinus officinalis	Camphor (28), 1,8-cineole (22), α-pinene (11), endoborneol (6), camphene (6), verbenone (5)
Satureja montana	Carvacrol (76), *p*-cymene (2), borneol (2), thymoquinone (1), 1-octen-3-ol (1)
Thymus vulgaris	Thymol (49), *p*-cymene (29), γ-terpinene (7), carvacrol (4)
Thymus zygis	Thymol (74), *p*-cymene (9), γ-terpinene (7), carvacrol (4)

TABLE 6 | Ixodicidal activity of the main components (% abundance ≥ 10) of the active essential oils on *Hyalomma lusitanicum* larvae.

Compound	% Mortality[a] (20 μg/mg)	LD_{50} (CL)[b]	LD_{90}^{a} (CL)[b]
α-Pinene	0	>20	>20
Limonene	6.87 ± 1.84	>20	>20
Linalool	9.73 ± 5.02	>20	>20
1,8-Cineole	3.70 ± 3.70	>20	>20
Camphor	15.60 ± 4.73	>20	>20
p-Cymene	5.70 ± 2.97	>20	>20
Carvacrol	100	1.42 (1.34–1.54)	1.76 (1.62–1.92)
Thymol	100	2.94 (2.08–3.54)	6.16 (5.30–7.84)
Piperitenone	100	1.77 (1.63–1.92)	2.19 (2.03–2.40)
Piperitenone oxide	100	0.88 (0.81–0.96)	1.09 (1.02–1.19)
Menthone	8.50 ± 4.44	>20	>20
Menthol	31.4 ± 13.6	>20	>20
Carvone	5.00 ± 2.67	>20	>20

[a] *Values (in percent) are the means of three replicates corrected according to Schneider–Orelli's formula (Püntener, 1981).*

[b] *Lethal doses (upper–lower 95% confidence limits) calculated to give 50% (LD_{50}) or 90% (LD_{90}) mortality by Probit analysis.*

reports showed that *T. zygis* EO (rich in thymol) was ovicidal, larvicidal, antifeedant, and repellent against the insect *Plutella xylostella* (Sangha et al., 2017), but this is the first report on its ixodicidal activity. *T. zygis* is distributed in the Iberian Peninsula and north of Africa (Morales Valverde, 1997), the thymol chemotype being of interest (Pérez-Sánchez et al., 2008). Therefore, the high content of thymol (75%) and the effects on ticks of the EO from the *T. zygis* line tested here support further agronomic development.

The EO from *S. montana* (carvacrol) was the most effective insect antifeedant and the second most effective ixodicidal agent tested in this study. The essential oil of *S. montana* is characterized by carvacrol, thymol, *p*-cymene, and linalool (Velasco and Perez-Alonso, 1983; Silva et al., 2009; Dunkic et al., 2012). *S. montana* EO has reported repellence to *Frankiniella occidentalis* (Picard et al., 2012), is toxic against *Leptinotarsa decemlineata* larvae and adults (Usanmaz-Bozhuyuk and Kordali, 2018), larvicidal against *Culex quinquefasciatus* (Benelli et al., 2017b), and toxic to *Drosophila suzukii* adults (Park et al., 2016). The population of *S. montana* used in this work, rich in carvacrol, has already been included in an agronomic development program for the production of biopesticides (Navarro-Rocha et al., 2020). However, this is the first report on the ixodicidal activity of this EO.

The *M. suaveolens* population selected for this work was rich in piperitenone oxide/piperitenone. This EO was the second most effective ixodicidal extract tested here (more effective than *T. vulgaris*), along with *S. montana*, and showed stronger antifeedant effects against *M. persicae* than *T. vulgaris*. *M. suaveolens* is native of Africa, temperate Asia, and Europe (Abbaszadeh et al., 2009). There are three chemotypes described for *M. suaveolens*: pulegone, piperitenone oxide, and piperitenone oxide/piperitone oxide (Oumzil et al., 2002; BoŽović et al., 2015). Previously, *M. suaveolens* EOs (pulegone and menthone) showed ovicidal and larvicidal effects against the tick *Hyalomma aegyptium* (Laghzaoui et al., 2019). This species' EOs also have reported insecticidal

TABLE 7 | Antifeedant activity of the main components (% abundance >10) of the active essential oils on *Spodoptera littoralis* larvae, *Myzus persicae*, and *Rhopaslosiphum padi* apterous adults in choice tests.

Compound	*S. litorallis*	*M. persicae*	*R. padi*
	%FI[a]	%SI[b]	
	EC_{50} (CL)[c]		
α-Pinene	67.3 ± 8.9	53.9 ± 10.2	34.9 ± 8.1
	∼37	>50	>50
Limonene	44.8 ± 14.5	29.3 ± 7.7	31.15 ± 0.55
	>50	>50	>50
Linalool	45.3 ± 7.2	27.3 ± 7.6	48.4 ± 8.3
	>50	>50	>50
1,8 Cineole	36.0 ± 8.7	56.0 ± 8.5	21.9 ± 7.2
	>50	>50	>50
Camphor	22.6 ± 6.0	37.6 ± 7.0	38.5 ± 7.6
	>50	>50	>50
p-Cymene	8.61 ± 6.09	20.24 ± 6.50	35.0 ± 7.5
	>50	>50	>50
Carvacrol	55.8 ± 11.8	86.4 ± 3.2*	90.6 ± 5.3*
	∼50	15.5 (11.1–18.8)	14.6 (11.7–18.2)
Thymol	52.4 ± 10.1	81.8 ± 7.7*	92.1 ± 2.6*
	∼50	7.6 (4.1–8.7)	18.6 (4.1–23.3.5)
Piperitenone	91.8 ± 4.9*	56.2 ± 2.4	nt
	1.45 (0.2–9.9)	∼50	
Piperitenone oxide	90.1 ± 3.7*	91.1 ± 5.3*	75.0 ± 6.5*
	5.0 (1.8–13.5)	8.6 (3.0, 24.5)	∼25.0
Menthone	29.2 ± 9.8	72.8 ± 9.2*	61.6 ± 6.7
	>50	∼34	>50
Menthol	35.6 ± 14.3	34.6 ± 8.7	45.4 ± 8.7
	>50	>50	>50
Carvone	52.9 ± 12.7	31.0 ± 9.8	51.5 ± 8.6
	∼50	>50	>50

[a] *Percent feeding (FI) inhibition at a dose of 100 μg/cm². Values are the means of five to seven replicates per dose. Values with asterisk are significantly different according to Wilcoxon paired rank test (P < 0.05).*

[b] *Percent setting (SI) inhibition at a dose of 100 μg/cm². Values are the means of 20 replicates.*

[c] EC_{50} *(95% lower–upper confidence limits), concentration needed to produce 50% feeding/setting inhibition.*

effects against stored-product pests such as *Sitophilus oryzae* (piperitenone oxide and piperitenone oxide/piperitenone chemotypes) (Zekri et al., 2013), *Rizopertha dominica* (piperitenone/pulegone/piperitone) (Benayad et al., 2012), and *Triboleum castaneum* (menthone/pulegone) (Kasrati et al., 2015) and larvicidal activity against *C. quinquefasciatus* (piperitenone oxide) (Pavela et al., 2014). Antifeedant effects against *L. decemlineata* and *M. persicae* have been reported for a piperitenone oxide/piperitone epoxide *Mentha* chemotype (Kimbaris et al., 2017). However, this is the first report on the ixodicidal effects of a piperitenone oxide/piperitenone *M. suaveolens* chemotype. The ixodicidal and antifeedant effects (stronger than those of *T. vulgaris*) of the *M. suaveolens* EO tested in this work support further agronomic development of this species for the production of biopesticides.

The chemotype of *O. vulgare* subsp. *virens* (*p*-cymene, carvacrol, and linalool) tested here showed ixodicidal effects similar to *T. vulgaris*, but was less antifeedant, affecting only the aphid *M. persicae*. *O. vulgare* have reported toxic or repellent activities against *I. ricinus* (Soutar et al., 2019) and acute and fumigant toxicity against aphids including *M. persicae* (Ikbal and Pavela, 2019). However, this is the first report on the ixodicidal and aphid antifeedant effects of *O. vulgare* subsp. *virens* EO. *O. vulgare* is a widespread species native to the Mediterranean, the Euro-Siberian, and the Irano-Turanian regions and is one of the most traded and consumed spice plants (Lukas et al., 2015). *O. vulgare* subsp. *virens* is a heterogeneous subspecies characterized by essential oils rich in acyclic and/or cymyl compounds (Lukas et al., 2015). Since the effects of the EO from *O. vulgare* subsp. *virens* tested here were similar to these of *T. vulgaris*, its further development for the production of biopesticides against arthropods is not supported. However, we suggest the valorization of its essential oil production residues (biomass: hydrolate) as a potential source of biopesticidal ingredients.

The moderate ixodicidal EOs were from *L. angustifolia* (linalyl acetate/linalool), *M. piperita* (menthone/menthol), *M. spicata* (carvone/1,8-cineole), *R. officinalis* (camphor/1,8-cineole/α-pinene), and *A. herba-alba* (camphor/1,8-cineole/*p*-cymene). All these EOs were less effective than that of *T. vulgaris*.

The EO from *L. angustifolia* tested here (linalyl acetate/linalool) showed moderate ixodicidal effects against *H. luxitanicum* larvae, lower than the effects of *T. vulgaris*, without significant insect antifeedant effects. Previous reports have shown interference with the host-seeking behaviors of *H. marginatum* and *D. reticulatus* for this species' EO (Mkolo and Magano, 2007; Štefanidesová et al., 2017) and toxicity to *Rhipicephalus* (*Boophilus*) *annulatus* (Pirali-Kheirabadi and Teixeira da Silva, 2010) for this species' EO. Additionally, a similar EO from the hybrid *Lavandula* × *intermedia* (rich in linalyl acetate and linalool) was also toxic to *H. lusitanicum* larvae and moderately antifeedant to *S. littoralis* (Ortiz de Elguea-Culebras et al., 2018). *L. angustifolia*, distributed in the sub-Mediterranean region, has a great economic importance in perfumery, cosmetics, food, pharmaceutical industries, and aromatherapy (Demasi et al., 2018). However, our results do not support its agronomic production as a biopesticide when compared to *T. vulgaris*, but suggest the valorization of its essential oil production residues (biomass: hydrolate) as a source of biopesticidal ingredients.

M. piperita (menthone/menthol) was moderately ixodicidal against *H. lusitanicum* and showed moderate antifeedant effects against *S. littoralis*. In previous works, *M. piperita* EO showed moderate repellency against adults of *D. reticulatus* (Štefanidesová et al., 2017), larvicidal effects against *R. microplus* (de Souza Chagas et al., 2016), and toxicity against aphids including *M. persicae* (Ikbal and Pavela, 2019). *M. spicata* (carvone/1,8-cineole) also had moderate larvicidal effects against *H. lusitanicum* and moderate-low antifeedant effects on *S. littoralis*. *M. spicata* EO has been reported as a moderate

repellent against adults of *D. reticulatus* (Štefanidesová et al., 2017) and toxic to stored-product pests (Irfan et al., 2009; Kedia et al., 2014; Eliopoulos et al., 2015; Nubia et al., 2016), *L. decemlineata* (Saroukolai et al., 2014), and *S. littoralis* (Pavela, 2005), while a carvone/limonene chemotype of *M. spicata* was not antifeedant or toxic to *S. littoralis*, *M. persicae*, and *R. padi* (Santana et al., 2014). *Mentha* oils are used commercially as biopesticide ingredients because of their various effects against insects, the most commercialized being the *Mentha* species spearmint (*M. spicata*), peppermint (*M. piperita*), and *M. arvensis* (Singh and Pandey, 2018). Since our results showed lower effects than *T. vulgaris*, we suggest the valorization of their commercial essential oil production residues (biomass: hydrolate) as an additional source of biopesticidal ingredients.

Rosmarinus officinalis (camphor/1,8-cineole/α-pinene) showed moderate ixodicidal effects in this work. Previous reports have shown a moderate post-ingestive toxicity to *S. littoralis* for a similar *R. officinalis* EO (Santana et al., 2014). *R. officinalis* EOs rich in 1,8-cineole were toxic to larvae of *Hyalomma scupense* (Djebir et al., 2019) and *I. ricinus* nymphs (Elmhalli et al., 2019), while an EO rich in α-pinene showed low–moderate toxicity against larvae of *R.* (*B.*) *microplus* (Martinez-Velazquez et al., 2011). This plant is cultivated worldwide as a food flavoring and preservative due to its antioxidant and antimicrobial potential (Borges et al., 2019). Our results showed lower effects for *R. officinalis* than *T. vulgaris*. However, being a commercial plant available worldwide, we suggest the valorization of its essential oil production residues (biomass: hydrolate) as a source of biopesticidal ingredients.

The chemotype of *A. herba-alba* (camphor/1,8-cineole/*p*-cymene) tested in this work showed low–moderate larvicidal effects against *H. lusitanicum*. *A. herba-alba* is a medicinal and aromatic shrub that grows wild in arid areas of the Mediterranean Basin, being abundant in the Iberian Peninsula (Mohamed et al., 2010), showing chemical diversity (Salido et al., 2004). An *A. herba-alba* EO rich in piperitone showed repellency against *I. ricinus* nymphs (El-Seedi et al., 2017), and a thujone/camphor chemotype was antifeedant and moderately toxic to *S. littoralis* (Santana et al., 2014). Our results showed lower effects for a camphor/1,8-cineole *A. herba-alba* chemotype than those reported or *T. vulgaris*. Given the chemical diversity of *A. herba-alba* wild populations, we suggest further research on chemotype–bioactivity correlations for this plant species prior to its selection for agronomical development.

Considering the plant species' rank based on the ixodicidal and antifeedant effects of their EOs, we propose the plant populations of *S. montana*, *T. zygis*, and *M. suaveolens* tested here for further agronomical development as biopesticide ingredients for the control of ticks and insects. These EOs (*S. montana*, *T. zygis*, and *M. suaveolens*) have additional biopesticidal effects such as strong nematicidal action against root-knot nematodes (*Meloidogyne javanica*), with *S. montana* being the most effective ($LC_{50} = 0.041$ μg/μl) (Andrés et al., 2012).

To further understand the effects of the active EOs, their main components (**Figure 1**) were also tested against the selected targets. The most effective larvicidal and antifeedant compounds were piperitenone oxide, carvacrol, piperitenone, and thymol. The activity of piperitenone oxide and piperitenone explained the effects of *M. suaveolens* EO. Thymol explained the effects of the EOs from *T. zygis* and *T. vulgaris*, while carvacrol was responsible for the effects of *S. montana* and *O. vulgare* subsp. *virens*.

These compounds have reported ixodicidal and/or insecticidal effects. Piperitenone epoxide and piperitenone showed strong larvicidal and repellent effects against *Aedes albopictus* (Giatropoulos et al., 2018). Piperitenone was antifeedant to *L. decemlineata* and *S. littoralis* (Kimbaris et al., 2017). However, there are no reports on the acaricidal effects of these compounds. Thymol was larvicidal to *H. lusitanicum* (Navarro-Rocha et al., 2018), and carvacrol was toxic to *Rhipicephalus turanicus* (Coskun et al., 2008) and moderately toxic to *Hyalomma marginatum* adults (Cetin et al., 2010). These compounds were repellent to *Amblyomma americanum* (Carroll et al., 2017) and showed strong toxicity against *I. ricinus* larvae and repellency against *I. ricinus* larvae and *A. americanum* nymphs (Carroll et al., 2017; Tabari et al., 2017). Carvacrol and thymol also have reported behavioral and toxic effects against several insect species, including the ones targeted here. Specifically, thymol was antifeedant to *M. persicae* (Navarro-Rocha et al., 2018). Thymol and carvacrol were antifeedant to *S. littoralis* fourth-instar larvae (Pavela, 2011) and affected the olfactory sensilla of female *S. littoralis* adults (Anderson et al., 1993). Additionally, carvacrol and thymol showed acute toxicity to *S. littoralis* third-instar larvae (Pavela, 2014), and carvacrol was toxic to *M. persicae* (Petrakis et al., 2014).

The rest of the tested compounds were not ixodicidal or antifeedant. Therefore, the activity of the moderately active EOs (*L. angustifolia*, *M. piperita*, *M. spicata*, *R. officinalis*, and *A. herba-alba*) cannot be explained by their main components (linalyl acetate, linalool, menthone, menthol, limonene, camphor, 1,8-cineole, *p*-cymene, α-pìnene, and carvone), suggesting synergistic effects. *p*-Cymene was among the most frequent synergists found, interacting with 22 terpenes commonly present in EOs (Pavela et al., 2014). Therefore, synergistic interactions among EO components could explain their ixodicidal effects.

CONCLUSION

This work has demonstrated the ixodicidal and insect antifeedant effects of EOs from experimentally cultivated AMPs. The EOs grouped as strong ixodicidal agents corresponded to *T. zygis*, *T. vulgaris*, *S. montana*, *M. suaveolens*, and *O. vulgare* subsp. *virens*. Similarly, the EOs grouped as strong antifeedants corresponded *to S. montana*, *T. zygis*, and *T. vulgaris*, followed by *O. vulgare* subsp. *virens* and *M. suaveolens*. The moderate ixodicidal EOs were from *L. angustifolia*, *M. piperita*, *M. spicata*, *A. herba-alba*, and *R. officinalis*.

The most effective larvicidal and antifeedant compounds were piperitenone oxide, carvacrol, piperitenone, and thymol, explaining the effects of *M. suaveolens*, *T. zygis*, *T. vulgaris*, *S. montana*, and *O. vulgare* subsp. *virens* EOs. The rest of the tested compounds were not ixodicidal or antifeedant. Therefore, the activity of the moderately active EOs (*L. angustifolia*,

M. piperita, *A. herba-alba*, *R. officinalis*, and *M. spicata*) cannot be explained by their main components (linalyl acetate, linalool, menthone, menthol, limonene, camphor, 1,8-cineole, *p*-cymene, α-pìnene, and carvone), suggesting synergistic effects.

T. zygis, *S. montana*, and *M. suaveolens* were better ixodicidals and *S. montana* was a better antifeedant than *T. vulgaris*. Therefore, we propose the plant populations of *S. montana*, *T. zygis*, and *M. suaveolens* tested here for further development as biopesticide ingredients for the control of ticks and insect pests.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AG-C conceptualized the study. AG-C, AO, MG, JN-R, and FV curated the data. MG, FV, and MA did the formal analysis. AG-C, AO, and FV helped with funding acquisition and resources. MG, JN-R, FV, AO, MA, and AG-C did the investigation. AO, FV, AG-C, and MA helped with the methodology. FV and AG-C wrote the original draft. AG-C, AO, MA, FV, and JN-R did the writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Larvicidal Activity of Essential Oils From *Piper* Species Against Strains of *Aedes aegypti* (Diptera: Culicidae) Resistant to Pyrethroids

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The continuous and indiscriminate use of insecticides has been responsible for the emergence of insecticide resistant vector insect populations, especially in *Aedes aegypti*. Thus, it is urgent to find natural insecticide compounds with novel mode of action for vector control. The goal of this study was to investigate the larvicidal activity of essential oils (EOs) from *Piper* species against *A. aegypti* characterized as resistant and susceptible strains to pyrethroids. The EOs from leaves of 10 *Piper* species were submitted to the evaluation of larvicidal activity in populations of *A. aegypti* in agreement with the (World Health Organization, 2005) guidelines. The resistance of the strains characterized by determining the lethal concentrations (LCs) with the insecticide deltamethrin (positive control). The major compounds of the EOs from *Piper* species was identified by GC-MS. The EOs from *Piper aduncum*, *P. marginatum*, *P. gaudichaudianum*, *P. crassinervium*, and *P. arboreum* showed activity of up to 90% lethality at 100 ppm (concentration for screening). The activities of the EOs from these 6 species showed similar LCs in both susceptible strain (Rockefeller) and resistant strains (Pampulha and Venda Nova) to pyrethroids. The major compounds identified in the most active EO were available commercially and included β-Asarone, (*E*)-Anethole, (*E*)-β-Caryophyllene, γ-Terpinene, *p*-Cymene, Limonene, α-Pinene, and β-Pinene. Dillapiole was purified by from EO of *P. aduncum*. The phenylpropanoids [Dillapiole, (*E*)-Anethole and β-Asarone] and monoterpenes (γ-Terpinene, *p*-Cymene, Limonene, α-Pinene, and β-Pinene) showed larvicidal activity with mortality between 90 and 100% and could account for the toxicity of these EOs, but the sesquiterpene (*E*)-β-Caryophyllene, an abundant component in the EOs of *P. hemmendorffii* and *P. crassinervium,* did not show activity on the three populations of *A. aegypti* larvae at a concentration of 100 ppm. These results indicate that *Piper'*s EOs should be further evaluated as a potential larvicide, against strains resistant to currently used pesticides, and the identification of phenylpropanoids and monoterpenes as the active compounds open the possibility to study their mechanism of action.

Keywords: essential oils, *Piper*, larvicides, vector control, *Aedes aegypti*, insecticide resistance

INTRODUCTION

Aedes aegypti (Linnaeus, 1762) (Mattingly et al., 1962) is a mosquito species known to transmit arboviruses such as dengue, chikungunya, and zika virus worldwide. It is a diurnal mosquito extremely adapted to urban and domestic environments (Maciel-de-Freitas et al., 2012). The rapid increase in rates of urbanization in tropical regions, lack of basic infrastructure and limited or non-existent sanitation, associated with favorable climatic conditions for the mosquito's development, have contributed to the expansion of the occurrence range of arboviruses transmitted by females of *A. aegypti* (Rebêlo et al., 1999; Carvalho and Moreira, 2016). Therefore, we have witnessed the increasing transmission of Dengue (DENV), Zika (ZIKV), and Chikungunya (CHIKV) virus in these regions.

Despite the FDA approval of a dengue vaccine (Dengvaxia) in 2019, its efficiency is restricted to people who have been previously infected by dengue and not as disease prevention for a large portion of the population. Therefore, the control of *A. aegypti* populations still represents the best line of defense. This strategy has focused on controlling the mosquito's population by means of using insecticides such as the larvicide Pyriproxyfen (Juvenil Hormone Analog–JHA), the adulticides malathion (organophosphate) and Cielo®, an insecticide containing imidacloprid (neonicotinoid) and paletrine (pyrethroid) (Valle et al., 2019). The main larvicide used worldwide was the organophosphate temephos, but by the end of 1990's, it led to the development of resistance in *A. aegypti*. In fact, in the last decades, the indiscriminate use of synthetic insecticides (for example, domestic use of pyrethroid insecticides available in the retail market, especially in epidemic periods), together with the lack of coordinated programs in multi-endemic areas, have led to the emergence of populations of *A. aegypti* resistant to different insecticides used (Maciel-de-Freitas et al., 2012; Macoris et al., 2018).

The resistance of *A. aegypti* in Brazil studied from 2005 to 2012 was characterized by the frequency and distribution of the resistance of this vector (Valle et al., 2019). The phenotypes for populations resistant to pyrethroids throughout the country have been characterized and are associated to the changes in biochemical and target site mutations V410L, G923V, I1011M, V1016I, and F1534C (Brengues et al., 2003; Saavedra-Rodriguez et al., 2007; Martins et al., 2009a,b; Lima et al., 2011; Araújo et al., 2013; Lins et al., 2014; Maciel-de-Freitas et al., 2014; Bellinato et al., 2016; Collet et al., 2016; Dolabella et al., 2016; Haddi et al., 2017; Viana-Medeiros et al., 2017; Garcia et al., 2018; Valle et al., 2019; Costa et al., 2020).

In the case of larval resistance in *A. aegypti*, two studies were carried out in the city of Belo Horizonte (MG, Brazil) (Belinato et al., 2013; Valle et al., 2019). The resistance ratio 95 (RR_{95}) to the insecticide Temephos was quantified in mosquito populations in 2005 (Belinato et al., 2013) and 2008 (Valle et al., 2019), in which resistance was observed ($RR_{95} = 5.4$ and $RR_{95} = 10.8$, respectively).

Essential oils (EOs) are odoriferous and volatile compounds found stored in plants structures, such as glands, secretory trichomes, secretory ducts, secretory cavities, or resin ducts (Ciccarelli et al., 2008; Bezić et al., 2009; Liolios et al., 2010; Morone-Fortunato et al., 2010). The production of these volatiles in plants is associated with the ecological role they display in nature, such as protecting plants against pathogens and herbivores and attraction of pollinating insects (Grodnitzky and Coats, 2002; Csekea et al., 2007; Bakkali et al., 2008). The emission of plant volatiles is associated with several messages they convey to the surrounding interacting organisms, such as volatiles used in the attraction of pollinating insects, kairomones in response to herbivores, attraction of parasitoids when damaged by herbivores, controlling the growth of pathogens in aerial parts or roots, and so forth (Grodnitzky and Coats, 2002; Csekea et al., 2007; Bakkali et al., 2008; Raveau et al., 2020). EOs from the plants *Cymbopogon* spp., *Ocimum* spp. and *Eucalyptus* spp. are well-known for their application as insect repellents and the active principles are associated with the presence of α-Pinene, Limonene, Citronellol, Citronellal, Camphor and Thymol (Nerio et al., 2010). Additionally, some plant species have been adopted in push-pull strategies for controlling insect pests in agricultures thanks to their emission of specific volatile compounds with repellent or attractive properties that lead pests away from cultivated plants and onto toxic "trap crops" (Cook et al., 2007; Alkema et al., 2019).

Several applications of volatiles of *Piper* species have been suggested because of their high potential for pest control, and due to the green technologies involved in the extraction process and the expected low environmental impact (da Silva et al., 2017; Salehi et al., 2019). The evaluation of the larvicidal activity of EOs of species of the genus *Piper* in *A. aegypti* has already been studied for the species: *Piper humaytanum*, *P. permucronatum*, *P. hostmanianum*, *P. gaudichaudianum* (de Morais et al., 2007), *P. augustum*, *P. corrugatum*, *P. curtispicum*, *P. darienense*, *P. grande*, *P. hispidum*, *P. jacquemontianum*, *P. longispicum*, *P. multiplinervium*, *P. reticulatum*, *P. trigonum* (Santana et al., 2016), *P. marginatum* (Autran et al., 2009; Santana et al., 2015), *P. klotzschianum* (Nascimento et al., 2013), *P. aduncum* (de Almeida et al., 2009; Oliveira et al., 2013; Santana et al., 2015; Scalvenzi et al., 2019), *P. corcovadensis* (da Silva et al., 2016), *P. sarmentosum* (Hematpoor et al., 2016), *P. betle* (Vasantha-Srinivasan et al., 2018; Martianasari and Hamid, 2019), *P. arboreum* (Santana et al., 2015), and *P. capitarianum* (França et al., 2021). Besides, non-volatile compounds from *Piper* species such as amides and lignans have also been described as larvicidal (Cabral et al., 2009; Kanis et al., 2018).

Despite the large number of studies with EOs from *Piper* species against larvae of *A. aegypti*, there is no assessment of their effect on strains of mosquitoes resistant to synthetic insecticides. Therefore, considering the limited number of safe chemical approaches for controlling *A. aegypti* as vectors in the field, the aim of this work is to investigate the larvicidal activity of essential oils of *Piper* species and to identify the active principle against populations of *A. aegypti* that are either susceptible or resistant to pyrethroids.

TABLE 1 | Voucher number, sampling site, and yield of essential oil from *Piper* species.

Species	Voucher #	Sites	EO yield (%)[a]
P. aduncum L.	K-0057	Campus–USP[b]	2.5
P. marginatum Jacq.	K-0223	Campus–USP	1.10
P. gaudichaudianum Kunth	K-0031	Serra do Japi[c]	0.98
P. crassinervium H.B.and K.	K-0091	Campus–USP	1.80
P. arboreum Aubl.	K-0053	Campus–USP	0.74
P. hemmendorffii C. DC.	K-1086	Campus–USP	0.86
P. cernuum Vell.	K-0137	Campus–USP	0.69
P. lucaenum var. *grandifolium* Kunth.	K-0486	Campus–USP	0.70
P. lindbergii C.DC.	K-2325	Serra do Japi	0.65
P. amalago L.	K-0110	Serra do Japi	0.66

[#]*K (Voucher number Kato-XXXX).*
[a]*From fresh leaves.*
[b]*University of São Paulo.*
[c]*Parque Municipal Serra do Japi, Jundiaí, Brazil.*

MATERIALS AND METHODS

Plant Material

The leaves of 10 species of plants belonging to the genus *Piper: Piper aduncum*, *P. marginatum*, *P. gaudichaudianum, P. crassinervium, P. arboreum*, *P. hemmendorffii*, *P. cernuum*, *P. lucaenum* var. *gradifolium*, *P. lindbergii*, and *P. amalago*, were collected in the period of January to June 2018. The vouchers were deposited at the Herbarium of USP–University of São Paulo for identification (**Table 1**). All collections were made under permits #59161-1 and 010/2018-R from the Sistema de Autorização e Informação em Biodiversidade–SISBIO and Fundação Serra do Japi, respectively.

Collection and Insect Rearing

In this study, three strains of *A. aegypti* larvae were used. The Rockefeller strain is a susceptible reference lineage (SRL) for all assays. Two other strains were collected in the regions of Pampulha (19° 51′ 04″ S; 43° 58′ 46″ W) and Venda Nova (20° 11′ 51″ S; 44° 1′ 40″ W) in Belo Horizonte, Minas Gerais, Brazil in the period of June 2018. The strains Pampulha (Pamp) and Venda Nova (VN) were evaluated and certified as resistant to pyrethroids.

The mosquitoes were kept and raised in the insectarium of the Laboratory of Physiology of Hematophagous Insects of the Federal University of Minas Gerais in accordance with the recommendations of the Ethics Committee (CEUA-UFMG) (protocol number 01/2017). The insects were maintained under controlled conditions of temperature (27 ± 1°C), photoperiod 12:12 h (L:D), and relative humidity (75%).

After the eggs hatched in dechlorinated water, the larvae and pupae were kept in plastic vats, containing fish food *ad libitum*. The adult insects were kept in cylindrical cages 30 × 90 cm with mesh on the top and with continuous access to cotton soaked in 10% sucrose solution. The females' blood meals were performed weekly on hamsters (*Mesocricetus auratus*) previously anesthetized with 0.2 mL of Thiopental® (50 mg/mL) and placed with the trichotomized abdomen on the screen of the cages so that the females could perform the blood meal for 1 h. The eggs were obtained 2 days after the meal using filter paper soaked in dechlorinated water in dark plastic pots, from which they were removed and kept in new plastic pots until the hatching time for testing.

Extraction of Essential Oils

The essential oils (EOs) were extracted from fresh leaves of each species, submitted to hydrodistillation in a Clevenger type apparatus for 4 h, using 300–500 g of fresh leaves and 500 mL of distilled water (Santos et al., 2012; Fanela et al., 2015). The EOs were collected and dried with anhydrous sodium sulfate and stored in amber bottles in a refrigerator at 4°C until the experiments were performed. The yield of EOs from *Piper* species are shown in **Table 1**.

Analysis of Essential Oils and Fractions by GC-MS

EOs samples were diluted 20 times in ethyl acetate (HPLC grade, Honeywell) and analyzed using a Shimadzu GCMS- QP2010 equipped with an HP-5ms column (length 30 m, ID 0.25 mm, film thickness 0.25 μm, Agilent) using Helium as a carrier gas (1.55 mL/min) and 1 μL of each sample was injected at 250°C with a 1:20 split. Detector temperature was set at 260°C with electron impact ionization energy of 70 eV and a scan range of *m/z*35-400 Da at 2500 spectra s^{-1}. The oven program started at 40°C for 2 min, and the temperature was increased at 5°C min^{-1} to 260°C and held for 2 min. Individual volatile compound peaks were identified using extracted ion traces of three specific reference ions and quantified by the peak area of the most abundant ion trace per compound using a custom-made analysis method in the GC-MS Postrun Analysis software (Shimadzu). Relative % of each compound was calculated by comparing the % peak area in relation to the total sum of peak areas within a sample. The identification of compounds was conducted by calculating Arithmetic retention indexes (RIs) in relation to a series of alkane standards (C8-C40, Supelco) injected using the same GC-MS method as used for the samples, according to Van Den Dool and Kratz (1963). Compound mass spectra and RIs were compared to those available in the Adams and Wiley databases (Adams, 2007), in previous studies of these *Piper* species, and confirmed by comparison to authentic standards, when available.

LC_{50} and LC_{95} for Larvicidal Activity of Deltamethrin Insecticide

The characterization of the larvae to be used in the tests as susceptible or resistant was made by assaying them with the technical grade insecticide deltamethrin (Bayer Brazil, 99.1%). The dose-response tests were performed in the range of 10–90% mortality. Thirty L3-L4 larvae (F1 generation) were separated per dose (in triplicate), requiring a minimum of 8 doses to perform the curve. The larvae were placed in 500 ml cups containing 249 ml of dechlorinated water, along with 1 ml of the

insecticide in the desired concentration, diluted in ethanol P.A. For the control group, 30 L3-L4 larvae were used in 250 mL of dechlorinated water. The concentrations of pyrethroid used for assaying Rockefeller, Venda Nova and Pampulha strains were 0.2–0, 9, 2.0–9.5, and 1.0–8.0 mg/mL, respectively.

Mortality was recorded every 10 min during the first hour and 24 h after the start of the test, as recommended by the World Health Organization (1981). Larvae that did not spontaneously move, even if subjected to mechanical stimulation, were considered dead. The LC calculation was performed using the Polo Plus program (see item on statistical analysis). The LC of the field population was divided by the LC of the Rockefeller strain to obtain the Resistance Ratio. The population was considered resistant when RR_{95} was >3 (Valle et al., 2019).

Qualitative Larvicidal Bioassays of *Piper* Essential Oils

The tests were performed in accordance with World Health Organization (2005), with modifications. The EOs of 10 species of *Piper* were diluted in a final volume of 100 ml of dechlorinated water with 2% dimethyl sulfoxide (DMSO), in a concentration of 100 ppm of each oil. Then, 30 larvae (L3–L4) from the three strains, Rockefeller, Venda Nova, and Pampulha were placed in the containers. Each experiment was carried out in three bottles (technical triplicate), being repeated five times on different days (five biological repetitions). The larvae of the control and vehicle control groups were exposed only to dechlorinated water and with 2% DMSO, respectively. Mortality was recorded at 24 h after the start of the test. Larvae not responding to mechanical stimulation were considered dead and the EOs with 90 to 100% larvicidal activity were considered active (Cheng et al., 2003; Dias et al., 2014; Intirach et al., 2016; Muturi et al., 2017).

Determination of LC_{50} and LC_{90} of *Piper* Essential Oils

EOs that showed preliminary larvicidal activity (90 to 100%) had their lethal concentrations (LCs) determined. Thus, 30 larvae L3-L4 from the three strains were submitted to different concentrations in a range of 10–90%, in a final volume of 100 mL of dechlorinated water. Each dose was assayed in duplicate, with three repetitions (biological triplicate) on different days. The larvae of the control and vehicle-control groups were exposed to dechlorinated water and with 2% DMSO, respectively, and the mortality was recorded 24 h after the start of the test.

Assays With the Major Compounds From the EOs

The major compounds from EOs characterized by GC-MS, *E*-Anethole (Sigma-Aldrich: 4180-23-8), γ-Asarone (Cayman Chemical: 11681) and (*E*)-β-Caryophyllene (Cayman Chemical: 21572), γ-Terpinene (Sigma-Aldrich: 86478), p-Cymene (Sigma-Aldrich: 121452), Limonene (Sigma-Aldrich: 45423), α-Pinene (Sigma-Aldrich: 147524), and β-Pinene (Sigma-Aldrich: 402753) were acquired commercially. Pure Dillapiole was obtained by fractionation using the Isolera Flash Chromatography system (Biotage INC). The EO of *P. aduncum* (0.5 ml) was loaded on the silica samplet and the flash chromatography was performed in the SNAP Ultra 25 g silica column using a gradient of hexane and ethyl acetate. The gradient started with 20% of ethyl acetate, after 2 min increased to 28% and in a linear increase reached 33% 12 min. Sixty fractions were collected and dillapiole was present in the fractions 30–35. The samples obtained from this fractionation were analyzed by GC-MS and a fraction with purity higher than 98% was selected for the assays.

For the larvicidal assays (qualitative bioassays), the pure compounds were diluted in dechlorinated water and 2% DMSO, in a final volume of 50 mL, to a final concentration of 100 ppm (screening concentration), containing 15 larvae of *A. aegypti* L3-L4 per cup. For the determination of LC_{50} and LC_{90}, the methodology was used as described above, using 15 larvae of *A. aegypti* L3-L4 per cup.

Statistical Analysis

The data were organized in spreadsheets using Microsoft Excel software (Office 2007). Lethal concentrations (LC) 50%, 90%, 95% and slope were obtained through Probit analysis with the aid of the Polo Plus software (Raymond, 1985). Significant differences in the LC_{50} and LC_{90} values were based on non-overlap of 95% confidence intervals (Hematpoor et al., 2017; Wang et al., 2019).

RESULTS

Determination of LC_{50} and LC_{95} for the Insecticide Deltamethrin

Based on the bioassays with Deltamethrin, the strains of Pampulha and Venda Nova were shown to be resistant to this insecticide, with the population of Pampulha (RR_{95} = 26.073) being more resistant than the population of Venda Nova (RR_{95} = 20.512). The resistance observed in these populations of *A. aegypti* for Deltamethrin is expected for pyrethroids in general, because of the similarity of the mode of action. The Rockefeller strain, defined as the susceptibility reference strain (LRS), has been maintained in the laboratory since 1881, without contact with insecticides and genetically isolated from external populations (Organização Pan-americana de Saúde (OPAS), 2005). The values of LC_{50} and LC_{95} with their 95% confidence intervals are listed in **Table 2**.

Larvicidal Activity of Essential Oils

The EOs of the leaves of 10 species of the genus *Piper* obtained from hydrodistillation were tested against the three strains (resistant and susceptible) of *A. aegypti*. EOs from 5 out of 10 species were considered active: *Piper aduncum*, *P. marginatum*, *P. gaudichaudianum*, *P. crassinervium*, and *P. arboreum*, with larvicidal activity of 90–100% at 100 ppm (**Table 3**).

EOs of these five species had their lethal concentrations, LC_{50} and LC_{90} investigated. Thus, the L3-L4 larvae of the two populations and LRS were submitted to different concentrations to achieve larval mortality in a range of 10–90%. After 24 h of exposure the EOs from *P. aduncum*, *P. gaudichaudianum*, and *P. marginatum* had the lowest LC_{50} compared to *P. crassinervium* and *P. arboreum*. The EOs from *P. aduncum* was the most

TABLE 2 | LC_{50} and LC_{95} for the technical grade deltamethrin insecticide (Bayer Brazil, 99.1%) in larvae of *Aedes aegypti*.

Strains	LC_{50} (mg/L) (95% CI)	RR_{50} (95% CI)	LC_{95} (mg/L) (95% CI)	RR_{95} (95% CI)	Slope (SD)
Rockefeller*	0.16 (0.14–0.189)	1	0.751 (0.55–1.22)	1	3.55 ± 0.45
Venda Nova	3.65 (3.21–4.08)	21.94 (18.36–26.23)	9.905 (8.29–12.80)	20.51 (14.44–29.12)	3.79 ± 0.41
Pampulha	2.94 (2.42–3.47)	17.67 (14.17–22.02)	12.590 (9.20–21.13)	26.07 (16.07–42.28)	2.60 ± 0.36

*95% CI, 95% confidence interval; LC_{50}, 50% lethal concentration; LC_{95}, 95% lethal concentration; RR_{95}, 95% resistance ratio; SD, standard deviation. *SRL—susceptibility reference lineage.*

TABLE 3 | Mortality percentage of *Aedes aegypti* larvae in resistant and susceptible strains to pyrethroids treated with essential oils of *Piper* species.

Species	Strains		
	Susceptible	Resistant	
	Rockefeller	Venda Nova	Pampulha
P. aduncum L.	100.00	100.00	100.00
P. marginatum Jacq.	100.00	97.11	98.44
P. gaudichaudianum Kunth	99.33	90.22	94.88
P. crassinervium H.B. and K.	96.22	91.11	92.66
P. arboreum Aubl.	93.11	90.66	90.00
P. hemmendorffii C. DC.	66.44	43.11	36.00
P. cernuum Vell.	45.11	42.00	30.44
P. lucaenum Kunth.	16.22	18.88	24.00
P. lindbergii C. DC.	12.00	13.55	10.22
P. amalago L.	2.66	6.22	2.22

active with LC_{50} (23.50 ppm) for Rockefeller, and LC_{50} of 25.11 ppm and 26.39 ppm to Venda Nova and Pampulha, respectively (**Table 4**).

Identification of Essential Oil Compounds by GC-MS

The EOs of the 10 species were analyzed by GC-MS and main constituents were identified based on library search, retention index (RI), and use of standard compounds when available, and expressed as relative percentage of each constituent (**Table 5**). In summary, the major compounds were identified as phenylpropanoids, sesquiterpenes and monoterpenes. The complete list of all compounds, the retention indexes, and the relative percentage of each one, for all 10 species of *Piper* analyzed is shown in **Table 6** and the GC-MS chromatograms for all species is shown in the **Supplementary Figures 1–3**.

Evaluation of the Larvicidal Activity of the Main Compounds in the EOs

The commercially available compounds *(E)*-Anethole, β-Asarone, (*E*)-β-Caryophyllene, γ-Terpinene, *p*-Cymene, Limonene, α-Pinene and β-Pinene and Dillapiole, obtained by fractionation of EO of *P. aduncum* using flash chromatography were submitted to further evaluation to determine whether they were involved as the active compounds in the EOs. Thus, pure standards were diluted in water and 2% DMSO, in a final volume of 50 mL, to a final concentration of 100 ppm (screening concentration). Out of the nine compounds evaluated, only (*E*)-β-Caryophyllene did not show activity on *A. aegypti* larvae in the three strains at the screening concentration. Nevertheless, the phenylpropanoids (Dillapiole, *(E)*-Anethole and γ-Asarone) and monoterpenes (γ-Terpinene, *p*-Cymene, Limonene, α-Pinene and β-Pinene) showed larvicidal activity in the range of 90–100% (**Table 7**). Additionally, when comparing the LC_{50} and LC_{90} of the three phenylpropanoids, Dillapiole displayed the lowest LC_{50} for the three strains, followed by *(E)*-Anethole and γ-Asarone (**Table 8**). Among the five monoterpenes tested, Limonene and γ-Terpinene showed the lowest LC_{50} for the three strains (**Table 8**).

DISCUSSION

Studies focusing on the investigation of EOs from plants from the perspective of discovering new ovicides, larvicides, adulticides and repellents have been an important strategy for controlling agricultural pests, vectors of medical-veterinary importance or urban viruses (Santos et al., 2012; Phukerd and Soonwera, 2014; Govindarajan et al., 2016; Benelli et al., 2017; Muturi et al., 2017; Luz et al., 2020a). Regarding the urban diseases in tropical regions, *A. aegypti* is considered one of the main targets since it has great dispersal capacity, is the vector of DENV, ZIKV, CHIKV viruses, and has developed a remarkable resistance to commercially available insecticides (Smith et al., 2016).

The availability of two strains of *A. aegypti* resistant to Deltamethrin (**Table 1**) prompted us to seek alternatives to control these populations by screening bioactive EOs from plant species. Despite Deltamethrin not having been used to control *A. aegypti* in Brazil, it is a stable molecule with a well-known mechanism of action, and it is a standard pyrethroid in studies with insecticide resistance.

In this article, we adopted the WHO methodology (2005) to perform larvicidal tests against *A. aegypti* (Dias et al., 2015; Luz et al., 2020a). However, as the World Health Organization does not establish criteria to recognize larvicidal activity, in the present study, we choose the level of 90–100% of larvicidal activity for selecting active EOs as previously suggested (Cheng et al., 2003; Dias et al., 2014; Intirach et al., 2016; Muturi et al., 2017). Based on this criterion, EOs from five *Piper* out of 10 species tested showed larvicidal activity (**Table 3**). The efficiency of EOs from *Piper* species as botanical insecticides against various arthropods, including mosquito larvae of the species *A. aegypti* has been previously demonstrated. For instance, EOs of *P. marginatum*

TABLE 4 | Lethal concentrations of essential oil of *Piper* species against *Aedes aegypti* larvae resistant and susceptible strains to pyrethroids, during 24 h of exposure.

Species	Strains	Slope ± SD	LC_{50} (ppm) (95% CI)	LC_{90} (ppm) (95% CI)
P. aduncum	Rockefeller	4.5 ± 0.2	23.50 (20.92–26.60)	45.25 (37.46–62.61)
	Venda Nova	4.2 ± 0.2	25.11 (22.92−27.80)	50.29 (42.37–65.56)
	Pampulha	4.3 ± 0.2	26.39 (24.69–28.40)	52.08 (45.58–62.60)
P. gaudichaudianum	Rockefeller	4.3 ± 0.2	37.88 (29.58–46.21)	75.20 (58.19–142.75)
	Venda Nova	5.6 ± 0.3	54.01 (49.50–58.95)	91.41 (79.56–115.2)
	Pampulha	3.9 ± 0.2	41.35 (34.57–49.01)	86.61 (67.51–148.57)
P. marginatum	Rockefeller	4.1 ± 0.2	39.91 (34.94–45.11)	80.85 (66.94–112.42)
	Venda Nova	5.0 ± 0.2	41.72 (37.05–46.80)	87.27 (72.22–120.23)
	Pampulha	4.0 ± 0.2	45.77 (43.65–48.05)	96.06 (87.07–108.70)
P. arboreum	Rockefeller	5.4 ± 0.3	51.63 (47.68–55.72)	89.13 (78.69–108.22)
	Venda Nova	5.6 ± 0.3	54.01 (49.50–58.95)	91.41 (79.56–115.27)
	Pampulha	5.5 ± 0.3	56.22 (52.16–60.82)	96.01 (84.15–118.36)
P. crassinervium	Rockefeller	4.9 ± 0.3	59.03 (53.36–66.47)	106.81 (88.43–152.98)
	Venda Nova	5.1 ± 0.3	63.55 (58.34–70.79)	113.21 (95.01–154.04)
	Pampulha	5.1 ± 0.3	62.96 (57.94–69.77)	112.10 (94.58–150.34)

95% CI, 95% confidence interval; LC50, 50% lethal concentration; LC90, 90% lethal concentration; SD, standard deviation.

(Autran et al., 2009), *P. aduncum* (de Almeida et al., 2009; Oliveira et al., 2013), *P. gaudichaudianum* (de Morais et al., 2007), *P. arboreum* (Santana et al., 2015), and *P. capitarianum* (França et al., 2021) have displayed an efficient larvicidal action on *A. aegypti*. However, to the best of our knowledge, the present study is the first to demonstrate the bioactivity of EOs of the genus *Piper* and the main active compounds in essential oils in strains of pyrethroid-resistant *A. aegypti* larvae.

Among the five species that showed larvicidal activity against *A. aegypti*, *P. aduncum* had a lower LC_{50} compared to the other four *Piper* considered active, and previous reports for *P. aduncum* EO activity against larvae of *A. aegypti* led to variable values of LC_{50}: 46 ppm (Santana et al., 2015); 50.9 ppm (de Almeida et al., 2009), and up to 289.9 ppm (Oliveira et al., 2013). Our average LC_{50} value of 25 ppm for EO from *P. aduncum* against the resistant strains (VN and PAMP) and SRL, is similar to that described by Scalvenzi et al., 2019 which was 23.73 ppm.

The analysis of the LC_{50} and LC_{90} of the five *Piper* species active against PAMP, VL, and SRL strains (**Table 4**) indicated comparable LCs values among them, indicating activity of *Piper* sp. EOs regardless of insect resistance to commercial pyrethroids. Such similar larvicidal activity, in populations of *A. aegypti* resistant and susceptible to the organophosphate temephos, was observed with EOs of *Syzygium aromaticum* (Myrtaceae) and *Citrus sinensis* (Rutaceae) (Araújo et al., 2016), while a study of EO from *Petroselinum crispum* (Apiaceae) showed no significant differences of the LC_{50} for EO larvicidal activity against the pyrethroid resistant and susceptible strains of *A. aegypti* (Intirach et al., 2016). Our results agree with previous studies of plant EOs and highlights their potential of acting as efficient larvicides on mosquito strains that are resistant to different types of insecticides, whose use has already led to the development of resistant populations in Brazil (for e.g.: Temephos–Valle et al., 2019; pyrethroids—this study and Costa et al., 2020) and elsewhere.

TABLE 5 | Major constituents of the EOs of *Piper* species.

Species	Major compounds (class)[a]	RI	% rel.
P. aduncum	Dillapiole (P)	1632	81.01
P. arboreum	Germacrene D (S)	1484	18.58
	δ-Elemene (S)	1339	14.53
P. crassinervium	α-Pinene (M)	932	13.95
	β-Pinene (M)	975	12.09
	(*E*)-β-Caryophyllene (S)	1422	8.01
P. gaudichaudianum	α-Humulene (S)	1457	15.50
	Bicyclogermacrene (S)	1500	13.53
P. marginatum	(*E*)-Isoosmorhizole (P)	1462	35.23
	(*E*)-Anethole (P)	1286	21.67
P. hemmendorffii	Limonene (M)	1039	30.99
	β-Pinene (M)	975	10.08
	(*E*)-β-Caryophyllene (S)	1422	9.65
P. amalago	α-Pinene (M)	932	28.80
	(*E*)-Nerolidol (S)	1566	9.2
	p-Cymene (M)	1024	8.4
P. lindbergii	α-Pinene (M)	932	61.67
	α-Copaene (S)	1378	6.4
	Limonene (M)	1039	5.3
P. cernuum	α-Pinene (M)	932	16.6
	β-Pinene (M)	975	11.5
	Bicyclogermacrene (S)	1500	10.7
P. lucaenum	Bicyclogermacrene (S)	1500	27.47
	(*E*)-Cadina-1,4-diene (S)	1527	21
	β-Myrcene (M)	992	10.7

RI, retention index; % rel., relative percentage.
[a]*P, Phenylpropanoid; S, Sesquiterpene; M, Monoterpene.*

Although *Piper* species (e.g., *P. hemmendorffii*, *P. lindbergii*, *P. amalago*, *P. cernuum*) did not show any larvicidal activity, in previous studies their major compounds such as α-Pinene (Ali

TABLE 6 | Chemical composition of the essential oils of *Piper* species.

Compounds	RI[a]	RI[b]	PAD	PAR	PAB	PCR	PGA	PAM	PHE	PLB	PAP	PCE	PLU
α-Pinene[S]	932	932	0.2	1.0	1.5	14.0	–	0.8	3.2	28.8	61.7	16.6	–
Camphene[S]	947	946	–	–	–	–	–	0.4	–	–	1.7	0.1	2.2
β-Pinene[S]	975	974	0.3	0.6	–	12.1	–	0.7	10.1	3.0	1.4	11.5	–
Sulcatone	989	981	–	–	–	6.2	–	–	–	–	–	–	–
β-Myrcene[S]	992	988	–	1.7	–	0.6	–	–	0.7	5.9	0.3	1.0	10.7
α-Phellandrene[S]	1,004	1,002	0.1	0.5	3.7	0.5	–	–	–	0.7	–	0.2	1.4
2-Carene[S]	1,010	1,008	0.1	0.7	–	1.5	–	0.8	0.5	0.7	–	0.2	0.1
α-Terpinene	1,016	1,014	–	6.8	–	–	–	–	–	–	–	4.5	–
p-Cymene[S]	1,024	1,020	0.1	3.0	1.5	0.5	–	–	0.3	8.4	1.0	9.2	–
Limonene[S]	1,039	1,024	0.1	–	2.1	1.2	0.2	0.1	30.9	–	5.3	0.8	1.9
(*Z*)-β-Ocimene[S]	1,039	1,032	1.6	–	8.5	0.1	0.5	0.1	0.3	2.1	–	0.1	4.6
(*E*)-β-Ocimene[S]	1,049	1,044	3.4	–	4.9	0.1	0.7	0.2	3.2	0.3	–	0.3	–
γ-Terpinene	1,059	1,054	0.2	22.6	–	0.1	–	–	–	–	–	9.9	0.3
α-Terpinolene[S]	1,088	1,086	0.4	11.5	–	0.1	–	–	0.1	–	–	2.7	0.2
2-Nonanone	1,092	1,087	–	3.1	–	–	–	–	–	–	–	–	–
Linalool[S]	1,100	1,095	–	–	0.6	2.4	0.7	–	–	2.0	1.6	–	5.9
(*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT)[S]	1,117	1,114	–	–	1.1	0.7	–	–	0.6	–	–	0.3	–
Camphor [S]	1,144	1,141	–	–	–	–	–	0.2	–	–	1.1	–	–
Terpinen-4-ol	1,178	1,174	–	–	–	–	–	–	–	–	0.1	0.3	1.0
α-Terpineole	1,191	1,186	–	–	–	0.6	0.4	–	0.7	–	2.3	0.3	1.1
Methyl-chavicol	1,198	1,195	–	–	–	–	–	0.2	–	–	–	–	–
Oxygenated monoterpene 1*	1,201	–	–	–	–	–	0.1	–	–	–	–	–	–
Oxygenated monoterpene 2*	1,209	–	0.1	–	–	–	0.3	–	–	–	0.1	–	–
Verbenone	1,209	1,204	–	–	–	–	–	–	–	–	–	–	–
(*Z*)-Anethole	1,253	1,249	–	–	–	–	–	6.1	–	–	–	–	–
(+)-Piperitone	1,255	1,249	0.7	–	–	–	–	–	–	–	–	–	–
(*E*)-anethole[S]	1,286	1,282	–	–	–	–	–	21.7	–	–	–	–	0.1
Safrole[S]	1,289	1,285	–	4.1	–	–	1.4	–	–	–	–	–	–
δ-Elemene	1,339	1,335	0.1	0.5	14.5	2.1	0.6	–	0.1	1.7	1.1	0.4	1.5
α-Cubebene	1,352	1,345	–	–	0.3	0.7	0.7	–	0.3	–	0.1	0.3	1.7
α-Ylanglene	1,374	1,373	0.1	–	–	1.1	0.4	–	0.1	–	–	–	–
α-Copaene[S]	1,378	1,374	0.2	–	1.4	2.9	2.8	1.4	4.1	0.2	6.4	2.1	0.7
β-Bourbonene	1,387	1,387	–	0.5	0.3	0.5	0.1	0.2	–	2.2	0.2	0.6	2.4
β-Elemene	1,394	1,389	0.2	0.2	1.4	1.0	1.8	0.5	0.6	3.3	0.3	4.4	–
(*E*)-Caryophyllene	1,404	1,408	–	–	–	–	–	–	0.5	–	–	–	–
Methyl-eugenol[S]	1,406	1,403	–	4.0	–	–	–	0.1	–	–	–	–	–
α-Gurjunene[S]	1,412	1,409	0.1	–	–	0.3	0.7	–	0.2	1.0	–	0.1	0.2
(*E*)-β-Caryophyllene[S]	1,422	1,417	0.8	0.3	8.1	8.0	4.8	2.7	9.6	0.1	0.5	7.0	2.7
β-Gurjunene	1,432	1,431	0.2	0.2	1.0	2.2	1.4	0.2	0.9	4.1	0.6	0.5	0.3
α-Guaiene	1,436	1,437	–	–	0.4	0.2	0.8	–	–	0.8	–	–	0.2
(+)-Aromadendrene[S]	1,442	1,439	–	–	0.5	1.5	4.4	–	0.4	0.3	0.2	0.4	0.9
α-Himachalene	1,446	1,449	–	–	–	–	0.8	–	–	–	–	–	0.1
α-Humulene[S]	1,457	1,452	0.9	–	4.9	2.8	15.5	0.9	2.7	1.2	0.2	2.1	0.5
Isoosmorhizole[±]	1,462	1,466	–	–	–	–	–	15.4	–	–	–	–	–
Croweacin	1,463	1,457	1.1	10.4	–	–	–	–	–	–	–	–	–
(-)-Alloaromadendrene[S]	1,464	1,458	–	–	–	0.4	–	–	0.5	0.5	0.9	0.1	0.3
Dehydro-aromadendrane	1,466	1,460	–	–	–	0.4	1.6	–	–	1.9	0.9	–	0.3
γ-Muurolene	1,480	1,479	–	–	2.1	–	2.8	0.1	1.3	0.8	1.3	0.5	0.2
Germacrene D	1,484	1,481	2.7	2.1	18.6	–	4.0	0.1	2.7	2.8	–	5.2	3.4
β-Selinene	1,490	1,490	–	–	–	–	1.2	1.1	1.0	–	0.3	0.5	–

(Continued)

TABLE 6 | Continued

Compounds	RI[a]	RI[b]	PAD	PAR	PAB	PCR	PGA	PAM	PHE	PLB	PAP	PCE	PLU
α-Selinene	1,500	1,498	1.4	1.2	2.4	–	–	0.2	1.7	2.4	0.1	–	–
Bicyclogermacrene	1,500	1,500	2.3	2.0	3.8	4.3	13.5	0.1	1.7	–	–	10.7	27
α-Muurolene	1,503	1,500	0.1	0.6	1.3	3.0	4.2	0.4	0.9	1.9	1.0	–	0.5
α-Bulnesene	1,510	1,509	0.2	–	2.1	–	–	–	–	0.4	–	0.7	–
(*E*)-Isoosmorhizole[±]	1,512	1,517	–	–	–	–	–	35.2	–	–	–	–	–
γ-Cadinene	1,517	1,513	0.1	–	–	5.1	5.7	–	1.3	2.5	1.2	0.4	–
δ-Cadinene	1,522	1,522	1.2	–	1.2	–	–	1.0	–	–	–	–	–
Myristicin	1,524	1,518	1.1	–	–	–	–	–	–	–	–	–	–
(*E*) Cadina-1,4-diene	1,527	1,533	–	–	–	4.3	10.1	–	7.1	–	1.3	0.3	21
α-Cadinene	1,537	1,537	–	–	–	–	–	–	–	–	–	–	–
Germacrene B	1,561	1,559	0.2	–	2.1	0.4	1.2	–	0.6	–	–	0.1	–
(*E*)-Nerolidol[S]	1,566	1,561	0.1	–	1.0	5.2	0.6	–	0.9	9.2	–	0.9	–
Palustrol	1,572	1,567	–	–	–	–	0.3	–	0.3	–	–	0.1	0.2
γ-Asarone[S]	1,577	1,572	–	22.0	–	–	–	–	–	–	–	–	–
Spathulenol	1,581	1,577	0.1	–	0.3	0.2	1.9	–	0.8	2.2	0.3	0.7	3.1
(-)-Caryophyllene oxide[S]	1,587	1,582	–	–	0.6	0.8	–	0.1	4.3	1.1	3.4	0.8	0.9
Veridiflorol	1,596	1,592	0.3	–	0.3	0.4	1.5	–	0.3	0.7	0.5	0.6	0.9
Guaiol	1,601	1,600	–	–	–	5.2	–	–	–	1.5	–	–	0.9
Humulene epoxide II	1,613	1,608	–	–	–	–	1.0	–	0.3	0.2	–	–	–
β-Asarone	1,623	1,616	–	–	–	–	–	0.4	–	–	0.3	–	–
Methoxy-4,5-(methylenedioxy)-propiophenone isomer[±]	1,627	1,627	–	–	–	–	–	2.3	–	–	–	–	–
Dillapiole[S]	1,632	1,620	81.0	–	2.2	1.7	–	–	–	–	–	0.1	–
epi-α-Muurolol	1,646	1,640	0.3	–	1.5	1.1	3.2	–	1.1	1.8	0.6	0.6	0.5
Torreyol	1,650	1,644	–	–	0.6	3.3	2.6	–	1.1	0.4	0.3	0.4	0.2
α-Cadinol	1,659	1,652	–	0.1	2.9	–	5.3	–	1.7	3.1	0.9	1.2	0.5
α-Asarona[S]	1,682	1,675	–	–	–	–	–	0.9	–	–	–	–	–
Apiole[S]	1,686	1,677	0.2	–	–	–	–	–	–	–	–	–	–
2-Methoxy-4,5-(methylenedioxy)-propiophenone[±]	1,717	1,713	–	–	–	–	–	4.7	–	–	–	–	–

*RI[a], Retention Index calculated against C8-C40 n-alkanes on the HP-5 m column; RI[b], Retention index from literature (Adams, 2007); PAD, Piper aduncum; PAR, Piper auritum; PAB, Piper arboreum; PCR, Piper crassinervium; PGA, Piper gaudichaudianum; PMA, Piper marginatum; PHE, Piper hemmendorffii; PAM, Piper amalago; PLB, P. lindbergii; PCE, Piper cernuum; PLU, Piper lucaenum; [S]Compound identity confirmed with an authentic standard, the remaining compounds were identified by comparing the RI and mass spectra with the Adams and Wiley databases (see text for details). [±]Compound IR corresponds to those found for Piper marginatum in Andrade et al. (2008). *Unidentified compounds.*

et al., 2014), β-Pinene (Lee and Ahn, 2013; Ali et al., 2014), (*E*)-Nerolidol (Ali et al., 2013), Limonene (Cheng et al., 2013; Lee and Ahn, 2013; Rocha et al., 2015; Nascimento et al., 2017), (*E*)-β-Caryophyllene (Ali et al., 2014, 2015), and β-Myrcene (Cheng et al., 2013; Lee and Ahn, 2013) presented larvicidal activity against *A. aegypti*. This suggests that minor compounds might negatively interfere with oil larvicidal activity, opening new possibilities to study synergisms between compounds, as their interactions are long-lasting and complex, especially because minor compounds often present biological effects.

Out of the major phenylpropanoid compounds tested, Dillapiole, (*E*)-Anethole and γ-Asarone (**Table 7**), only (*E*)-Anethole was previously reported as an active compound in essential oils against *A. aegypti* larvae. The LC_{50} interval found to (*E*)-anethole (28.0–30.6 ppm) (Rocha et al., 2015), overlaps the confidence interval found for the three strains in the present study (25.60–44.52 ppm) (**Table 8**). However, in a study carried out by Pandiyan et al. (2019) the LC_{50} confidence interval, was 48.89–51.50 ppm for the (*E*)-anethole, which is a higher value than that found in our study.

The sesquiterpene (*E*)-β-Caryophyllene was the only compound that did not show potential larvicidal activity (**Table 7**). In fact, this data is in accordance with that found by Luz et al. (2020b), but different to the LC_{50} values found by Ali et al. (2014) (26, μg/mL), Lee and Ahn (2013) (38.58 μg/mL), and Borrero-Landazabal et al. (2020) (29.28 μg/mL).

Larvae treated with *Piper* EOs that showed larvicidal activity, were completely damaged, compared with control groups, particularly in the chest and segments of the abdominal region. Specifically, the midgut region was destroyed, and content became dark. These visual observations after the exposure period to *Piper's* EOs or to major active compounds, indicate morphological (structural) changes in the larva. Therefore, the similar values of LCs in resistant and susceptible strains suggest

a mode of action unrelated to the known biochemical and target site mutations in resistant strains.

The chemical profile of the compounds described in the EOs of the *Piper* species investigated here (**Table 6**) has already been described in previous studies. For instance, Dillapiole is a typical compound for *P. aduncum* (Pino et al., 2004; de Almeida et al., 2009; Guerrini et al., 2009; Volpe et al., 2016; Scalvenzi et al., 2019); Germacrene D for *P. arboreum* (Machado et al., 1994; Mundina et al., 1998; Navickiene et al., 2006; Perigo et al., 2016; Santana et al., 2016); α-Pinene, β-Pinene, and (*E*)-β-Caryophyllene for *P. crassinervium* (Morandim et al., 2010; Morandim-Giannetti et al., 2010; Perigo et al., 2016); α-Humulene and Bicyclogermacrene for *P. gaudichaudianum* (Von Poser et al., 1994; Andrade et al., 1998; Morandim-Giannetti et al., 2010; Sperotto et al., 2013); α-Pinene for *P. amalago* (Potzernheim et al., 2006; Perigo et al., 2016) and *P. cernuum* (Bernuci et al., 2016; Perigo et al., 2016).

In case of *P. lucaenum*, the major compound Bicyclogermacrene described in our study was replaced by α-pinene in another study (Marques et al., 2015). In fact, large chemical variability in EOs of *Piper* species has already been reported (Andrade et al., 2008). For instance, EOs from 22 samples of *P. marginatum* leaves collected in different areas and ecosystems of the Brazilian Amazon, separated by up to 1000 km, exhibited different major compounds depending on the place of origin. In our study, while the species *P. marginatum* had (*E*)-Anethole as a major compound, analysis of other specimens led to the characterization of 3,4-methylenedioxy propiophenone (Macêdo et al., 2020), and (*Z*)- or (*E*)-Asarone, and Patchouli alcohol (Autran et al., 2009) as major compounds. Such variability can result from different environmental conditions,

TABLE 7 | Percentage of dead larvae after 24 h of exposure to major compounds of the studied *Piper* species at concentration 100 ppm.

Major compounds of the studied *Piper* species	Strains		
	Susceptible	Resistant	
	Rockfeller	Venda Nova	Pampulha
Dillapiole	100	100	100
E-Anethole	98.89	96.67	100
γ-Asarone	98.89	96.67	97.78
(*E*)-β-Caryophyllene	0	0	0
γ-Terpinene	94.44	93.3	100
p-Cymene	91.1	92.2	90
Limonene	100	100	100
α-Pinene	90	91.1	100
β-Pinene	95.5	97.7	91.1

TABLE 8 | Evaluation of lethal concentrations of major compounds in *Aedes aegypti* larvae of resistant and susceptible strains to pyrethroids during 24 h exposure to major compounds of *Piper* species.

Compounds	Strains	Slope ± SD	LC_{50} (ppm) (95% CI)	LC_{90} (ppm) (95% CI)
Dillapiole	Rockfeller	2.6 ± 0.2	15.06 (11.94–18.33)	46.16 (35.62–68.71)
	Venda Nova	2.6 ± 0.2	15.75 (12.01–19.74)	47.96 (35.21–77.11)
	Pampulha	2.6 ± 0.1	17.60 (14.24–21.28)	54.56 (41.77–82.48)
E-Anethole	Rockfeller	4.0 ± 0.3	34.41 (25.60–42.10)	71.03 (54.85–136.85)
	Venda Nova	4.3 ± 0.3	38.20 (29.33– 47.26)	75.39 (57.74–153.58)
	Pampulha	3.9 ± 0.3	38.98 (33.57–44.52)	82.72 (67.24–120.97)
γ-Asarone	Rockfeller	3.7 ± 0.3	32.65 (29.91–35.20)	71.92 (64.20–83.85)
	Venda Nova	3.3 ± 0.1	37.85 (34.73–40.96)	92.52 (79.59–114.69)
	Pampulha	3.3 ± 0.3	36.23 (31.02–41.20)	88.34 (71.06–130.59)
	Rockfeller	2.7 ± 0.2	25.29 (21.25–29.30)	74.77 (59.26–108.25)
γ-Terpinene	Venda Nova	2.6 ± 0.2	24.58 (21.87–27.26)	76.33 (64.23–96.71)
	Pampulha	2.7 ± 0.2	25.00 (22.41–27.58)	72.55 (61.87–89.92)
	Rockfeller	3.6 ± 0.3	44.80 (41.23–48.48)	100.71 (88.53–119.53)
p-Cymene	Venda Nova	3.4 ± 0.3	49.25 (45.30–53.52)	115.51 (99.70–141.21)
	Pampulha	3.5 ± 0.3	47.39 (43.60–51.43)	109.83 (95.47–132.59)
	Rockfeller	3.1 ± 0.2	21.86 (18.43–25.33)	55.02 (45.22–72.76)
Limonene	Venda Nova	3.2 ± 0.2	23.23 (18.76–27.97)	57.94 (45.53–85.24)
	Pampulha	3.0 ± 0.2	21.92 (17.01–27.27)	58.38 (44.19–93.46)
	Rockfeller	4.2 ± 0.2	44.17 (37.68–50.50)	71.92 (74.59–112.61)
α-Pinene	Venda Nova	4.1 ± 0.2	45.17 (37.72–52.43)	92.52 (76.84–124.16)
	Pampulha	3.9 ± 0.2	45.70 (39.48–51.80)	96.49 (82.01–122.45)
β-Pinene	Rockfeller	2.8 ± 0.1	32.97 (26.97–38.92)	93.11 (75.49–126.12)
	Venda Nova	2.8 ± 0.1	33.35 (26.31–40.40)	95.70 (75.13– 138.87)
	Pampulha	2.6 ± 0.1	35.13 (26.02–44.53)	105.59 (78.01–179.01)

95 % CI, 95% confidence interval; LC_{50}, 50% lethal concentration; LC_{90}, 90% lethal concentration; SD, standard deviation.

soil composition, development, biotic factors, and plant genetic diversity (Gobbo-Neto and Lopes, 2007; Silva et al., 2019; Mollaei et al., 2020).

CONCLUSION

Our results suggest the promising role of the EOs of these five species of *Piper* as an alternative in controlling *A. aegypti* mosquito larvae of susceptible and insecticide resistant strains. The efficacy of these EOs suggest their use as alternative bioinsecticides in the management of insecticide resistant mosquitoes. Despite the ease of obtaining EOs by hydrodistillation, which is an advantage together the green appeal of such products, their high chemical variability may represent a potential drawback for product development unless a rigorous cultivation control or full understanding of the regulatory processes in the biosynthesis of these phenylpropanoids are achieved.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AP, MK, and GP designed the research carried. AP, MK, GP, LY, and MS interpreted the data and contributed to writing the manuscript. AS, RP, and WN contributed to methodology and investigation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.685864/full#supplementary-material

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Allelopathic, Phytotoxic, and Insecticidal Effects of *Thymus proximus* Serg. Essential Oil and Its Major Constituents

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The chemical profile of *Thymus proximus* essential oil (EO) and its allelopathic, phytotoxic, and insecticidal activity was evaluated. Carvacrol, p-cymene, and γ-terpinene were detected as the major components of the EO, representing 85.9% of the total oil. About 50 g fresh plant material of *T. proximus* in a 1.5-L air tight container completely inhibited the seed germination of *Amaranthus retroflexus* and *Poa anuua*. Meanwhile, the EO exhibited potent phytotoxic activity, which resulted in 100% germination failure of both the test species when 2 mg/ml (for *A. retroflexus*) and 5 mg/ml (for *Poa annua*) oil was applied. The EO also triggered a significant insecticidal activity on *Aphis gossypii* with a LC_{50} value of 6.34 ppm. Carvacrol was identified as the main active compound responsible for both the plant suppressing effect and the insecticidal activity of the EO. Our study is the first on the allelopathic, phytotoxic, and insecticidal activity of *T. proximus* EO, and the determination of the responsible compound, which indicated their potential of being further explored as environment friendly biopesticides.

Keywords: phytotoxicity, biopesticides, carvacrol, P-cymene, γ-terpinene

INTRODUCTION

Essential oils (EOs) are mixtures of plant-derived secondary metabolites that are extensively applied in food preservation and medical practices for thousands of years (Majewska et al., 2019; Suteu et al., 2020; Giunti et al., 2021). Many aromatic plants are known for their extraordinary ability to produce a large amount of EOs that can repel grazers, kill pests, or inhibit the growth of competing plants growing in the neighborhood (Willmer et al., 2009; Aungtikun et al., 2021; Han et al., 2021; Sousa et al., 2021). Due to these qualities, certain EOs obtained from aromatic plants, including their major constituents, have the potential to be used as environmentally compatible alternatives to synthetic pesticides and herbicides. Successful commercialized examples include clove oil, which is the main active ingredient in the herbicide Burnout II (Bonide Products Inc., Oriskany, NY, USA), and cinmethylin, the phytotoxin 1,4-cineole's derivative that can be detected in EOs of many plants (Grayson et al., 1987; Ahuja et al., 2015). On the other hand, the commercial production of pest management products based on plant EOs appears to have lagged significantly behind, indicating a major disconnect between academic research and industrial practice (Isman, 2017).

However, there are some commercial pesticides that contain plant EOs. For example, a commercial product named as "Rice Weevil Eradication" (manufacturer: Hub Club, Siheung, Korea) containing cinnamon [*Cinnamomum cassia* (L.) J. Presl] oil as its active ingredient (Yang et al., 2020). Ecotrol Plus, the flagship agricultural product produced and marketed by KeyPlex Co. (Winter Park, FL, USA), introduced in 2003, contains 10% rosemary oil, 2% peppermint oil, and 5% geraniol as active ingredients.

Worldwide, synthetic chemicals are used in agriculture; however, the extensive application has triggered resistance in pests, not to mention that they can cause many problems not only to the environment but also to human health. Compared to synthetic chemicals, plant-derived natural compounds have the advantages of fast biodegradability, low risk for subsequent pest/weed resistance, and relatively weak toxicity to non-target organisms (Chandler et al., 2011; Isman, 2015; Pavela and Benelli, 2016).

To the best of our knowledge, environment friendly agricultural chemicals are particularly important to drylands, which are characteristic for low precipitation and simple, fragile soil microbiota, which almost unavoidably cause slow degradation rate of synthetic chemicals; the accumulation of synthetic compounds subsequently might result in acute and chronic toxicity to human and herds and pose threat on the environment such as suppressing the growth of desert plants, which lead to increased wind soil erosion (Pavela, 2015). Due to the occurrence of resistance of agricultural pests to synthetic chemicals, farmers have to either increase the amount of application or switch to a new type of pesticides and herbicides, which may considerably increase the costs of maintaining dryland farms (Benhalima et al., 2004; Ahmad and Jaiswal, 2015). In addition, dryland harbors very rich natural resource of medicinal aromatic plants. Many desert aromatic plants belonging to the genus *Thymus* are known for their outstanding ability to produce high productivity and quality of EOs, which have been widely used in pharmaceutical, food, and cosmetic applications (Stahl-Biskup and Saez, 2002; Imelouane et al., 2009). *Thymus proximus* Serg, for example, a dense and robust shrub distributes over a wide range of mountainous regions and predominantly scatters in northwest China and Central Asia dryland (Wu et al., 1983), was found to have antimicrobial, antioxidant, and other biological activities (Jia et al., 2010). *T. proximus* is known for its high productivity of EOs, and like reports on some other desert aromatic plants including *Thymus* species growing in the drylands, its EO might have the allelopathic effect that can either act directly as volatile allelochemicals or accumulate in the soil to impact the growth of neighboring plants (Barney et al., 2009; Inderjit et al., 2011; Ali et al., 2014, 2015; Alexa et al., 2018; Vaiciulyte and Loziene, 2020).

Although some biological activities, such as antimicrobial activity of *T. proximus* EO, have been reported before, its allelopathic, phytotoxic, and insecticidal activities are not studied, and the bioactive compound(s) remains unclear. The objectives of our study include: (i) evaluation of the phytochemical profile of the EO produced by the desert plant *T. proximus* growing in Xinjiang province of China; (ii) assessment of the allelopathic, phytotoxic, and pesticidal effects of the EO and its major components; and (iii) determination of the major active component responsible for the biological activities of the EO.

MATERIALS AND METHODS

Plant Material

Aboveground *T. proximus* Serg. material (flowering shoot) was collected in Tianshan mountains (Lat 43.4268°N, Lon 87.1764°E, with an elevation of 2,006 m) in Xinjiang Province, China in June, 2019. Specimens were identified by Professor Li Wenjun, and a voucher specimen (XJBI018367) was deposited at the Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences Ownbey Herbarium.

Extraction of the EO

About 200 g of fresh materials of *T. proximus* was hydrodistillated for 4 h using a Clevenger-type apparatus to extract the EO, and this procedure was repeated three times (altogether 600 g plant material was used) to yield enough oil for the gas chromatography/mass spectroscopy (GC/MS) analysis and the following bioassay. The oil was then dried using anhydrous Na_2SO_4 and kept at 4°C.

GC/MS Analysis

The GC/MS analysis was performed to determine the chemical profile of *T. proximus* EO using a 7890A/5975C GC/MS system (Agilent Technologies, Palo Alto, CA, USA) equipped with a (5%-phenyl)-methylpolysiloxane phase column (30 m × 0.25 mm; film thickness 0.25 μm), DB-5MS (Agilent J&W Scientific, Folsom, CA, USA). The experimental conditions were programmed as follows: Helium (carrier gas) at a flow rate of 1 ml/min; the oven temperature was first held at 50°C for 10 min and then programmed from 50 to 120°C at a rate of 1.5°C/min and from 120 to 240°C at 20°C/min and then held for 5 min; injector and detector temperature: 280°C; sample volume: 0.1 μl; split ratio: 50:1; mass spectra: 70 eV, mass range: *m*/*z* 40–800 amu. Identification of the compounds was determined by comparison of their mass spectra and retention indices (RIs), which were determined by the linear interpolation relative to retention times of a standard mixture of C_7-C_{40} *n*-alkanes with the data given in National Institute of Standards and Technology (NIST) and published literature (Oladipupo and Adebola, 2009; Shao et al., 2018).

Allelopathic Effect

Fresh stems and leaves of *T. proximus* were arranged into plastic containers (13.5 × 13.5 × 8.5 cm, volume 1.5 L) at the following ratios: 0 g, 6.67 g, 13.33 g, and 26.67 g/L containers. Their allelopathic potential was assessed by performing bioassays against *Amaranthus retroflexus* L. and *Poa annua* L., which grow in the same habitat alongside *T. proximus*. Seeds of receiver species were surface sterilized with 2% sodium hypochlorite before use. Distilled H_2O (5 ml) was added to each Petri dish (ϕ 9 cm, lined with a layer of filter paper), followed by sowing of 20 seeds. Each container received one Petri dish that was placed onto

the plant material. Containers without plant materials (0 g) were used as the control. All containers were kept open for 5 min each day to allow in the fresh air. *A. retroflexus* and *P. annua* seedlings were measured after 5 and 7 days of incubation, respectively, due to relatively slow development of *P. annua* seedlings. Three replicates were prepared for the bioassay and in total 50 seedlings were measured (Williamson and Richardson, 1988; Wei et al., 2019; $n = 50$).

Phytotoxic Effect of the EO and Its Major Components

Amaranthus retroflexus and *P. annua* were used to evaluate the phytotoxic activity of the EO and its major ingredients. p-Cymene, γ-terpinene, and carvacrol (purity 98%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Seeds of the test species were surface sterilized with 2% sodium hypochlorite before application of the oil and its major components. *T. proximus* oil and the major components were first dissolved in dimethyl sulfoxide (DMSO, 0.1% v/v final concentration) and then diluted with the distilled water containing Tween 80 (final concentration 0.02%) to yield solutions at 0.25, 0.5, 1, 2, 5, and 10 mg/ml for the assay. Previously, DMSO has been adopted in similar bioassays due to the fact that essential oils and oil constituents are soluble in it, and that DMSO does not pose significant inhibitory effect on test plants (Tanveer et al., 2012; Pinto et al., 2015). The mixture of the three major constituents was prepared by combining p-cymene, γ-terpinene, and carvacrol at the ratio of 44.3:33.2:8.5, which was identical to their relative percentage in the EO to test their possible synergistic/antagonistic effect.

About 5 ml of solutions were added to each Petri dish (ϕ 9 cm; controls received 5 ml of distilled H_2O containing 0.1% DMSO and 0.02% Tween 80), followed by sowing of 10 test seeds. Petri dishes were sealed with parafilm and kept in a growth cabinet at 25°C with a photoperiod L:D = 16:8. *A. retroflexus* and *P. annua* seedlings were checked and measured after 5 and 7 days of incubation, respectively, due to relative slow development of *P. annua* seedlings. Five replicates were performed for the assays, and in total 50 seedlings were measured ($n = 50$; Shao et al., 2018).

Insecticidal Activity of the EO

Thymus proximus oil, p-cymene, γ-terpinene, carvacrol and their mixture (ratio of 44.3:33.2:8.5, the relative percentage in the EO) at 2.5, 5, 10, 20, 50, and 100 ppm was impregnated into the Whatman No.2 filter paper (Maidstone, Kent, United Kingdom) discs (1 × 1 cm), which were then taped onto the inner side of the lid of each Petri dishes (9 cm in diameter) to avoid direct contact between the EO/major components and *Aphis gossypii* Glover. Thirty adults of *A. gossypii* were placed onto a healthy fresh black nightshade (*Solanum nigrum*) leaf on a layer of moist filter paper. All Petri dishes were covered and kept in an incubator (25 ± 2°C, photoperiod L:D = 16:8) for 2 days. Mortalities of the adults were determined at 24-h intervals after treatment. Three replicates were performed to measure the insecticidal activity, which was expressed as percent mean mortalities of the adult *A. gossip* (Laborda et al., 2013; Zhou et al., 2019).

Statistical Analyses

The bioassay experiment followed a completely randomized design with five replications and 50 seedlings for each treatment. Results were expressed as mean ± SE of the mean. One-way ANOVA ($p < 0.05$) was applied using the IBM SPSS statistical package version 21.0 (IBM SPSS, Armonk, NY, USA) for Windows to examine whether the difference of the allelopathic, phytotoxic, and insecticidal effects of the EO produced by *T. proximus*, their major constituents, that is, p-cymene, γ-terpinene, and carvacrol and their mixture tested at different concentrations was significant; then all data were further processed using the Fisher's least significant difference (LSD) test at $p < 0.05$ level to compare the difference among treatments. The inhibitory concentration required for 50% inhibition (IC_{50}/LC_{50}) values were calculated using the PROBIT analysis (SAS/STAT User's Guide; SAS Institute Inc., Cary, NC, USA).

RESULTS

Essential Oil Yield and Composition

The EO of *T. proximus* was obtained by the traditional hydrodistillation method using fresh aboveground plant materials. The yield was 0.35% (v/w, volume/fresh weight). Eventually, 18 compounds were determined, which accounted for 98.51% of the total oil, whereas 1.49% of the oil remained unclassified. The most abundant components were p-cymene (44.26%), γ-terpinene (33.17%), and carvacrol (8.47%), which represented 85.9% of the total oil. Monoterpene hydrocarbons accounted for 86.60% of the total oil, whereas oxygenated monoterpenes and sesquiterpene hydrocarbons represented 10.05 and 1.86% of the total oil, respectively (**Table 1**).

Allelopathic Potential

The allelopathic effect of volatile organic compounds (VOCs) released by *T. proximus* was investigated by arranging fresh aboveground plant parts into air-tight plastic containers; *A. retroflexus* (dicot) and *P. anuua* (monocot), which are found growing in the same habitat alongside *T. proximus*, were selected as the test species VOCs released by *T. proximus* at 6.67 g/L containers suppressed radical elongation of *A. retroflexus* and *P. anuua* by 40.1 and 31.1%, respectively, and 13.33 g/L treatment resulted in the reduction of the root elongation by 48.8 and 63.6% for *A. retroflexus* and *P. anuua*, respectively. About 26.67 g/L treatment basically prohibited the seed germination of two test species. *P. annua* (monocot) was apparently more sensitive compared to *A. retroflexus* (dicot); the IC_{50} values were 14.69 and 14.42 g for *A. retroflexus* and *P. annua* roots, and 20.595 and 16.316 g for shoots, respectively (**Figure 1**).

Phytotoxic Activity Bioassay

Phytotoxic activity of the EO (concentrations applied ranging from 0.25 to 5 mg/ml) and its major components was assessed by comparing their plant regulatory effect on the seedling growth of *A. retroflexus* and *P. annua*. For *A. retroflexus,* p-cymene promoted the root development of *A. retroflexus* at 0.5 mg/ml; however, the inhibitory activity was observed with the increase of concentration, and 5 mg/ml treatment resulted in 93.54%

TABLE 1 | Chemical composition of *Thymus proximus* essential oil.

Compounds	RI[a]	RI[b]	Area (%)	Identification
α-Thujene	913	924	1.76	MS, RI
α-Pinene	928	938*	0.93	MS, RI
(-)-Camphene	946	952	0.66	MS, RI
4-Thujene	965	969	0.3	MS, RI
β-Pinene	976	982	1.41	MS, RI
δ-Carene	1,006	1,004	2.52	MS, RI
p-Cymene	1,011	1,012	44.26	MS, RI
1,5-Dimethyl cyclooctadiene	1,018	1,017	0.49	MS, RI
β-(Z)-Ocimene	1,038	1,041	0.63	MS, RI
γ-Terpinene	1,047	1,056	33.17	MS, RI
Terpinolene	1,050	1,084	0.47	MS, RI
Borneol	1,142	1,173	0.61	MS, RI
Carvacrol	1,273	1,287	8.47	MS, RI
2-Ethyl-4,5-dimethylphenol	1,281	1,300	0.53	MS, RI
Thymol	1,284	1,287	0.05	MS, RI
Durenol	1,324	1,319	0.39	MS, RI
Caryophyllene	1,401	1,415	1.08	MS, RI
β-Bisabolene	1,495	1,489	0.78	MS, RI
Monoterpene hydrocarbons			86.6	
Oxygenated monoterpenes			10.05	
Sesquiterpene hydrocarbons			1.86	
Total identified			98.51	

RI[a], Retention index measured relative to n-alkanes (C_7-C_{40}) using a DB-5MS column; RI[b], Retention index from literature; MS, mass spectra.

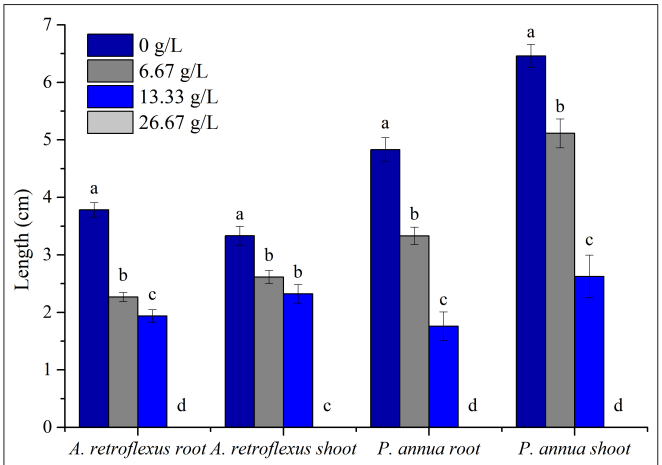

FIGURE 1 | Allelopathic effect of volatile organic compounds (VOCs) released by *Thymus proximus* on the root and shoot elongation of *Amaranthus retroflexus* and *Poa annua*. Means with different letters indicate significant differences at $p < 0.05$ level according to the Fisher's least significant difference (LSD) test.

reduction on the root development. γ-Terpinene exhibited relatively stronger activity against *A. retroflexus*, inhibiting the root length by 52.35% at 2 mg/ml, and 89.55% at 5 mg/ml. The third major constituent, carvacrol, showed remarkably stronger activity compared with the other two compounds, which completely suppressed the seed germination at the lowest concentration tested (0.25 mg/ml). The mixture of these three major components exhibited stronger activity than p-cymene and γ-terpinene but much weaker activity than carvacrol, which reduced the root length by 33.92% at 0.5 mg/ml, and 98.94% at 1 mg/ml. When the concentration reached 2 mg/ml, the seed development was completely prohibited. In conclusion, the EO exerted more potent activity than p-cymene and γ-terpinene but much weaker activity than carvacrol; the strength of the EO was comparable but still somewhat weaker than the mixture; the IC_{50} values of p-cymene, γ-terpinene, mixture, and the EO were 4.52, 3.78, 2.06, and 2.60 mg/ml, respectively (**Figure 2**, **Table 2**).

Similarly, for the monocot plant *P. annua*, carvacrol exhibited the most potent activity, which completely suppressed its seed germination at 0.25 mg/ml, the lowest concentration applied in the assay. The EO exhibited comparable activity compared with p-cymene and γ-terpinene, whose IC_{50} values were 3.65, 3.89, and 3.63 mg/ml, respectively, and the mixture showed much stronger activity with the IC_{50} value of 1.62 mg/ml (**Figure 2**, **Table 2**).

However, the seedling growth exhibited a similar pattern as the root development to a lesser extent. Carvacrol caused complete failure of the seed development of *P. annua* and *A. retroflexus* at the lowest concentration tested (0.25 mg/ml), whereas the other components and EO started to inhibit shoot growth of *A. retroflexus* at 0.25 mg/ml as well, representing 13.32, 20.37, 23.02, and 35.36% for p-cymene, γ-terpinene, a mixture of main components, and EO, respectively. When the concentration

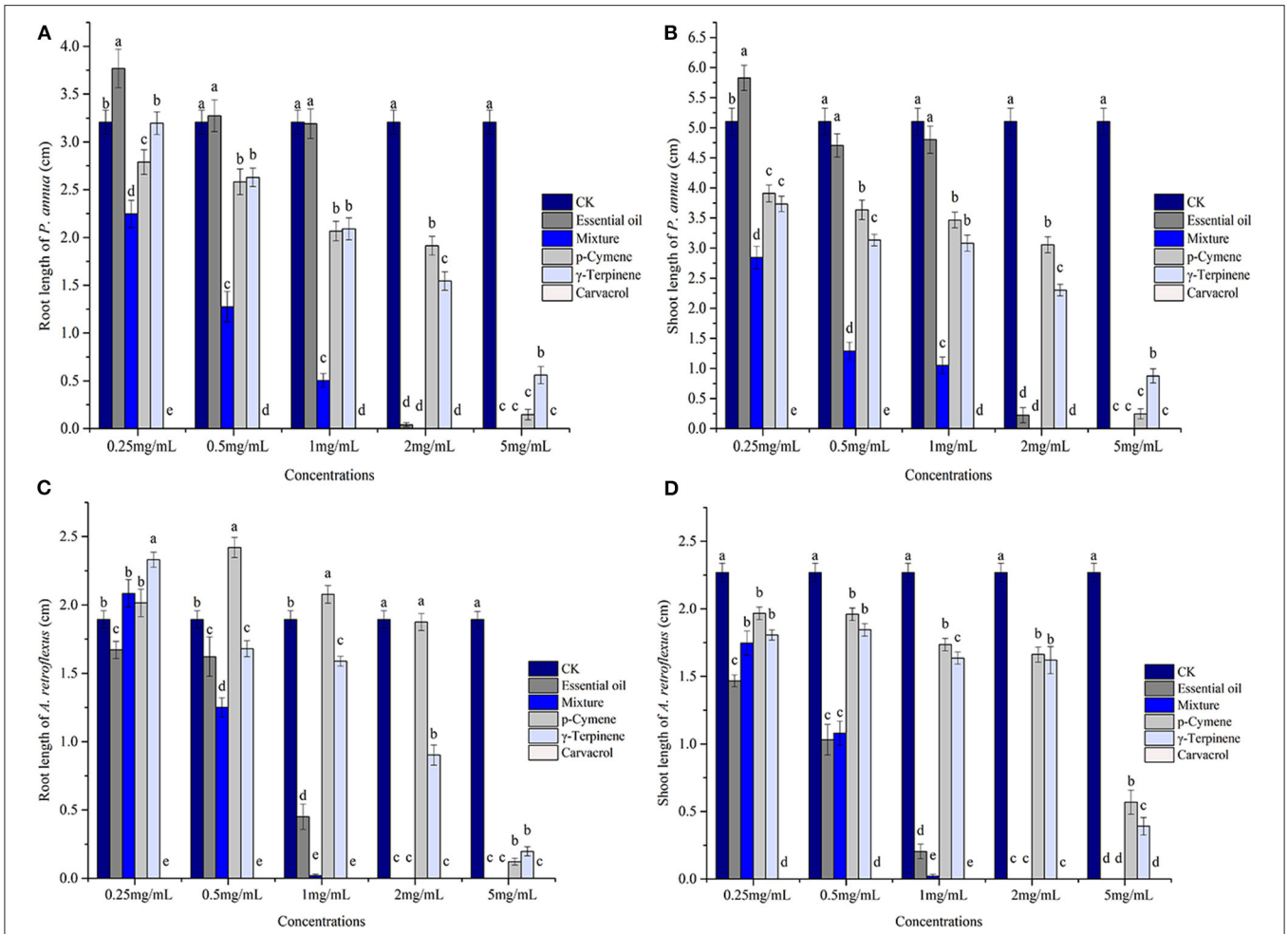

FIGURE 2 | Phytotoxic effects of *Thymus proximus* essential oil (EO) and its major constituents, p-cymene, γ-terpinene, carvacrol, and their mixture on the seedling growth of *Poa annua* and *Amaranthus retroflexus* ($n = 50$). Different letters represent a significant difference at $p < 0.05$ level according to the Fisher's LSD test. **(A)** root length of *P. annua*; **(B)** shoot length of *P. annua*; **(C)** root length of *A. retroflexus*; and **(D)** shoot length of *A. retroflexus*.

raised to 5 mg/ml, the EO and mixture of major ingredients completely inhibited the shoot growth, while p-cymene and γ-terpinene suppressing the shoot growth of *A. retroflexus* by 74.93 and 82.76%, respectively. The effect of *P. annua* is similar to *A. retroflexus*, with IC_{50} values of 3.93, 3.55, 0.83, and 3.35 mg/ml for p-cymene, γ-terpinene, and mixture of principal components and EO, respectively (**Figure 2**, **Table 2**). The dose–response curve of the phytotoxic activity was shown in **Figure 3**.

Insecticidal Activity

The insecticidal activity of *T. proximus* EO was determined on adjusted mortality rates of *A. gossypii* at concentrations ranging from 2.5 to 100 ppm. Results showed that *T. proximus* EO had obvious behavioral avoidance and lethal action on *A. gossypii*. The EO, its major components, and their mixture killed all the tested insects at the dose of 100 ppm after 24 h of exposure. The mortality rates of *T. proximus* under 2.5, 5, 10, 20, and 50 ppm, the EO treatments reached 15, 15.33, 40.67, 93.33, and 99.00%, respectively, after 24 h of exposure to the oil. Carvacrol showed the strongest activity against *A. gossypii* with a LC_{50} value of 0.1 ppm, compared with the LC_{50} values of 9.63, 5.69, 6.8, and 7.34 ppm for the EO, p-cymene, γ-terpinene, and the mixture of three major components, respectively (**Table 3**). The dose–response curve of the pesticidal activity was shown in **Figure 4**.

DISCUSSION

A large body of literature has reported the chemical composition of the EOs produced by *Thymus* species, which were frequently found to have abundant thymol, carvacrol, p-cymene, γ-terpinene, caryophyllene oxide, etc. (Kabouche et al., 2005; Hazzit et al., 2006; Sanja and Milka, 2015; Zeynep et al.,, 2018; Behnaz et al., 2020). Marla et al. (2008) investigated the chemical composition of *T. vulgaris* EO and found it was rich in thymol (57.7%), p-cymene (18.7%), and carvacrol (2.8%). Behnaz et al. (2017) evaluated the EOs produced by14 *Thymus* accessions belonging to 10 species and found that their major components were thymol (12.4–79.74%), carvacrol (4.37–42.14%), geraniol

TABLE 2 | Regression analyses of the phytotoxic effect of *Thymus proximus* essential oil (EO), its major constituents p-cymene, γ-terpinene, and carvacrol, and their mixture on the root and shoot growth of *Amaranthus retroflexus* and *Poa annua*.

Test plants	EO/major components	Regression equation	r^2	IC_{50} (mg/ml)	95% CL
A. retroflexus root	p-Cymene	$y = 15.753x^2 - 71.642x + 51.737$	0.951	4.52	3.93–5.11
	γ-Terpinene	$y = 26.643x - 50.704$	0.958	3.78	3.25–4.31
	Carvacrol	–	–	–	–
	Mixture	$y = -10.857x^2 + 93.768x - 97.324$	0.965	2.06	1.48–2.64
	Essential oil	$y = 26.221x - 18.216$	0.873	2.60	2.05–3.15
A. retroflexus shoot	p-Cymene	$y = 6.3663x^2 - 24.564x + 34.084$	0.927	3.03	2.71–3.35
	γ-Terpinene	$y = 7.3667x^2 - 30.733x + 46.836$	0.903	4.27	3.94–4.60
	Carvacrol	–	–	–	–
	Mixture	$y = -7.4577x^2 + 64.903x - 37.786$	0.961	1.67	1.23–2.11
	Essential oil	$y = -4.7013x^2 + 45.681x - 9.1479$	0.964	1.54	1.17–1.91
P. annua root	p-Cymene	$y = 6.143x^2 - 18.292x + 28.067$	0.938	3.89	3.49–4.29
	γ-Terpinene	$y = 19.82x - 21.954$	0.982	3.63	3.24–4.02
	Carvacrol	–	–	–	–
	Mixture	$y = 17.99x + 20.915$	0.903	1.62	1.25–1.99
	Essential oil	$y = 4.8065x^2 + 4.751x - 31.199$	0.853	3.65	2.93–4.37
P. annua shoot	p-Cymene	$y = 7.4384x^2 - 29.138x + 49.486$	0.923	3.93	3.57–4.29
	γ-Terpinene	$y = 3.335x^2 - 7.1789x + 33.397$	0.965	3.55	3.28–3.82
	Carvacrol	–	–	–	–
	Mixture	$y = 13.68x + 38.628$	0.889	0.83	0.55–1.11
	Essential oil	$y = 31.638x - 55.906$	0.865	3.35	2.71–3.99

r^2: adjusted coefficient of determination.
IC_{50}: the inhibitory concentration required for 50% inhibition.
95% CL: 95% confidence limits.
(–): not calculable.

(0.3–22.44%), and p-cymene (0.8–12.86%). As of the EO of *T. proximus*, Jia et al. (2010) identified 60 compounds from the EO of *T. proximus*, which accounted for 99% of the total oil, with p-cymene (25.4%), γ-terpinene (18.0%), and thymol (28.0%) being the most abundant components, which was consistent with our results. It is noteworthy to mention that there are various factors that might affect the chemical profile of plant-derived EOs including but not limited to species variety, growth period, geographic locality, surrounding climate, stress, and post-harvest processing, etc. (Raut et al., 2014).

Many plants are capable of synthesizing and releasing VOCs that are found to play key roles in attracting seed-disperser and pollinators, defense against pathogenic fungi and herbivores, interplant signaling, and allelopathic action (Pichersky and Gershenzon, 2002; Dudareva et al., 2013; Adebesin et al., 2017). Wei et al. (2019) reported that VOCs produced by *Atriplex cana* Ledeb. negatively affected the seedling development of *A. retroflexus* and *P. annua*, with 80 g of fresh *A. cana* leaves and stems in a 1.5-L airtight container almost completely prohibited the seed germination of the test plants. Volatiles emitted from the leaves of star anise (*Illicium verum* Hook. f.) totally inhibited the seedling growth of *Lactuca sativa* L., and the major volatile compounds were identified as α-pinene, β-pinene, camphene, 1,8-cineole, D-limonene, camphor, and L-fenchone (Kang et al., 2019). Tang et al. (2019) reported the allelopathic activity of VOCs released by the exotic invasive weed *Xanthium sibiricum* and found that at 80 g fresh plant materials in a 1.5-L airtight container, root growth of receiver plants *A. retroflexus* and *P. annua* was reduced by 49.1 and 69.6%, respectively. The release of volatile allelochemicals into the surroundings are believed to be able to facilitate the dominance of the donor species; and interestingly, herbivore-infested plants are found to produce volatiles to mediate plant–plant interactions by triggering the expression of volatiles of neighboring unattacked plants to decrease their susceptibility to herbivores (Ruther and Kleier, 2005).

There have been a number of reports on the phytotoxic effects of EOs and their constituents, especially monoterpenes, on the seed germination and seedling growth of the test species (Langenheim, 1994; Vokou et al., 2003; Nishida et al., 2005; Salamci et al., 2007). EOs produced by *Thymus* species have also been studied for their phytotoxicity. Ali et al. (2015) found that EOs obtained from different plant parts of *T. algeriensis* inhibited both the shoot and root growth of *Medicago sativa* L. and *Triticum astivum* L. seedlings at the lowest tested concentration (0.1 mg/ml). *T. daenensis* Celak. EO significantly suppressed the seedling development of *A. retroflexus*, and 600 μl/l oil almost completely prohibited its seedling growth (Kashkooli and Saharkhiz, 2014). Another species of the *Thymus* genus, *T. eigii*, showed significant herbicidal activity against *L. sativa*, *Lepidium sativum* L., and *Portulaca oleracea* L., with 0.5 mg/ml oil completely suppressed the seed germination of all the tested

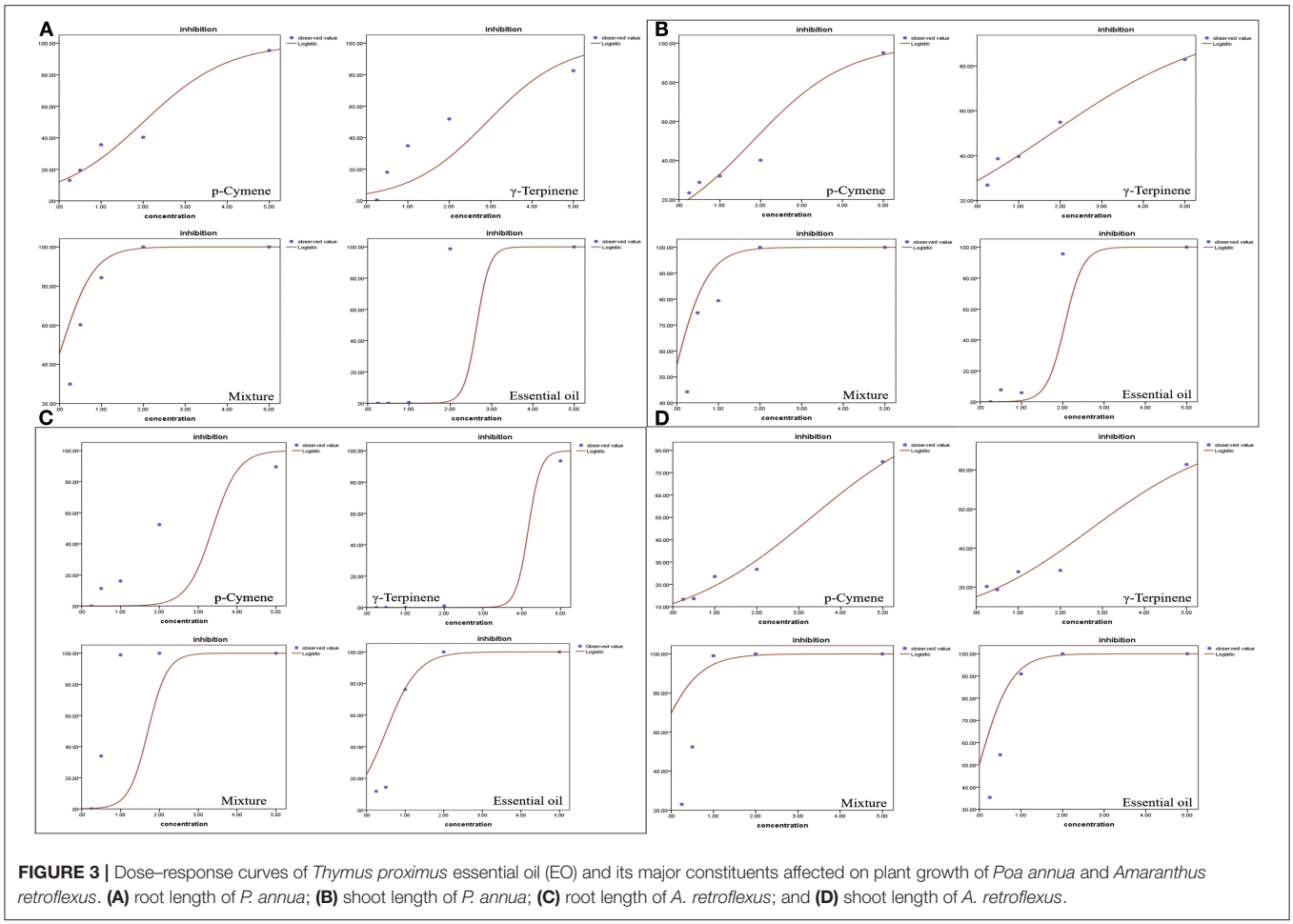

FIGURE 3 | Dose–response curves of *Thymus proximus* essential oil (EO) and its major constituents affected on plant growth of *Poa annua* and *Amaranthus retroflexus*. **(A)** root length of *P. annua*; **(B)** shoot length of *P. annua*; **(C)** root length of *A. retroflexus*; and **(D)** shoot length of *A. retroflexus*.

TABLE 3 | Toxicity of *Thymus proximus* essential oil (EO), p-cymene, γ-terpinene, carvacrol, and their mixture against *Aphis gossypii* adults.

EO/major components	Regression equation	r^2	LC_{50} (ppm)	95% CL
EO	y = 0.053 + 3.202x	0.922	9.63	4.29–16.63
p-Cymene	y = 0.95 + 3.882x	0.939	5.69	3.75–8.04
γ- Terpinene	y = 0.729 + 4.348x	0.997	6.8	6.16–7.47
Carvacrol	y = 1.94 + 0.963x	0.896	0.1	0.00–0.46
Mixture	y = 0.398 + 2.79x	0.831	7.34	3.81–12.58

r^2_{adj}: adjusted coefficient of determination.
LC_{50}: 50% lethal concentration of A. gossypii.
95% CL: 95% confidence limits.

species (Zeynep et al.,, 2018). Sara et al. (2019) investigated the herbicidal action of *T. fontanesii* EO and found 0.03% *T. fontanesii* oil inhibited the seed germination by 100% on *Sinapis arvensis*, *Avena fatua*, *Sonchus oleraceus*, and *Cyperus rotundus*. In another study comparing the strength of phytotoxicity of 12 EOs produced by the Mediterranean aromatic plants conducted by Rolim et al. (2010), thyme, balm, vervain, and caraway EOs were found to be more active on germination and radicle elongation of receiver species; among them, thyme oil completely inhibited the seed germination of *Lepidium sativum*, *Raphanus sativus* L., and *L. sativa* at 1.25 μg/ml. Synowiec et al. (2017) also performed a study comparing the phytotoxicity of 12 EOs and detected that *T. vulgaris*, *Carum carvi* L., *Mentha piperita* L., and *Salvia officinalis* L. oils possessed the most potent activity, with the ED_{50} values for thyme oil ranging between 0.06 and 1.03 g/L against seven tested plants, which was comparable to our findings (Synowiec et al., 2017). *Thymus pulegioides* L. EO with high content of α-terpinyl acetate inhibited the seed germination and radicle growth for high economic productivity forage grass monocotyledon *Poa pratensis* L (Vaiciulyte et al., 2021). Different extraction methods also cause differences in EO activity. *Thymus decussatus* EO extracted using hydrodistillation method inhibited

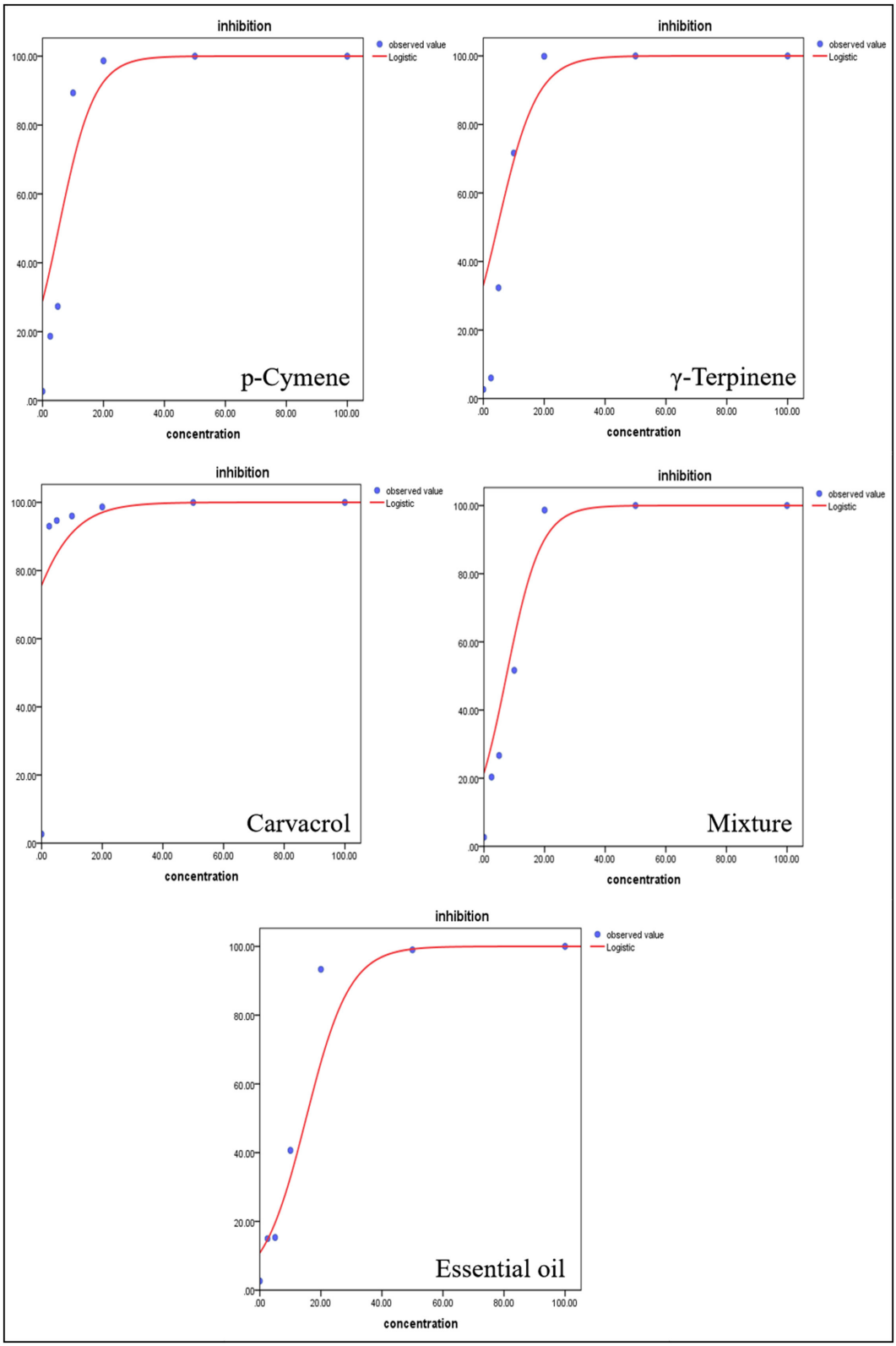

FIGURE 4 | Dose–response curves of *Thymus proximus* essential oil (EO) and its major constituents against *Aphis gossypii* adults.

the seed germination, shoot growth, and root growth of lettuce by 86.6, 87.4, and 89.9%, respectively, whereas the EO extracted using the microwave-assisted techniques method inhibited *L. sativa* by 77.7, 8 5.8, and 84.6% at 100 μl/L, respectively (Saleh et al., 2020).

The phytotoxic effect of a particular EO can be mainly ascribed to certain toxic component(s). Monoterpene compounds have been reported to show strong inhibitory effects on the seed germination of many crops and weeds (López et al., 2008; Li et al., 2011; Ali et al., 2015). The outstanding phytotoxic activity of thymol has been previously studied. Thammyres et al. (2018) found that thymol exhibited phytotoxicity at different concentrations (0.375–3 mmol/L), as reflected on the reduced germination rate of tested monocot and dicot species. Kordali et al. (2008) found carvacrol and thymol prohibited the seed germination and seedling development of *A. retroflexus*, *Chenopodium album,* and *Rumex crispus* L., whereas p-cymene did not exert a significant phytotoxic activity (8.6 mg/Petri dishes). Consistent with this study, Vasilakoglou et al. (2013) reported thymol completely restrained the seed germination of rigid ryegrass (*Lolium rigidum* Gaudin) at 160 nl/cm^3 or above, whereas p-cymene was found to be only slightly phytotoxic. Martino et al. (2010) tested the antigerminative activity of thymol, p-cymene, and γ-terpinene, along with other monoterpenes and found that thymol negatively affected the radicle elongation of garden cress significantly at 10^{-3} M, p-cymene suppressed the root growth of garden cress at 10^{-4} M; however, γ-terpinene did not exert any significant effect at tested concentrations. Meanwhile, the isomer of γ-terpinene, that is, α-terpinene, was detected to be phytotoxic against maize seedlings by reducing the root growth, changing the root border cells number, increasing the pectin methyl esterase activity, and upregulating the repel expression in the roots (Wang et al., 2019). In conclusion, thymol exhibited much stronger phytotoxic activity compared with p-cymene and γ-terpinene, implying its role as the major active compound responsible for phytotoxicity of the oil.

Natural products with plant origin can play crucial roles in pest management practice (Faraone et al., 2015; Barua et al., 2020; Basaid et al., 2020; Chen and Oi, 2020). Previously, EOs synthesized by *Thymus* species have been demonstrated to possess insecticidal activity. *T. serpyllum* L. and *T. vulgaris* EOs presented a LC_{50} of <10 mg/dm^3 against the pest (*Acanthoscelides obtectus* say) of kidney bean (*Phaseolus vulgaris* L.) after 24 h of exposure (Regnault-Roger et al., 1993). Isman et al. (2001) tested 21 EOs for their insecticidal action *via* topical administration to third instar larvae of the tobacco cutworm, *Spodoptera litura*, and EOs of *Satureja hortensis*, *Origanum creticum*, and *T. serpyllum* exhibited over 90% larval mortality at 24 h at 100 μg/larva; the LD_{50} value for *S. hortensis* (48.4 μg) was comparable to that for *T. vulgaris* (46.9 μg). Park et al. (2017) detected that the LC_{50} values of *T. vulgaris* EO against *Pochazia shantungensis* nymphs using the leaf dipping bioassay was recorded as 57.48 and 75.80 mg/L for adults using the spray bioassay method. Pavela (2005) tested the insecticidal activity of *T. mastichina* and *T. vulgaris* EOs against *Spodoptera littoralis* larvae and determined their LD_{50} values were 19.3 and 22.9 ml/m^3. *Thyme* (*T. vulgaris*) EO was also found to show high activity against *Lycoriella ingenua* at 20×10^{-3} mg/ml air (Park et al., 2008). EO produced by *T. satureioides* had moderate toxicity with the LD_{50} value of 0.31 μl/cm^2 and the LD_{90} of 0.77 μl/cm^2 against the important stored-product pest insect *Tribolium castaneum* (Kasrati et al., 2015). Ali et al. (2015) found the EOs obtained from all organs of *T. algeriensis* possessed strong insecticidal activity (LC_{50} = 44.25–112.75 μl/L air) against cotton leafworm larvae (*Spodoptera littoralis*). In a recent study, EOs of *T. spinulosus* and *T. longicaulis* were assayed for their insecticidal toxicity, and their LC_{50}/LD_{50} values were detected in the range of 39.6–87.1 μg/larva, 21.7–62.4 μl/L, and 35.9–147.3 μg/adult, for *Culex quinquefasciatus*, *Spodoptera littoralis*, and *Musca domestica,* respectively; it is noteworthy to mention that they found the most active samples were those with the highest amounts of thymol (Pavela et al., 2019). *A. gossypii* were reported to be susceptible to a variety of EOs. For example, the strength of *Santalum austrocaledonicum* Vieill EO was comparable to imidacloprid (a neonicotinoid insecticide) against *A. gossypii* infesting Rose of Sharon (*Hibiscus syriacus* L.) with 98.8% mortality (Roh et al., 2015). In another study, *Melaleuca styphelioides* Sm. EO exhibited strong fumigant toxicity on adults and nymphs of *A. gossypii*; 263.18 μl/L air EO let to 100% mortality of this insect (Albouchi et al., 2018).

Previous works have demonstrated the insecticidal activity of carvacrol, p-cymene, and γ-terpinene; in fact, carvacrol was speculated to be the main insecticidal compound of the EOs (Pavela and Sedlák, 2018; Pavela et al., 2019). Park et al. (2017) measured the insecticidal activity of thymol, carvacrol, citral, 2-isopropylphenol, 3-isopropylphenol, and 4-isopropylphenol against *Pochazia shantungensis* adults, and their LC_{50} values were 28.52, 56.74, 89.12, 71.41, 82.49, and 111.28 mg/L, respectively. Dias et al. (2019) evaluated the toxicity of thymol, cinnamaldehyde, carvacrol, eugenol, and trans-anethole on *Mahanarva spectabilis* eggs, nymphs, and adults, and they found that treatments with eugenol, carvacrol, and thymol showed the highest mortalities, presenting efficiencies higher than 85% after 48 h of application. Traboulsi et al. (2002) found that the compounds thymol, carvacrol, (1R)-(+)-α-pinene, and (1S)-(−)-α-pinene showed potent toxicity (LC_{50} 36–49 mg/L), whereas menthone, 1,8-cineole, linalool, and terpineol (LC_{50} 156–194 mg/L) were less toxic to the mosquito *Culex pipiens* molestus. On the other hand, p-cymene and γ-terpinene also showed effective insecticidal activity. Cetin et al. (2010) detected that γ-terpinene triggered ≥90% knockdown against adult *Hyalomma marginatum* at 105 min through 3 h, meanwhile at 24 h only about 87% of the ticks were dead. Another study found that both γ-terpinene and terpinen-4-ol exhibited a significant insecticidal effect on *Spodoptera littoralis* and *A. fabae*; however γ-terpinene was more toxic than terpinen-4-ol, with the LC_{50}/LD_{50} values being 23.94 g/L, 18.03 g/L for γ-terpinene, and 32.94 g/L, 20.77 g/L for terpinen-4-ol, respectively (Abbassy et al., 2009). Silva et al. (2018) tested the activity of p-cymene and γ-terpinene against *Rhipicephalus microplus* and revealed their LC_{50} values were 1.41 and 3.08 mg/ml, respectively. Tak and Isman (2017) tested the insecticidal activity of thymol, p-cymene, and their mixture against *Trichoplusia ni*, and the LC_{50} values were 244.3, 875.4, and 534.8 μg/insect after 24 h of treatment, respectively; they also suggested that

p-cymene seemed to enhance penetration of thymol through the integument. There was a study comparing the insecticidal activity of 11 Apiaceae plant EOs and their components on adult male and female *Blattella germanica*, and p-cymene and γ-terpinene were found to exhibit significant fumigant toxicity against adult *Blattella germanica*, whereas p-cymene exerted potent contact toxicity against adult *Blattella germanica* (Yeom et al., 2012). In the case of *T. proximus* EO, we discovered that the insecticidal activity of p-cymene, γ-terpinene, and the mixture of three major constituents was much weaker than carvacrol, which suggested that carvacrol might be the main responsible insecticidal compound in the oil. Moreover, these results supported the speculation that carvacrol was the major insecticidal compound of some EOs (Pavela and Sedlák, 2018; Pavela et al., 2019).

Among the three major constituents, carvacrol was found to possess much stronger biological activity compared with p-cymene and γ-terpinene, although they are similar aromatic monoterpenoids. A recent study revealed that monoterpenoids can induce cell membrane dysfunction and interfere with cell metabolism, and OH^-- and O^--radicals are considered to react with cellular components affecting homeostasis (Scariot et al., 2020). By comparing the chemical structures of carvacrol and p-cymene, it is speculated that the hydroxyl group of carvacrol might be critical for its potent activity. In another report, carvacrol was found to possess herbicidal activity due to its ability to incite membrane leakage (Chaimovitsh et al., 2017). On the other hand, it is noteworthy to mention that it is also possible that minor components in the oil might play important roles in the activity. Furthermore, due to the fact that EOs are composed of small molecules that can easily evaporate in the air, the optimization of the formula is necessary so as to stabilize the oils and their constituents.

CONCLUSION

Essential oils are valuable sources of providing candidate compounds as potential environment friendly pesticides and herbicides, which can be utilized in pest and weed control safely due to their ability to degrade in nature, and the fact that they are less toxic to the environment. Our study is the first report on the allelopathic, phytotoxic, and pesticidal activities of the EO extracted from the aromatic plant *T. proximus* and on the determination of the major active compound, that is, carvacrol, to be responsible for the biological activity of the oil, implying their potential value of being explored as pesticides and herbicides. Limitations of our work include that only fumigant method was used to evaluate the insecticidal activity of *T. proximus* EO and its constituents against *A. gossypii*, and leaf dipping method needs to be performed in the future on more insects such as stored product pests.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SZ drafted the manuscript and wrote the manuscript. CH drew the scheme and edited the manuscript. CW performed the literature survey. CheZ performed the correction. NK edited and improved the scheme artwork. ChiZ and HS conceptualized, wrote, and edited the manuscript, and they performed the literature survey and ideation of the scheme. All authors contributed to the article and approved the submitted version.

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REVIEW
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Azadirachtin-Based Insecticide: Overview, Risk Assessments, and Future Directions

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In the context of the major crop losses, pesticides will continue to play a key role in pest management practice in absence of practical and efficient alternatives; however, increasing awareness regarding environmental and human health impacts of conventional pesticides as well as the development of resistance and cross-resistance reduced their availability and promoted the search for alternative control strategies and reduced-risk pesticides. Among the various alternatives, a drastic re-emergence of interest in the use of plant-derived compounds, called allelochemicals, was noted and demand for an organic product is rising. Currently, azadirachtin, a tetranortriterpenoid derived from the neem seed of the Indian neem tree [*Azadirachta indica* A. Juss (Meliaceae)], is one of the prominent biopesticides commercialized and remains the most successful botanical pesticide in agricultural use worldwide. Azadirachtin is a powerful antifeedant and insect growth disruptor with exceptional low residual power and low toxicity to biocontrol agents, predators, and parasitoids. This review summarizes the state of the art on key azadirachtin insecticidal activities and risk assessment, identifies knowledge gaps that could serve as the basis for future research direction and highlights limitation in agricultural use and the development of novel strategies by the use of nanotechnology to control its release rate and improve its stability and sustainability.

Keywords: azadirachtin, *Azadirachta indica*, nanotechnology, alternative pest control, agroecosystems

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INTRODUCTION

The United Nations predicts that the global population will increase from 7.7 billion in 2019 to 9.7 billion in 2050 (United Nations, 2017), this evolution is the main factor that will increase the demand for food production which is expected to continue to grow and is projected to increase by 25–70% in 2050 to meet the increasing human demand (Hunter et al., 2017; Silva, 2018). Annual crop losses caused by insects, weeds, and diseases are estimated between 20 and 40 percent, similar to those of 50 years ago due to the intensification of agricultural production together with the effects of climate change (FAO, 2017). To safeguard and improve food security, crop protection from pests is required and aimed to avoid or prevent crop losses or to reduce them to an economically acceptable level (Karuppuchamy and Venugopal, 2016).

Over the years and since the 1950s, conventional synthetic insecticides have played a crucial role in increasing agricultural productivity (Aktar et al., 2009; Popp et al., 2013). In the context of the major crop losses, pesticides will continue to play a key role in pest management practice in absence of practical and efficient alternatives. Indeed, the beneficial outcome from the use of

pesticides remains vital for avoiding hunger and food insecurity and meeting the demand of today and future generations especially in the developing countries (Deravel et al., 2014); however, the extensive use of pesticides generates human and environmental health risk and hazards (Carson, 1962; Aktar et al., 2009; Nicolopoulou-Stamati et al., 2016; Jars et al., 2018) and a growing resistance to targeted pests by exerting selection pressure on insect pests (Harrop et al., 2014; Helps et al., 2017).

After the publication of the Silent Spring by Rachel Carson (Carson, 1962), and to attenuate the negative impacts of pesticides in the environment and public health, search for alternative control strategies and reduced risk pesticides became a real challenge (Pimentel, 1997; Khater, 2012). Consequently, a drastic re-emergence of interest in the use of natural pesticides known as biopesticides was noted (Cantrell et al., 2012; Kumar, 2015; Mishra et al., 2018; Haddi et al., 2020). Although there is no formally agreed definition, biopesticides are eco-friendly pest management agents based on living organisms or natural products (Chandler et al., 2011). They may be derived from animals (ex: nematodes), microorganisms (ex: *Bacillus thuringiensis*), plants (ex: *Azadirachta*) as well as certain minerals (Damalas and Koutroubas, 2018). If biopesticides are gaining popularity as reduced environmental impact alternatives to conventional synthetic pesticides, the biopesticides market remains small (5%) to the worldwide pesticide market (Olson, 2015). However, this segment of the industry is experiencing rapid growth in recent years with a compound annual growth rate of 8.64% and is projected to outpace that of chemical pesticides (Olson, 2015; Damalas and Koutroubas, 2018).

The main advantages of biopesticides are that they are inherently less toxic than conventional pesticides by offering more targeted action against specific pests (Damalas and Koutroubas, 2018). Indeed, conventional pesticides which exert their effects on the nervous system of insects often affect a broad spectrum of pests along with bird and mammalian species (Thakora, 2006). Furthermore, biopesticides often are effective in very small quantities and decompose quickly, resulting in lower exposures and largely avoiding the pollution problems caused by conventional pesticides (FAO). When using as a component of integrated pest management (IPM) programs, biopesticides can supplement the conventional pesticides and greatly reduce their use and offer potentially higher crop yields (Thakora, 2006; Damalas and Koutroubas, 2018).

Recently, among the biopesticides, plants with pesticidal properties have been the subject of an increasing number of academic researches as a potential option for environment friendly pest management tools for developing sustainable agricultural practices and promote human and environmental safety (Isman, 2006; Cantrell et al., 2012; Hikal et al., 2017). Plants, the most common source of biopesticides, produce a great variety of secondary metabolites potentially applicable in IPM programs (Céspedes et al., 2014).

Growing attention has been given to the neem tree, *Azadirachta indica* A. Juss. (Meliaceae), as the most prominent biopesticide (Isman and Grieneisen, 2014; Aribi et al., 2020). In Asia, the neem tree is regarded as a wonder tree and has been used for centuries in Ayurvedic medicine as one of oldest medical systems in humanity (Biswas et al., 2002; Pasquoto-Stigliani et al., 2017). Among its many attributed properties, it acts as an antidiabetic, immunostimulant, antimicrobial, antiviral, cholesterol-lowering agents, contraceptive and anticancer remedy, and it has long been revered by ancient Indian people and is entitled "village drugtore" (Tinghui et al., 2001; Hummel et al., 2016; Moga et al., 2018; Blum et al., 2019). Additionally, aqueous extracts of powdered neem kernels have been used as an insecticide in India for about 2,000 years for the control of insect pests (Schmutterer, 1995). In recent time, and following the isolation of azadirachtin, the major active compound, that is mainly responsible for the insecticidal activity of neem, the use of neem-based insecticide has increased in the last 30 years (Chaudhary et al., 2017; Pasquoto-Stigliani et al., 2017). Currently, azadirachtin is one of the prominent biopesticides commercialized and remains the most successful botanical pesticide in agricultural use worldwide (Isman and Grieneisen, 2014; Chaudhary et al., 2017; Aribi et al., 2020); however, its mechanisms of action still unclear and remain to be clarified especially in relation to the neurophysiological and the possible long-term activities.

THE NEEM TREE

Neem is an evergreen fast-growing tree native to India and Burma, it grows in arid, semiarid, and tropical regions (Schmutterer, 2002). Today, the neem tree is widely distributed throughout tropical and subtropical Asia, Africa, Australia, and South America (Kumar et al., 2016). Neem products have been obtained from several species of neem trees belonging to the Meliaceae family. *A. indica.* Juss, is the most important species of this group considered a renewable resource of various useful domestic, medicinal, and agricultural products (Kumar et al., 2016). All parts of the tree (leaf, flower, seed kernel, wood, bark, and twig), are a source of biologically active ingredients, and the maximum of activity is associated with the seed kernel (Kumar et al., 2016). More than 300 different phytochemicals have been reported from different parts of the neem tree (Gupta et al., 2017) and over 130 of these compounds belongs to limonoid-type triterpenoids that are endowed with potent medicinal and insecticidal properties (Chen et al., 2018); However, the chemical composition of neem is far to be completely elucidated, as evidenced by the novel compounds reported each year (Nicoletti et al., 2016; Chen et al., 2018). The most important neem limonoids include azadirachtin, nimbolide, salannin, nimbin, deacetylnimbin, mahmoodin, epoxy-azadiradione, deacetylgedunin, and gedunin (Nagini, 2014; Gupta et al., 2017). These compounds have been shown to possess many useful properties of which, antifeedancy, insecticidal, and insect growth disruption are used in the management of pest (Schmutterer, 1995). Most of the triterpenoids of neem were found in very small quantities in various parts of the tree and account for the total bioactivity of the neem seed extract (Mordue et al., 2010). Azadirachtin A is the major active component and is responsible for 72 to 90% of the biological activity (Schmutterer, 1990; Mordue et al., 2010).

AZADIRACHTIN: PROPERTIES AND INSECTICIDAL ACTIVITIES

Azadirachtin is a complex tetranortriterpenoid with 16 chiral carbon centers, derived from the mevalonic acid pathway in the neem tree (Hansen et al., 1993; Aarthy et al., 2018). It is a highly oxidized tetranorterpenoid natural product related to limonin, the bitter principle of citrus fruits and known as limonoids (Benuzzi and Ladurner, 2018). Azadirachtin A is considered as the main constituent and azadirachtin commercial formulations, available on the world market for insect control in organic farming, contain a stated amount of azadirachtin A (**Table 1**) (Benuzzi and Ladurner, 2018). It has a complex molecular structure and following the determination of its correct structure in 1985 (Kraus et al., 1985), the first total synthesis of this molecule was published two decades after the discovery of the compound (Jauch, 2008). Azadirachtin is a broad-spectrum insecticide (**Figure 1**), its acts as a feeding deterrent, insect growth disruptor (IGD), and sterilant and is used to control various agricultural pest species, including Coleoptera, Heminoptera, Diptera, Orthoptera, and Isoptera (Morgan, 2009). The toxicity of azadirachtin varies among insect orders and is influenced by the different penetration rates and activities of detoxifying enzymes (**Table 2**).

The chemical complexity of azadirachtin minimizes the potential risk of insect resistance (Mordue et al., 2010). Feng and Isman (1995) reported development of resistance to pure azadirachtin over 40 generations in the peach potato aphid *Myzus persicae* but no resistance was reported with neem seed extract. Bomford and Isman (1996) also showed habituation to pure azadirachtin in the tobacco cutworms with less sensitivity to the antifeedant properties of azadirachtin, but not to neem with the same absolute amount of azadirachtin. This might account for avoiding desensitization to commercial neem-based insecticides containing additional non- AZA-compounds (Bomford and Isman, 1996). Azadirachtin A is very well-received by the root system, and, subsequently, it is systematically distributed through the xylem into the green parts of plant tissues and stored in leaves in an unchanged form. In addition, a very low content of azadirachtin A in plant tissues may protect significantly plant damage against phytophagous pest larvae (Pavela, 2016).

In addition, azadirachtin has displayed remarkable selectivity with low mammalian toxicity (Mordue et al., 2010). According to Raizada et al. (2001), azadirachtin has shown an LD_{50} value of more than 5,000 mg/kg which falls into class U (Unlikely to present an acute hazard) of the WHO (2009) toxicity rating. Azadirachtin is registered in the United States as a general-use pesticide with a toxicological class Environmental Protection Agency (EPA) of IV (relatively non-toxic). Azadirachtin seems to be selective, non-mutagenic, and readily degradable and has also been reported as safer for non-target organisms and beneficial organisms (Medina et al., 2004; Cordeiro et al., 2010; Mordue et al., 2010; Celestino et al., 2014; Dai et al., 2019); however, the presumed safety of azadirachtin has been questioned, especially, in relation to natural enemies and pollinators (Barbosa et al., 2015; Lima et al., 2015; Xavier et al., 2015; Bernardes et al., 2017, 2018; Francesena and Schneider, 2018). Nevertheless, semi-field and field studies may enable to reliably predict potential side effects of azadirachtin on non-target insects. However, azadirachtin is still considered as one of the best alternatives to conventional insecticides in IPMprograms and considered as one of the most promising plant compounds for pest control organic agriculture (Tomé et al., 2013; Bezzar-Bendjazia et al., 2017). Despite the progress on the physiological and biological activities and agricultural application of azadirachtin, its exact mechanism of action, especially, at the molecular level is not yet fully understood (Lai et al., 2014; Dawkar et al., 2019).

TABLE 1 | Commercial azadirachtin-based products available worldwide.

Products	Manufacturer	Azadirachtin Percent %
Agroneem plus	Agro logistic systems Inc	0.15
Azagro	India MART	1
AzaGuard	BioSafe systems	3
Azamax	E.I.D parry Ltd	1.2
AzaPRO	CANN-CARE company	1.2
Azasol	ARBORJET Inc	6
Debug TRES	AGROLogistic systems	3
EcoZin plus	AMVAC chemical Corp.	1.2
Fortune aza	Fortune biotech Ltd	3
MOLT-X	BIOWORKS Inc	3
Neem Azal TS	Trifolio-M GmbH	1
Neemarin	AZA-Direct Gowan company LLC	0.15
Neemfol	Gassin pierre	5
Neemix	Certis	0.25
Ornazin	SEPRO corporation	3

Effects on Neuro-Endocrine Activity

In insects, 20-hydroxyecdysone (20E) and juvenile hormone (JH) play a central role in the regulation of growth and development (Bensebaa et al., 2015), and the hormonal balance determines the outcome of each developmental transition (Dubrovsky, 2005). Therefore, any interference with hormonal homeostasis leads to interrupted development and is considered as a potential specific target for pest control (Pener and Dhadialla, 2012). Azadirachtin is known as an antagonist of these two principles hormones; its major action was its ability to modify or suppress hemolymph ecdysteroid and JH titers through inhibition of the secretion of morphogenetic peptide hormone (PTTH) and allatotropins from the *corpus cardiacum* complex and this account for its well-documented IGD effects defined mostly as reduced pupation, malformation or a failure of adult emergence (Mordue and Blackwell, 1993; Bezzar-Bendjazia et al., 2017). Furthermore, this compound is known to cause degenerative structural changes of the nuclei in all endocrine glands (prothoracic gland, *corpus allatum,* and *corpus cardiacum*) responsible for controlling molting and ecdysis in insect which would contribute to a generalized disruption of neuroendocrine function (Mordue et al., 2010). Azadirachtin applied on the diet at 74 ppm affects the growth, suppresses ecdysis, and inhibits ecdysteroids synthesis

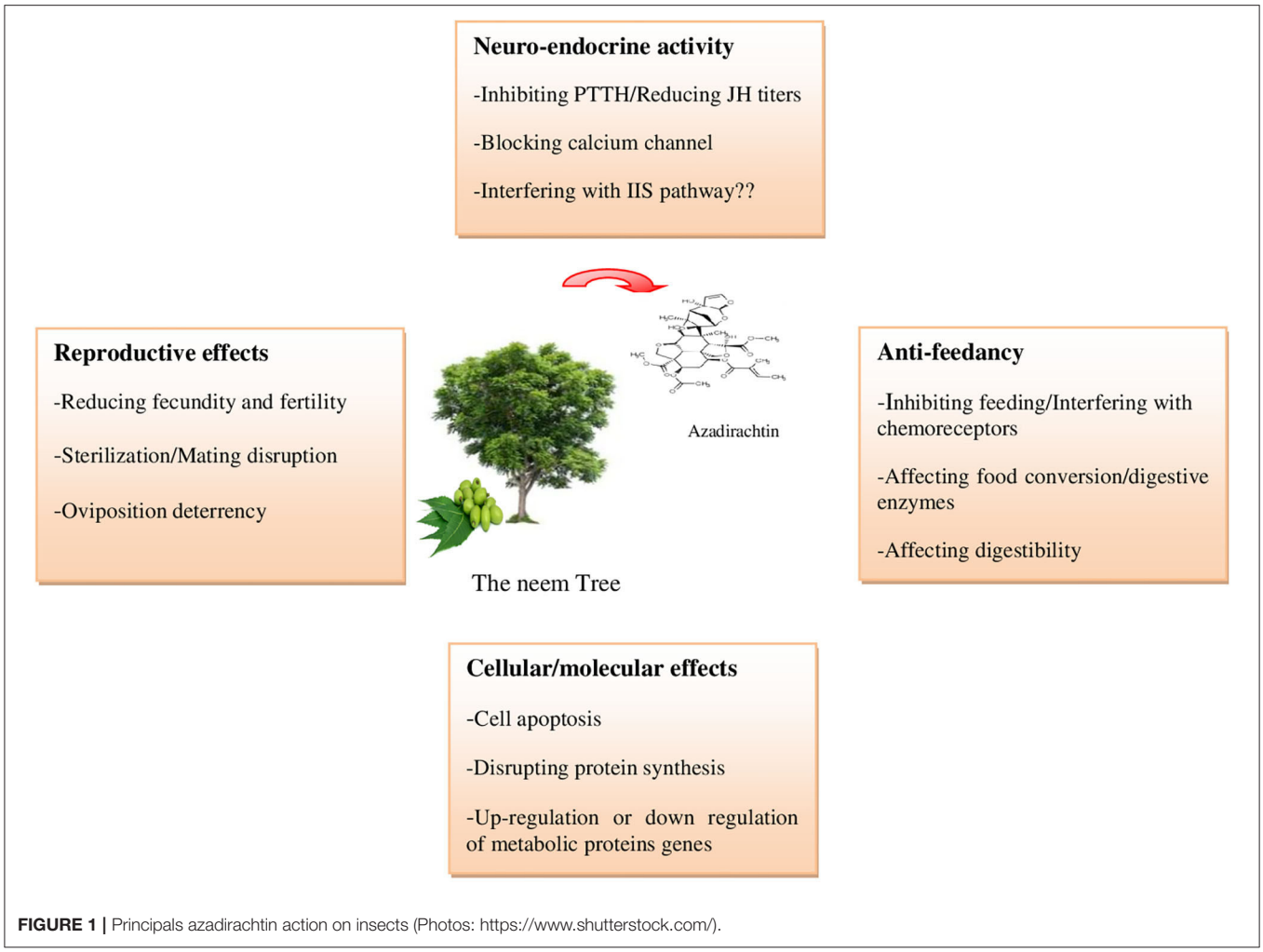

FIGURE 1 | Principals azadirachtin action on insects (Photos: https://www.shutterstock.com/).

TABLE 2 | Azadirachtin LC_{50} in some species of pest.

Species	Mode of application	Developmental stage	LC_{50} (ppm)	References
Pluttella xylostella	Orally	3rd instar larvae	0.63	Zada et al., 2018
Tuta absoluta	Orally	2nd instar larvae	5.62	Tomé et al., 2013
Tirathaba rufivena	Orally	Larval	28.79	Zhong et al., 2017
Helicoverpa armigera	Orally	3rd instar larvae	12.95	Abedi et al., 2014
Coridius viduatus	Orally	Adults	0.003	Aljedani, 2018
Megaselia scalaris	Dipping	1st instar larvae	13.79	Abdel-Gawad, 2018
Agonoscena pistaciae	Dipping	5th instar larvae	0.22	Izadi et al., 2012
Heteracris littoralis	Topically	4th instar larvae	101.20	Ghazawi et al., 2007
Lobesia botrana	Orally	1th instar larvae	2.1	Irigaray et al., 2010
Galleria mellonella	Topically	Immature stage	16.56	Er et al., 2017

in the larvae of *Ostrinia furnacafis* Guenée (Min-Li and Shin-Foon, 1987). In *Tenebrio molitor*, the injection of 1 μg of azadirachtin into freshly ecdysed pupae induced a significant depletion of levels of immunoreactive ecdysteroids affecting 20-hydroxyecdysone levels and suppressing the ecdysteroid peak that normally appears at the middle of the instar (Marco et al., 1990). A drastic reduction of hemolymph ecdysteroid titers was also reported in *Rhodnius prolixus* after a unique dose of azadirachtin (Garcia et al., 1990). In addition to its effects on morphogenetic PTTH, azadirachtin affects ecdysone 20-monooxygenase activity, the insect cytochrome P450-dependant hydroxylase responsible for the conversion of the steroid

hormone ecdysone to its more active metabolite, and 20E (Smith and Mitchell, 1988). Indeed, *in vitro* analysis of three insect species, homogenates of wandering third instar larvae of *Drosophila melanogaster,* fat body or midgut from last instar larvae of *Manduca sexta* and abdomens from adult female *Aedes aegypti*, incubated with radiolabelled ecdysone and azadirachtin revealed inhibition of the ecdysone 20-monooxygenase with a dose-dependent relationship (Smith and Mitchell, 1988); however, ingested or injected azadirachtin had no effect on ecdysone 20-monooxygenase activity in *Spodoptera frugiperda* (Yu, 2000). Besides its negative effects on molting hormone, azadirachtin induced a delay or a reduction in JH titters, primarily by hindering the release of the allatotropins and thereby blocking the synthetic and release processes of the JH (Mordue et al., 2010; Dhra et al., 2018).

Azadirachtin is reported to impair the growth and molting process of insects and induced robust developmental delays in the larva-to-pupa and the pupae-to-adult transition compromising their survival (Hasan and Ansari, 2011; Tomé et al., 2013; Lai et al., 2014; Bezzar-Bendjazia et al., 2016). In addition, growth and nutrient intake are functionally linked processes in development and growth and body mass are directly affected by nutrient uptake principally governed by the insulin/insulin-like growth factor signaling (IIS) pathway (Tennessen and Thummel, 2011). Lai et al. (2014) reported that the inhibition of growth and development in *D. melanogaster* after azadirachtin treatment was similar to those caused by disruption of the IIS pathway. In addition, azadirachtin can inhibit the excitatory cholinergic transmission and block partly the calcium channel (Qiao et al., 2014), and this might interfere with different endocrinological and physiological actions in insects.

Effects on Reproduction

The negative effects of azadirachtin on reproduction were reported in several insect orders (Pineda et al., 2009; Tine et al., 2011; Tomé et al., 2013; Boulahbel et al., 2015; Er et al., 2017; Oulhaci et al., 2018). Reduced fecundity and fertility has been recorded in many insects including *Spodoptera littoralis*, *D. melanogaster, Galleria mellonella, Dysdercus cingulatus, Tuta absoluta,* and *Helicoverpa armigera* (Pineda et al., 2009; Pandey and Tiwari, 2011; Tomé et al., 2013; Ahmad et al., 2015; Er et al., 2017; Oulhaci et al., 2018) and could be due to the interference of azadirachtin with yolk protein synthesis and or its uptake into oocytes (Boulahbel et al., 2015). In leaf-cutting ant queens *Atta sexdens*, azadirachtin affects oviposition, decreases, and inhibits vitellogenin reserve, which impact negatively the egg development (Amaral et al., 2018).

Sterility effects in females due to interference with vitellogenin synthesis and uptake into oocytes were also reported. A single injection of 10 μg of azadirachtin resulted in sterilizing effect on *Locusta migratoria migratorioides* with an arrest of terminal oocytes maturation and oviposition (Rembold and Sieber, 1981). In *Heteracris littoralis*, ovaries in azadirachtin-treated females showed complete shrinkage with oocyte growth arrest with disintegration and destruction in follicular cells and mitochondria (Ghazawi et al., 2007). In males, azadirachtin decreases significantly the number of cysts and the apical nuclei within the cysts in *D. melanogaster* (Oulhaci et al., 2018). The inhibition of spermiogenesis was also reported in *Mylabris indica* (Vivekananthan and Selvisabhanayakam, 2014) and *Heteracris littoralis* (Ghazawi et al., 2007).

For the normal progress of oogenesis and spermatogenesis, a proper balance between JH and 20E is needed, antagonist action of azadirachtin on these two principal hormones account for the deleterious effects on reproductive parameters. Indeed, the application of exogenous 20E after azadirachtin treatment can compensate for its depressive effects on *D. melanogaster* and restored normal values of yolk protein content in the fat body and ovaries (Boulahbel et al., 2015).

In addition, azadirachtin was finding to alter reproductive behavior in *D. melanogaster* by reducing mating success (Aribi et al., 2017; Oulhaci et al., 2018). The impact of azadirachtin on sex behavior and mating response to sexual pheromones was also reported in *Oncopeltus fasciatus* (Dorn et al., 1987) and the predator *Neoseiulus baraki* (Lima et al., 2015). Oviposition sites treated with azadirachtin or other neem-based compounds induce an oviposition repellence, deterrence, or inhibition in several species of insects after a probable detection of the bioinsecticide on the treated surface (Schmutterer, 1990; Dhar et al., 1996, Cordeiro et al., 2010; Tomé et al., 2013). Pure azadirachtin was also reported to deter the oviposition in *Nezara viridula* (Riba et al., 2003). A single larval exposure to a commercial formulation of azadirachtin, the Neem Azal, was found to reduce fecundity in *D. melanogaster* and enhance avoidances to this compound (Bezzar-Bendjazia et al., 2016). These effects were observed in the next non-exposed generations which can be used as repellent strategies in pest management programs (Ferdenache et al., 2019).

Anti-feedancy

Azadirachtin is usually associated with a marked antifeedant activity and even behavioral avoidance in a large number of insect species including hemipterans (Kumar and Poehling, 2007), lepidopterans (Charleston et al., 2006; Shannag et al., 2015), ortheopterans (Capinera and Froeba, 2007), coleopterans (Baumler and Potter, 2007), and dipterans (Kilani-Morakchi et al., 2017).

Insects use an olfaction system to search and locate potential food and thereafter contacting chemoreception, called primary antifeedancy, which could confirm its quality and provide a basis for food selection and discrimination (Lee et al., 2010). A signal to the brain provokes avoidance from further approach or feeding.

The primary antifeeding effect of azadirachtin seems to be mediated by gustatory chemosensillas and linked to inhibition on the rate of firing of sugar-sensitive cells of the gustatory chemoreceptors by activating bitter sensitive gustatory cells (Lee et al., 2010; Weiss et al., 2011; Delventhal and Carlson, 2016). Indeed, the sensitivity to primary antifeedancy of azadirachtin was reported in different species, which starve to death rather than ingest the biopesticide (Mordue and Nisbet, 2000). An internal feedback mechanism called secondary antifeedancy, including a long-term reduction in food intake, and deleterious effects on different insect tissues (muscles, fat body, gut epithelial cells), is also reported (Mordue et al., 2010; Khosravi and Sendi,

2013; Shannag et al., 2015). Third-instar larvae of *S. littoralis* orally treated with sublethal concentrations of azadirachtin display a reduction in food intake, conversion efficiency, and feeding behavior (Martinez and van Emden, 1999). In second instar larvae of *Spodoptera eridania*, short-term consumption (2 days) of food treated with Azatrol, a commercial formulation of azadirachtin, reduced relative consumption rate, the efficiency of conversion of ingested food, relative growth rate, approximate digestibility, and assimilation rate of food during the entire larval developmental period (Shannag et al., 2015). In *D. melanogaster*, a single topical application of azadirachtin on early third instars larvae decreased significantly the amount of larval food intake and disrupted the ability of the insect to digest food by interfering with digestive enzymes activities (Bezzar-Bendjazia et al., 2017). This effect is also observed in adults surviving the pre-imaginal treatment, which suggests a long-term antifeedancy and delayed effects through the developmental stage with a possible reinforcement of the insecticidal activity of azadirachtin (Kilani-Morakchi et al., 2017).

In addition, azadirachtin showed an agonistic effect on dopaminergic neurons and can induce aversive taste memory in *D. melanogaster*, and such memory is regulated by dopaminergic signals in the brain resulting in inhibition of proboscis extension response (PER) (Yan et al., 2017).

Cellular and Molecular Effects

Besides the above mentioned effects, accounting for its broad-spectrum activities, azadirachtin was also shown to cause upregulation of p53, resulting in cell cycle mediated cells apoptosis induction and cell proliferation inhibition in *S Spodoptera litura* S1-1 cell line (Huang et al., 2011). In the same species, Shu et al. (2018) demonstrated that azadirachtin induced structural alteration in the larval midgut by apoptosis activation including increased expression of caspase family members and apoptosis-binding motif 1 and the release of cytochrome c from mitochondria to cytoplasm, which may affect the digestion and absorption of nutrients. The induction of apoptosis through caspase-dependent pathways by azadirachtin was also reported in *S. frugiperda* cultures cell line Sf9 (Shu et al., 2015). Based on proteomic studies, Sun et al. (2018) reported that the molecular response mechanism of male infertility induced by azadirachtin in *S. litura* may be linked to regulation of many proteins in the pathway of focal adhesion exerting influences in detachment of cell attachment, the loss of cell-cell interactions, and inducing apoptosis at the pupal stage. Furthermore, many proteins in the adenosine monophosphate-activated protein kinase (AMPK) pathway were also changed at the adult stage after azadirachtin treatment as larvae (Sun et al., 2018). In *D. melanogaster*, a depolymerization of actin causing a cell arrest and apoptosis caspase-independent was reported after azadirachtin treatment (Anuradha et al., 2007; Anuradha and Annadurai, 2008).

At the cellular level, azadirachtin disrupts protein synthesis and secretion. In *Schistocerca gregaria*, injections of 3 μg azadirachtin/g body weight induce an inhibitory effect of the incorporation of radiolabelled glycine into the protein of the whole locust (Paranagama et al., 2004). Roberston et al. (2007) reported that the heat-shock protein, hsp 60, in cultured *Drosophila* Kc 167 cells could bind to azadirachtin A which might be associated with a failure of protein synthesis and release.

At the molecular level, azadirachtin alters or prevents the transcription and/or expression of several proteins. Ingestion of 10 ppm of azadirachtin in third instars larvae of *Ostrinia furnacalis* significantly affected the fat body by interfering with protein expression related to hemolymph lipid (Huang et al., 2007). Lai et al. (2014) reported that azadirachtin downregulated expression of genes of cuticular protein and amylase and upregulated gene odorant-binding protein 99b (Obp99b) in *D. melanogaster,* which may be related to the development, molting defects, and antifeedancy action of the biopesticide. Azadirachtin treatment was shown to increase superoxide dismutase activity (SOD) and malondialdehyde contents (MDA) in *D. melanogaster* and induce antioxidant enzymes, such as SOD, catalase (CAT), and gluthation S-transferase (GST), by an upregulation of gene expression to protect against oxidative damage caused by elevated and accumulation of reactive oxygen species (ROS) triggered by a stress response to azadirachtin (Zhang et al., 2018). Azadirachtin also inhibits the expression of ferritin and thioredoxin peroxidase genes, in the sweet potato of whitefly *Bemissia tabaci*, related to protective roles against oxidative stress (Asaduzzaman et al., 2016).

Recently, azadirachtin was found to regulate the growth of *S. frugiperda* by affecting the insect chitin synthesis pathway by a downregulation of 31 cuticle proteins and several other genes encoding important enzymes involved in insect chitin and hormone biosynthesis, such as, trehalase, chitin-synthase, chitin deacetylase, chitinase (Shu et al., 2020). The suppressed expression of chitin biosynthesis and cuticle genes by azadirachtin might represent the molecular basis for the retardation of molting and growth.

Genes encoding enzymes responsible for key steps in hormone biosynthesis were also affected by azadirachtin. Azadirachtin also affected genes encoding key enzymes in hormone biosynthesis, such as genes encoding farnesol dehydrogenase, responsible for oxidization of farnesol, a precursor of JH named farnesal (Mayoral et al., 2009); the gene encoding an aldehyde dehydrogenase, which is responsible for converting farnesal into farnesoic acid and CYP15A1_C1, which converts the farnesoic acid to JH-III acid (Qu et al., 2015); the gene encoding JH epoxide hydrolase, responsible for JH degradation by hydrolyzing the epoxide of JH (Zhao et al., 2017); the gene encoding cytochrome oxidase-related proteins CYP307A1 and CYP314A1, which catalyze the 20-Hydroxyecdysone (Liu et al., 2019). All these changes in the expression levels of these key genes account for the disruption of the synthesis of JH and ecdysone, and therefore, interfere with the balance of these hormones, contributed to the growth inhibition.

RISK ASSESSMENTS

Azadirachtin-based pesticides act on a wide range of pestiferous insects from different orders as well as some ectoparasites which present high sensitivity to these compounds. The major property of azadirachtin is the blockage of neurosecretory peptides,

which regulate the synthesis and release of ecdysteroids and JH leading to disruption of endocrine events. The important roles of these hormones in arthropods physiology for normal development leave open the possibility that azadirachtin may pose a hazard to non-target species. Indeed, Barbosa et al. (2015) reported that long-term chronic exposure with azadirachtin may affect reproduction and behaviors of the bumblebee *Bombus terrestris* under laboratory conditions. Similarly, *in vitro* chronic exposure of azadirachtin affects stingless bee, *Partamona helleri*, by reducing the survival, development time, growth, and affecting reproductive organs but did not affect the larval food intake, the rate of emergence of queen and walking activity (Bernardes et al., 2018); however, the instability of azadirachtin and its low residual potential persistence makes these chronic conditions unexpected under semi-field and field situations. Azadirachtin was also found to be selective to the honeybee, *Apis cerana*, based on three essential risk assessment criteria [selectivity ratio, probit substitution method (%), and hazard ratio/risk quotient (Challa et al., 2019)].

In the case of predatory insects and parasitoids, azadirachtin, and neem-based insecticides show slight to moderate toxic effects and are considered to be harmless and with a certain degree of selectivity, especially for the adult insects (Raguraman and Kannan, 2014); however, pre-imaginal instars of beneficial organisms (nymphal/larval instars) are more susceptible to neem insecticides under laboratory conditions (Raguraman and Kannan, 2014). Hence, it is important to control the stage of parasitoids/predators used and the timing of application to avoid any toxicity in semi-field and field applications.

According to European Food Safety Authority (European Food Safety Authority, 2011), azadirachtin has moderate to high toxicity to aquatic organisms (acute LC_{50} = 0.048 mg azadirachtin A/L, chronic NOEC = 0.0047 mg azadirachtin A/L) and aquatic insects (chronic NOEC = 0.0016 mg azadirachtin A/L), with an aquatic half-life of around 30 days. The risk assessment for this compound focused on freshwater organisms as there are no marine or estuarine data. However, the risk values did not exceed the criteria and were predicted to be low when azadirachtin was used following the label instruction of the product (Goktepe et al., 2004; European Food Safety Authority, 2011).

Azadirachtin is not highly mobile in soil due to its oily composition. Its half-lives in soil are about few hours to 1 or 2 days reducing the risk to earthworms and soil macro-organisms. The hazard index of heavy metal contamination in vegetables after soil treatment with azadirachtin was <1 and does not exceed the WHO/FAO permissible limit in vegetables, suggesting it is safer for consumption (Egwu et al., 2019).

However, information regarding the fate, behavior, and toxicity of individual compounds, and the degradation of products are needed to complement its relatively favorable ecotoxicological profile (European Food Safety Authority, 2011). In general, European Food Safety Authority (2018) reported that the margin safety of the risk assessment performed for azadirachtin A is considered sufficient to estimate the risk from the whole azadirachtin. In addition, semi-field and field studies should be performed considering situations that may include acute and chronic exposure in the risk assessment setup.

FUTURE DIRECTIONS

Azadirachtin has a variety of physiological effects on many insect pests, such as antifeedancy (Qin et al., 2020), growth and development inhibition (Zhao et al., 2019), impairment of oocyte structure, inhibition of fecundity, and egg viability (Bezzar-Bendjazia et al., 2016; Amaral et al., 2018; Oulhaci et al., 2018; Ferdenache et al., 2019). Despite extensive studies of the mechanisms that highlight the physiological effects of azadirachtin, the behavioral effects remain more controversial (Charleston et al., 2006; Hasan and Ansari, 2011; Tomé et al., 2013).

The fitness and survival of insects strongly depends on successful localization of host plants, food source, mating partners, and oviposition sites. Many insect behaviors are heavily dependent on chemosensation, especially on the perception of olfactory and gustatory cues (Herrero, 2012; Depetris-Chauvin et al., 2015; Walker et al., 2016). In addition to these olfactory and gustatory cues, locomotion represents an integral part of insect behaviors as is essential for food-seeking, mating, and escape response (Zhu et al., 2020). The ability of insects to modify their behavior based on prior experience is essential for their survival (Chia and Scott, 2020). Increasing evidence has highlighted the critical role of early life experience in adult behavior in insects (Caubet et al., 1992; Bezzar-Bendjazia et al., 2016; Ferdenache et al., 2019). In addition, exposure to a stressor, such as pesticides, has been shown to prompt a range of behavioral effects which can be inherited to the next generation (Ferdenache et al., 2019; Lu et al., 2020). Recent work demonstrated for the first time that *D. melanogaster* can modulate its behavior based on previous experiences of early life (third instars larvae) with azadirachtin affecting oviposition site preference and food selection and enhancing avoidances of this compound in adults of parent generation as well as the non-exposed F1 generation (Bezzar-Bendjazia et al., 2016; Kilani-Morakchi et al., 2017; Ferdenache et al., 2019). These changes in insect behavioral responses are influenced by individual sensory experience and may leave an "imprinted" trace into adult life in accordance to experience-induced learning by changes in the neurophysiology of insects (Dukas, 2008; Little et al., 2019). Indeed, biogenic amines, octopamine (OA), serotonin (5-HT), and dopamine (DA) are known to convey the reinforcing cues for many different types of associative memory in *Drosophila* (Masek and Keene, 2016). Azadirachtin treatment was found to reduce OA, 5-HT, and DA levels in both the brain and the hemolymph of *Acherontia styx* (Awad et al., 1997). Furthermore, azadirachtin interferes with the amount of 5-HT in the endocrine organs and, mainly, in the brain of locusts (Banerjee and Rembold, 1992).

Moulin et al. (2020) reported that transient dysregulation of the dopaminergic signaling can produce behavioral alterations in *D. melanogaster* adults, which can then be carried to descendants. In addition, azadirachtin can excite different clusters of dopaminergic neurons, such as PPL1, and increase

dopamine release inducing aversive taste memory in *Drosophila* (Yan et al., 2017); however, the neurophysiological actions of azadirachtin remain to be clarified. In addition, insecticides are known to be able to provoke epigenetic alterations, which can be inherited in the next generations (Vandegehuchte and Janssen, 2011); this possible epigenetic alteration induced by azadirachtin treatment was never investigated. The comprehension of the mechanisms that induce the transgenerational conservation of the aversive effects of azadirachtin may contribute to better use of this compound in IPM programs.

In addition, azadirachtin had the potential to be used in synergy with other botanical compounds. Indeed, azadirachtin and clarified neem oil can significantly synergize the pyrethrum activity while reducing or eliminating the need for pipronyl butoxide as an agent to augment pyrethrum activity, which represents a significant cost advantage when compared with existing pyrethrum/pipronyl butoxide formulations (Chang et al., 1996). On the other hand, phenol compounds in neem were suspected to synergize with the main component (azadirachtin) in increasing the antifeedant activity on *S. litura* (Prianto et al., 2019). The use of azadirachtin in synergy with *B. thuringiensis* (Bandyopadhyay et al., 2014) and karanj (*Pongamia pinnata* Pierre) was also reported (Kumar et al., 2007). Azadirachtin was found to enhance the efficacy of *B. thuringiensis* in *Cydia pomonella*, *S. exigua*, and *Dendrolimus pini* (Konecka et al., 2019). This synergistic effect was observed between azadirachtin and multicapsid nucleopolyhedrovirus (SfMNPV) on the mortality of *S. frugiperda* (Pineda et al., 2014).

More studies are needed for synergism between azadirachtin and other insecticides to find combinations that can effectively control pests. Essential oil or their main compounds, especially compounds (linalool, borneol) with antifeeding activities, might represent a good candidate.

PRACTICAL PROBLEMS OF AZADIRACHTIN APPLICATION

If rapid degradation by sunlight and low persistence in the environment are considered as advantages of the use of azadirachtin and neem derived products, it also represents a problem for their use on a large scale and is disadvantageous from an agribusiness perspective, since they result in lower efficiency and necessitates a greater number applications (Pasquoto-Stigliani et al., 2017).

The chemical nature of the media containing azadirachtin formulation is important and influences its stability. Indeed, studies on the effect of various solvents on the stability of azadirachtin in extracts and formulations reported higher stability of azadirachtin in alcoholic and other aprotic solvents, which are neutral, as compared with protic solvents (Pereira et al., 2019). Furthermore, azadirachtin was most stable in mildly acidic solutions between pH 4 and 6 (Pereira et al., 2019).

The neem-based oil in water emulsion formulation by high shear mixing also improves stability and bio-efficacity of the biopesticide by a decrease of particle size of the emulsion with the increase of stirring time leading to excellent emulsion stability (Iqbal et al., 2020). In addition, the stability of neem oil-based microemulsion can be enhanced by the use of botanical synergists, such as aqueous extract of *Prosopis Juliflora* (Sharma et al., 2019).

The use of nanotechnology also represents a way to overcome such limitations, and the development of controlled-release formulations of botanical insecticides by polymeric encapsulation has been studied in recent years (Das et al., 2014; Pasquoto-Stigliani et al., 2017). Flores-Céspedes et al. (2015) reported that natural polymers, such as kraft lignin and alginate, protect azadirachtin against photodegradation and could be used to improve its stability and delivery to its site of action. These new procedures to encapsulate botanical pesticides provide several benefits including slow-release, enhanced stability of compounds, use of small dose, and masking of odor (Chaudhary et al., 2017). Poly(ε-caprolactone) nanocapsules loaded with neem oil are save to soil microbiota during 300 days of exposure and did not affect the net photosynthesis and stomatal conductance of maize plants, and present lower toxicity against non-target organisms (Pasquoto-Stigliani et al., 2017); however, the same nanocapsule containing a mixture of neem oil and oleic acid presented higher toxicity and led to negative effects. Recently, Shanmugapriya et al. (2019) demonstrated that azadirachtin loaded in silica nanoparticles at 500 ppm showed high mortality of adult *Bemisia tabaci* and can be used as an alternative to chemical pesticides.

Although nanotechnology is still at an early stage in the agricultural sector, it is clear that there is growing interest in its use; however, studying the toxicity of nano pesticides and understanding their mechanism of action in target organisms is a key factor in the selection of the best formulations for use in agricultural applications (Feng and Peng, 2012; Seugling et al., 2019; Jesser et al., 2020).

In addition, a sublethal dose of azadirachtin was reported to induce hormesis in the Mexican Bean Weevil, *Zabrotes subfasciatus*, with increased fecundity daily to compensate for azadirachtin-induced decreased longevity (Vilca Malqui et al., 2014). In addition, the population of *Z. subfasciatus* engendered from females exposed to azadirachtin present a higher rate of population increase and a higher net reproductive rate (Vilca Malqui et al., 2014). Similar results were reported in *Myzus persicae* exposed to sublethal concentrations of azadirachtin with a modest hormetic response under laboratory conditions (Cutler et al., 2007). Evidence-based toxicology under field conditions must be used to solidify the importance of hormesis to understand the risk of exposure to azadirachtin and neem-based compounds. In addition, new research tools, such as toxicogenomics and statistical modeling processes, must be designed to evaluate possible hormetic responses when devising pest management strategies.

CONCLUSION

Health and environmental concerns have influenced the use of safe and non-hazardous pest control measures. Azadirachtin-based insecticides have recently been promoted as an alternative

pest control method, especially in agroecological farming and organic agricultural systems. Azadirachtin has broad-spectrum activity for combating numerous pests in different crops, and it has not yet reached most of its potential utilization. Currently, information is sparse on the possible long-term and transgenerational effects of azadirachtin on insects; a better comprehension of this phenomenon could improve its use in IPM programs by reducing the concentrations used, frequency of application and targeting the best time of application, which might enhance its ecotoxicological profile.

In addition, the nanoencapsulation of this biopesticide provides a novel way to enhance its stability and sustainability, since they protect it against degradation and modulate its release.

AUTHOR CONTRIBUTIONS

SK-M wrote the manuscript. SK-M, HM-G, and KS contributed to the collection of the information and the discussion and revised the manuscript. All authors approved the final version of the manuscript.

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4-Ethylphenol, A Volatile Organic Compound Produced by Disease-Resistant Soybean, Is a Potential Botanical Agrochemical Against Oomycetes

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Oomycetes, represented by *Phytophthora*, are seriously harmful to agricultural production, resulting in a decline in grain quality and agricultural products and causing great economic losses. Integrated management of oomycete diseases is becoming more challenging, and plant derivatives represent effective alternatives to synthetic chemicals as novel crop protection solutions. Biologically active secondary metabolites are rapidly synthesized and released by plants in response to biotic stress caused by herbivores or insects, as well as pathogens. In this study, we identified groups of volatile organic compounds (VOCs) from soybean plants inoculated with *Phytophthora sojae*, the causal agent of soybean root rot. 4-Ethylphenol was present among the identified VOCs and was induced in the incompatible interaction between the plants and the pathogen. 4-Ethylphenol inhibited the growth of *P. sojae* and *Phytophthora nicotianae* and had toxicity to sporangia formation and zoospore germination by destroying the pathogen cell membrane; it had a good control effect on soybean root rot and tobacco black shank in the safe concentration range. Furthermore, 4-Ethylphenol had a potent antifungal activity against three soil-borne phytopathogenic fungi, *Rhizoctonia solani*, *Fusarium graminearum*, and *Gaeumannomyces graminis* var *tritici*, and four forma specialis of *Fusarium oxysporum*, which suggest a potential to be an eco-friendly biological control agent.

Keywords: 4-Ethylphenol, leaf volatile compounds, cell membrane damage, biological control, *Phytophthora*

INTRODUCTION

Oomycetes, encompassing *Phytophthora*, *Albugo*, *Pythium*, and a group of downy mildews that cause plant epidemics, have a negative impact on natural and farm ecosystems due to their strong pathogenicity and infectivity (Yutin et al., 2008; Kamoun et al., 2015). Besides the well-known potato late blight caused by *Phytophthora infestans*, which led to the Irish famine of the 19th

Century, *Phytophthora nicotianae* is a pathogen distributed worldwide, and it causes tobacco black shank and is responsible for many foliar and fruit diseases (Fang et al., 2016). Soybean root rot is caused by *Phytophthora sojae* and is the leading cause of global soybean production losses (Tyler, 2007). Oomycetes are phylogenetically different from fungi, forming an independent group, and are therefore resistant to many broad-spectrum fungicides (Tyler et al., 2006). Some of the fungicides effective against oomycetes, such as metalaxil, have resulted in the emergence of insensitive strains and resurgence events due to their single site of action (Randall et al., 2014). Interdisciplinary studies and consistent resources have been invested in finding new, effective alternatives for the integrated pest management of oomycete diseases (Gessler et al., 2011).

Novel pharmaceuticals against oomycetes should be explored for rational fungicide design, and further focus should be placed on developing alternative botanical agrochemicals to fight different pathogens that attack crops and related products (Drakopoulos et al., 2020; Liang et al., 2021; Wang et al., 2021). Environmentally friendly botanical fungicides are widely welcomed due to their higher efficiency, lower residue, and lower negative impact on the environment (Naz et al., 2018; Tschoeke et al., 2019). Plants are a rich natural source of active antimicrobial substances (Nino et al., 2012; Hu et al., 2018). For example, artemisinin, present in sweet wormwood, a Chinese medicinal plant, is the most effective antimalarial drug available (Tu, 2011).

Natural products have a long history as a source of novel agrochemicals (Yoon et al., 2013). Phytopathologists search for alternative botanical products to replace synthetic fungicides and effectively control plant diseases without significantly affecting crop yields (Bowers and Locke, 2000). For example, poacic acid, which is derived from grass lignocellulosic hydrolysates, inhibits the growth of the *Sclerotinia sclerotiorum* and *Alternaria solani* fungi and the oomycete *P. sojae* (Piotrowski et al., 2015). Pathogen cells treated with poacic acid suffer similar effects to those treated with cell wall-targeting synthetic drugs (Lee et al., 2018). There is considerable evidence for the protective effects of phytochemicals isolated from tissue exudates or volatiles against disease propagation (Drakopoulos et al., 2020; Liao et al., 2021; Wang et al., 2021). For example, grape cane ε-viniferin has antifungal activity against *Plasmopara viticola* and *Botrytis cinerea* (Schnee et al., 2013). Secomicromelin, coumarin, isomicromelin, and micromarin B, present in *Micromelum falcatum* fruits, inhibit the growth of *Pythium insidiosum* (Suthiwong et al., 2014). Cuminic acid, isolated from cumin seeds (*Cuminum cyminum* L.), inhibits *Phytophthora capsici* mycelial growth and zoospore germination (Wang et al., 2016). Gossypol, naturally present in cotton root tissues, has a strong inhibitory activity on *Pythium irregulare*, *Pythium ultimum*, and *Fusarium oxysporum* growth (Mellon et al., 2014).

Leaf volatile organic compounds (VOCs) are rapidly emitted when plants respond to biotic stress caused by herbivores or attacks by necrotrophic fungi (Scala et al., 2013; Matsui and Koeduka, 2016; Tanaka et al., 2018). VOC production is a basic defense mechanism for plants to enhance resistance or tolerance to upcoming stresses and may contribute to direct plant defense responses through their powerful antimicrobial activities (Jerkovic et al., 2012; Krajaejun et al., 2012; Ulloa-Benitez et al., 2016; Ricciardi et al., 2021).

Here, we analyzed soybean leaf volatiles produced in incompatible interaction and compatible interaction with *P. sojae*. A group of VOCs was identified by headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS–SPME–GC–MS), which were specifically present in the incompatible interaction. In Petri dish assays, 4-Ethylphenol, a volatile phenolic substance, inhibited the mycelial growth, sporangia formation, and zoospore germination of *P. sojae* and *P. nicotianae*. Additionally, it had potent antifungal activities against three soil-borne phytopathogenic fungi, *Rhizoctonia solani*, *Fusarium graminearum*, *Gaeumannomyces graminisvar*, and four *Fusarium oxysporum* forma specialis. We found that 4-Ethylphenol triggers mycelia malformation and cytoplasmic electrolyte leakage because of a disrupted or disintegrated plasma membrane. Finally, we analyzed the potential of 4-Ethylphenol as an oomycete biological control agent and confirmed its efficacy in controlling soybean root rot and tobacco black shank diseases in potted plants, and observed a positive effect on plant growth when present in low concentrations.

MATERIALS AND METHODS

Plant and *Phytophthora* spp. Cultivation

Soybean and tobacco plants were grown in a chamber at 25°C, with a cycle of 16 h of high light intensity and 8 h of darkness. *P. sojae* strain P6497 and *P. nicotianae* strain INRA-310 were grown on 10% V8 medium (10% V8 juice, 0.02% $CaCO_3$, and 1.5% agar) in the darkness at 25°C. Mycelia were cultured in V8 liquid medium. To observe zoosporangia, the mycelia were washed with sterile water five times and cultured in the darkness at 25°C for 6 h. When zoosporangia formed, zoospores were released after washing with sterile water at 10°C three times. Finally, the concentration of the zoospore suspension was adjusted to 10^5 CFU/mL.

Gas Chromatography–Mass Spectrometry Analysis

Soybean leaves (Williams and Williams-82 cultivars) inoculated with *P. sojae* were placed in a 20 mL headspace bottle, and the VOCs were analyzed with gas chromatography–mass spectrometry (GC–MS). An AOC-6000 Multifunctional Autosampler was used for solid-phase microextraction injection, and GCMS-TQ8040 NX was used for detection following the standard SPME parameters (SPME fiber: FIB-C-WR-95/10. The following parameters were used: aging temperature, 240°C; aging time (before extraction), 30 min; equilibration temperature, 40°C; equilibration time, 5 min; extraction time, 30 min; injection port temperature, 250°C; desorption time, 2 min; and aging time (after extraction), 5 min. The GC–MS/MS parameters used were: column, inert cap pure-wax, 30 m × 0.25 mm × 0.25 m; oven program, 50°C (5 min),

10°C/min_250°C (10 min); carrier gas pressure, 83.5 kPa; injection mode, split; split ratio, 5:1; ion-source temperature, 200°C; interface temperature, 250°C; detector voltage, tuning voltage + 0.3 kV; and acquisition mode, MRM.

Effect of 4-Ethylphenol on the Radial Growth of *Phytophthora* spp. Hyphae

Hyphal plugs of *P. sojae* and *P. nicotianae* were cultured in 10% V8 agra medium containing different concentrations of 4-Ethylphenol, or the same volume of sterile water as a control. The medium was incubated in the darkness at 25°C for 5 days; then, the colony diameter was measured, and the mycelium status was observed under the microscope. Each treatment was repeated three times.

Effects of 4-Ethylphenol on *Phytophthora* spp. Zoosporangium Formation and Zoospore Release

Washed mycelia were placed in different concentrations of 4-Ethylphenol. After incubation at 25°C for 6 h, the number of zoosporangia was observed and recorded under the microscope using Mallassez cell counting. 4-Ethylphenol was added to the sporangium-forming dishes, and after 2 h, the number of zoospores was measured under the microscope using Mallassez cell counting. Each treatment was repeated three times.

Effects of 4-Ethylphenol on *Phytophthora* spp. Zoospore Germination

The 0.1 mL zoospore suspension was evenly spread on V8 medium containing four different concentrations of 4-Ethylphenol. After incubation at 25°C for 4 days, the minimum concentration with no colony formation was determined by naked eye observation. Each treatment was repeated three times.

Effect of 4-Ethylphenol on *Phytophthora sojae* Virulence

Soybeans were planted in the dark for 7 days, and etiolated seedlings were immersed in 4-Ethylphenol solution for 1 h and then placed in zoospore suspension for infection. After 4 or 6 h of infection, the hypocotyls were collected and stained with a lactophenol–trypan blue dye solution. After 2 h of staining, samples were destained with chloral hydrate until they were translucent. The discolored epidermis was then removed with forceps, prepared, and observed under the microscope. Each treatment was repeated three times.

Damage of the *Phytophthora* spp. Cell Membrane by 4-Ethylphenol

Mycelia were cultured in V8 liquid medium for 3 days. Washed mycelia were placed in PBS buffer (pH 7.0) containing 4-Ethylphenol; DNA and protein concentrations were measured and recorded every 2 h. Each treatment was repeated three times.

Safety of 4-Ethylphenol on Soybean and Tobacco Plants

Different concentrations of 4-Ethylphenol (0–25 mg a.i./plant) were mixed with soil. The growth and development of soybean and tobacco plants were recorded at 7 and 14 days after treatment, respectively. Each treatment was repeated three times.

Efficacy of 4-Ethylphenol as a Soil Fumigant

Different concentrations of 4-Ethylphenol were mixed with soil and then sealed in plastic film for 15 days. After being air-cured for 2 days, soybean and tobacco were planted in the soil, and their growth and development were observed after 9 days. Each treatment was repeated three times.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 19.0. The difference among treatments was determined based on one-way analysis of variance (ANOVA), and means were subjected to Duncan's multiple range test with significance set at $P < 0.05$.

RESULTS

Detection of the VOC 4-Ethylphenol in Soybean Leaves

We investigated the VOCs produced in the leaves of susceptible soybean plants (Williams), lacking resistance genes to *P. sojae* (*Rps*), and resistant soybean plants (Williams82), containing the *Rps1k* resistance gene, by GC—–MS. The 4-Ethylphenol content in Williams82 was significantly higher than that in Williams leaves (**Supplementary Figure 1**), suggesting that 4-Ethylphenol is involved in the defense response of soybean to *P. sojae*.

Effect of 4-Ethylphenol on the Mycelium Growth of *Phytophthora* spp.

The radial diameter of *P. sojae* and *P. nicotianae* colonies grown on V8 medium supplemented with different concentrations of 4-Ethylphenol was determined through naked eye observation; the toxicity of 4-Ethylphenol to *Phytophthora* spp. was observed and calculated (**Figure 1A**). The average diameter of *P. nicotianae* colonies cultured for 5 days on V8 medium containing 0.4 mmol (57.66 mg/L) of 4-Ethylphenol was 20.52 mm, and the inhibition rate was 57.73% (**Figure 1B**). Additionally, the average diameter of *P. sojae* colonies cultured for 5 days on V8 medium containing 0.6 mmol (86.48 mg/L) of 4-Ethylphenol was 20.50 mm, and the antifungal rate was 54.14%. Medium containing 1 mmol (144.14 mg/L) of 4-Ethylphenol completely inhibited the growth of both oomycete species (**Figure 1C**).

Effect of 4-Ethylphenol on the Morphology and Cell Membrane of *Phytophthora* spp. Hyphae

Phytophthora spp. hyphae grew naturally in V8 medium, without increased terminal branching; the mycelium growing points

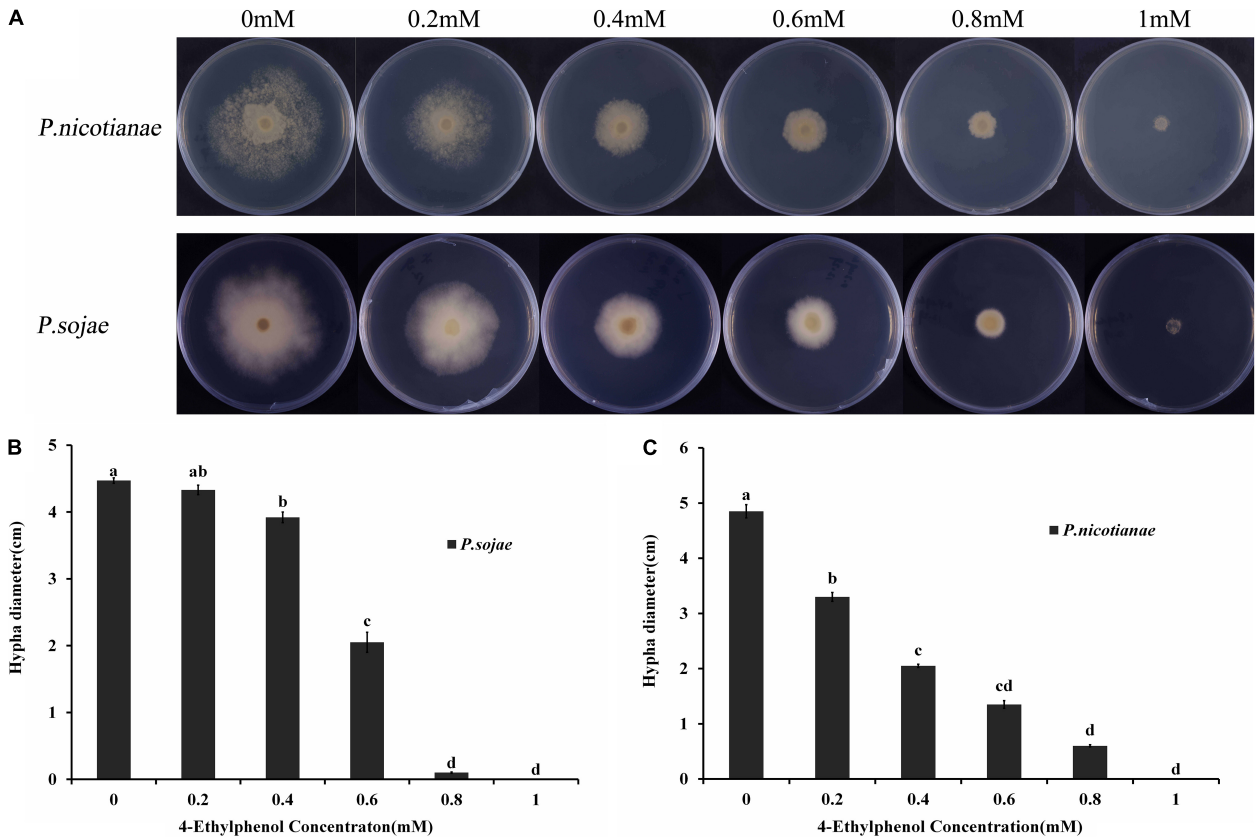

FIGURE 1 | Antifungal activity of 4-Ethylphenol against *Phytophthora* spp. **(A)** *P. sojae* and *P. nicotianae* were cultured on V8 medium with different concentrations of 4-Ethylphenol. **(B)** Colony diameters of *P. sojae* were measured after 5 days. **(C)** Colony diameters of *P. nicotianae* were measured after 5 days. The experiment was repeated three times with similar results.

were uniform, and the branches formed far from the top. After treatment with 0.4 mmol of 4-Ethylphenol, the growth of *P. sojae* and *P. nicotianae* hyphae was inhibited in the V8 liquid medium. The morphology of the mycelia changed in the presence of 4-Ethylphenol, and the branches at the end increased significantly and became disordered (**Figure 2A**). The results showed that 4-Ethylphenol inhibited growth by changing the morphology of *Phytophthora* spp. hyphae. To analyze the mechanisms of antimicrobial activity against *P. sojae* and *P. nicotianae*, we treated mycelia with 4-Ethylphenol and investigated the presence of leakage of cellular material. By monitoring the total DNA and protein concentrations in the media every 2 h, we observed that the contents increased significantly with time, and the intensity of leakage increased with 4-Ethylphenol concentration (**Figures 2B,C**). The leakage of cellular contents was likely due to the destruction of the cell membrane by 4-Ethylphenol.

Inhibition Effect of 4-Ethylphenol on the Formation of Sporangium and Zoospores of *Phytophthora* spp.

To study the effect of 4-Ethylphenol on *Phytophthora* spp. zoosporangia formation, we determined the number of zoosporangia and calculated the inhibition degree after treatments with different concentrations of the VOC. The formation of *P. sojae* zoosporangia was significantly reduced by 0.2 mmol of 4-Ethylphenol, while no zoosporangium was formed with 1 mmol. For *P. nicotianae,* 0.4 mmol of 4-Ethylphenol significantly reduced zoosporangium formation, whereas 0.8 mmol of 4-Ethylphenol completely inhibited it (**Figure 3A**).

Additionally, zoospore release from both *P. sojae* and *P. nicotianae* sporangia was significantly reduced with 0.4 mmol of 4-Ethylphenol, and completely inhibited with 0.8 mmol of 4-Ethylphenol (**Figure 3B**).

Effect of 4-Ethylphenol on *Phytophthora* spp. Zoospore Germination

To analyze the effect of 4-Ethylphenol on *Phytophthora* spp. zoospore germination, we spread a zoospore suspension evenly on 1% V8 medium containing different concentrations of the VOC. Compared with control, 0.4 mmol of 4-Ethylphenol significantly inhibited the germination of *P. sojae* zoospores; only a few zoospores could germinate and form separate colonies. At 0.8 mmol of 4-Ethylphenol, the germination of zoospores was completely inhibited. For *P. nicotianae*, 0.6 mmol of 4-Ethylphenol significantly inhibited zoospore germination, and 1 mmol completely inhibited it (**Supplementary Table 1**).

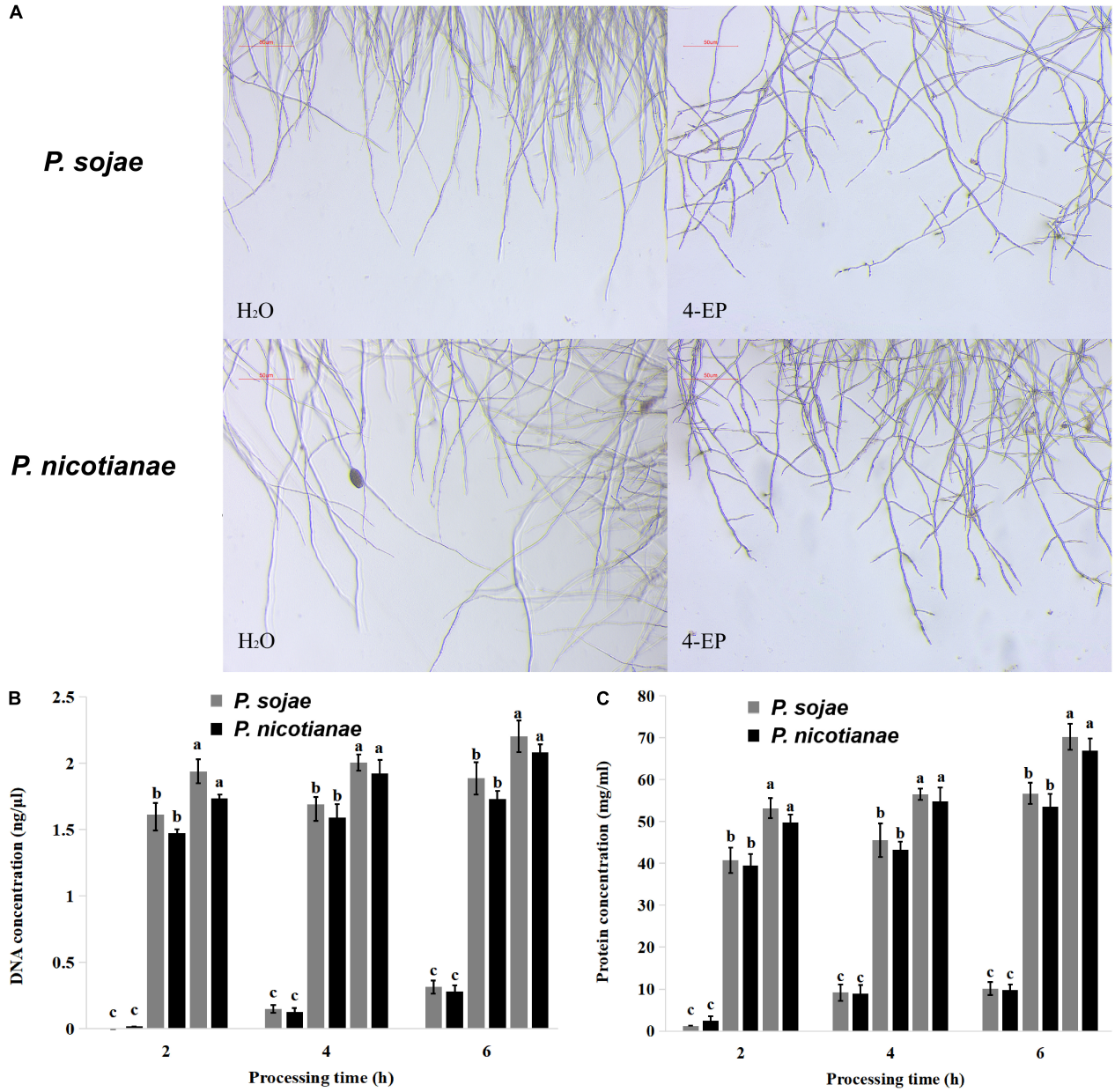

FIGURE 2 | Effect of 4-Ethylphenol on *Phytophthora* spp. morphology and DNA and protein leakage. **(A)** Effect of 4-Ethylphenol on the morphology of *P. sojae* and *P. nicotianae* treated with 4-Ethylphenol for 2 h; **(B)** effect of 4-Ethylphenol on DNA leakage of *P. sojae* and *P. nicotianae*; **(C)** effect of 4-Ethylphenol on protein leakage of *P. sojae* and *P. nicotianae*.

Effect of 4-Ethylphenol on *Phytophthora sojae* Zoospore Invasion

Phytophthora sojae zoospores treated with the control could attach to the epidermis of soybean hypocotyls, and some of them could form germ tubes at 4 h post-inoculation (hpi) (**Figure 4A**). At 6 hpi, more zoospores were attached to the epidermis, and the majority had germinated hyphae for infection (**Figure 4B**). Treatment with 4-Ethylphenol significantly reduced the zoospore adhesion ability, and only a few attached to the surface of soybean hypocotyls at 4 hpi (**Figure 4C**). At 6 hpi, the zoospore attachment and germination decreased significantly compared with the control (**Figure 4D**), indicating that 4-Ethylphenol could effectively inhibit zoospores from infecting soybean.

Effect of 4-Ethylphenol on Germination and Growth of Soybean and Tobacco Plants

To investigate the safety of 4-Ethylphenol for host plants, we planted soybean and tobacco seedlings in soil mixed with different concentrations of 4-Ethylphenol, and seedling heights

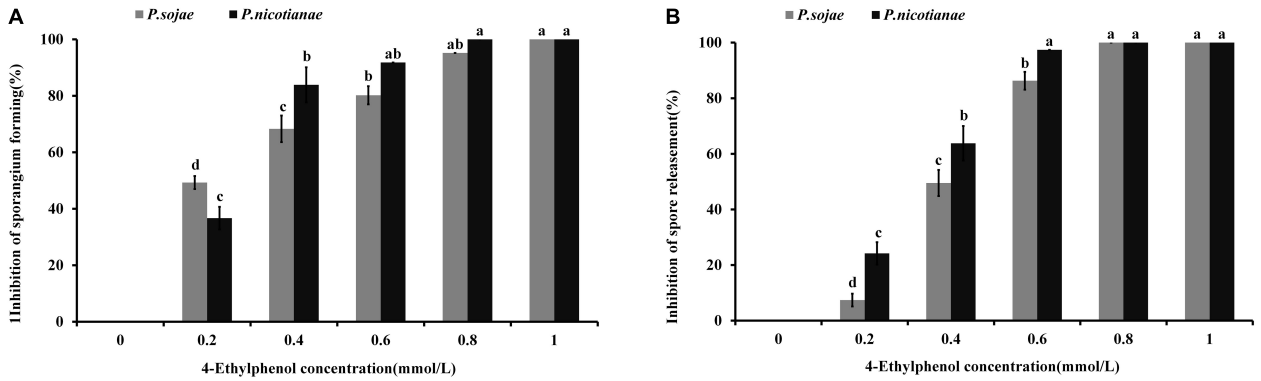

FIGURE 3 | Effect of 4-Ethylphenol on zoosporangium formation and zoospore release. **(A)** Inhibition rate of 4-Ethylphenol on *P. sojae* and *P. nicotianae* zoosporangium formation; **(B)** inhibition rate of 4-Ethylphenol on *P. sojae* and *P. nicotianae* zoospore release. The experiment was repeated three times with similar results.

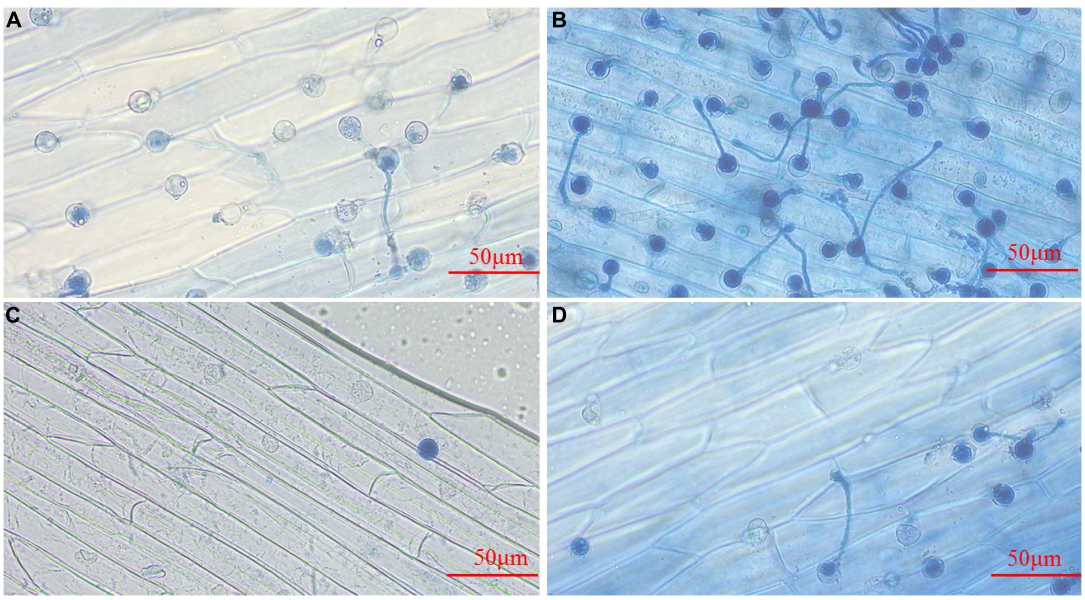

FIGURE 4 | Effect of 4-Ethylphenol on *P. sojae* zoospore invasion. Zoospore invasion after **(A)** 4 h of the control treatment (water), **(B)** 6 h of the control treatment (water), **(C)** 4 h of the 4-Ethylphenol treatment, **(D)** 6 h of the 4-Ethylphenol treatment. The control-treated *P. sojae* zoospores attached to the hypocotyls of yellow–yellow seedlings and grew many hyphae for infection, whereas the *P. sojae* treated with 4-Ethylphenol significantly reduced the adhesion and infection ability. The experiment was repeated three times, and similar results were obtained.

were measured 7 and 14 days after the treatment (**Figure 5**). The results showed that the germination rate of soybean plants under all tested concentrations reached 100%, indicating that 4-Ethylphenol did not affect soybean seed germination. The average height of soybean plants increased compared with the control with treatments at low concentrations, indicating that 4-Ethylphenol promoted the growth of soybean plants. The maximum concentration tested, 25 mg a.i./plant, had no obvious inhibitory effects on seedling height, indicating that 4-Ethylphenol did not affect the normal soybean growth (**Figure 5A** and **Supplementary Figure 2A**). Regarding tobacco plants, the average height of plants with 4-Ethylphenol was not significantly different from that of the control group after 7 and 14 days (**Figure 5B** and **Supplementary Figure 2A**). Tobacco seedlings treated with different concentrations grew normally and somewhat consistently.

Efficacy of 4-Ethylphenol Against Soybean Root Rot and Tobacco Black Shank Diseases

Based on the results from the concentration gradient safety test, we planted soybean and tobacco on soil mixed with 4-Ethylphenol and mycelia to observe the control effect on soybean root rot and tobacco black shank. The results showed that control-treated soybean plants (no pathogen) grew

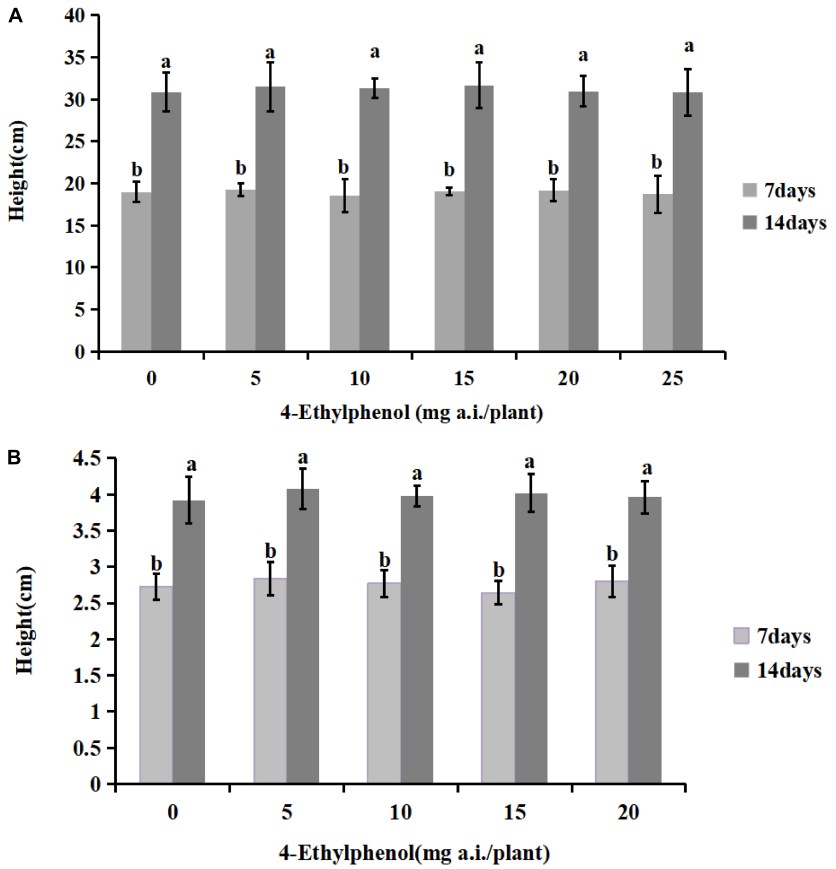

FIGURE 5 | Effects of 4-Ethylphenol on soybean **(A)** and tobacco **(B)** height. Data are the mean ± S.E. from three replicates per treatment.

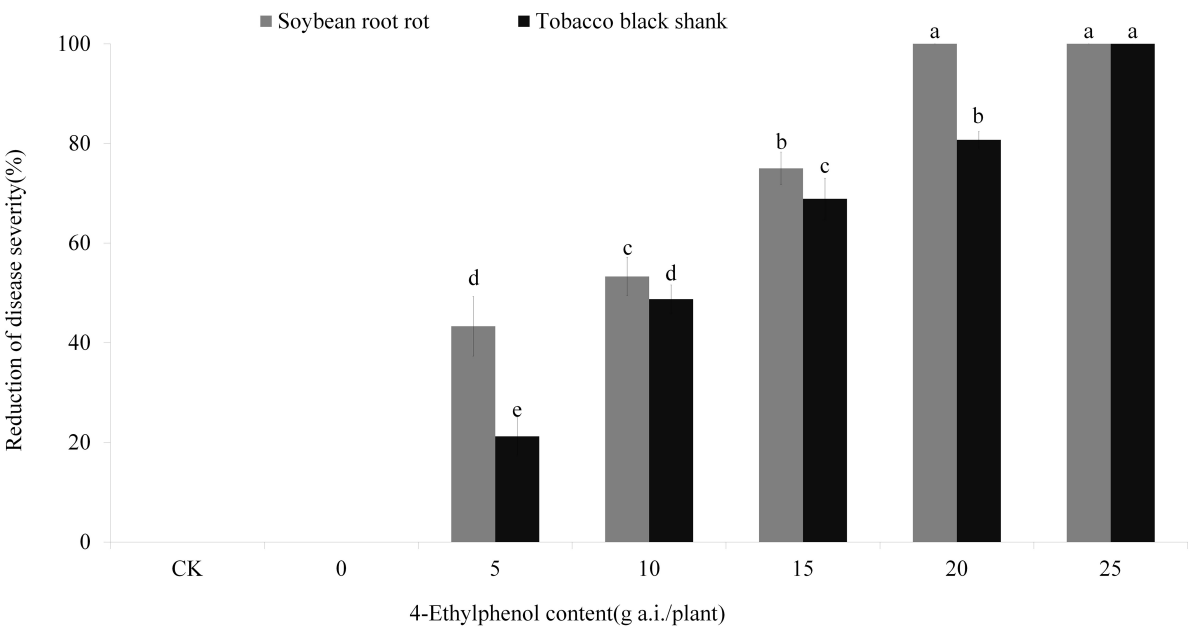

FIGURE 6 | Efficacy of 4-Ethylphenol on soybean root rot and tobacco black shank diseases. Data are the mean ± S.E. from three replicates per treatment.

normally, whereas those treated with *P. sojae*-containing soil had serious disease symptoms (**Figure 6** and **Supplementary Figure 3B**). With the increase in 4-Ethylphenol concentration, the disease index decreased gradually, and the relative control effect improved. When 20 mg a.i./plant of 4-Ethylphenol was mixed with soil, the control effect reached 100%, and soybean plants were healthy (**Figure 6** and **Supplementary Figure 3A**). Furthermore, tobacco plants grown without the pathogen grew normally, whereas adding *P. nicotianae* to the soil prompt quick plant death. Application of 4-Ethylphenol decreased the disease index gradually in a dose-dependent response and the tobacco plants were mostly healthy. When 25 mg of 4-Ethylphenol was applied per pot, all tobacco plants grew normally, and the control effect reached 100% (**Figure 6** and **Supplementary Figure 3B**).

DISCUSSION

Oomycetes are fungus-like organisms that include a group of notorious phytopathogens, namely *Phytophthora*, *Albugo*, *Pythium*, and downy mildews (Kamoun et al., 2015). Although oomycetes resemble filamentous fungi (Jiang and Tyler, 2012), they are phylogenetically related to diatoms and brown algae in the stramenopiles (Tyler et al., 2006; Haas et al., 2009; Thines and Kamoun, 2010; Tan et al., 2020). The well-documented Irish famine forced humans to investigate the causes of the potato late blight and discover the responsible microbial pathogen, *P. infestans* (Haas et al., 2009). Since the 1870s, breeding efforts for late blight resistance have failed to provide a durable, resistant cultivar (DeArce, 2008). To control late blight, farmers rely largely on fungicides with unknown modes of action (Rekanovic et al., 2012; Ferreira et al., 2014; Randall et al., 2014; Childers et al., 2015; Miao et al., 2016; Chen et al., 2018). Many fungicides are ineffective against oomycetes because of their phylogenetic differences (Siegenthaler and Hansen, 2021). Although some fungicides have had some effect on oomycetes, widespread use quickly caused fully insensitive races to emerge (Randall et al., 2014; Childers et al., 2015; Matson et al., 2015; Pang et al., 2016). Currently, *P. infestans* remains a major constraint to the global production of potato and tomato and is thus a constant threat to food security (Haverkort et al., 2008; Fisher et al., 2012). In addition to late blight, *Phytophthora* spp. soybean root rot and tobacco black shank are diseases that are distributed worldwide, which lack effective control methods (Dorrance et al., 2003; Cui et al., 2010; Ji et al., 2014; Li et al., 2017). Copper-based fungicides are effective in crop protection against oomycetes, but they are facing restrictions because of copper accumulation in the soil (Mackie et al., 2013; Wightwick et al., 2013; Thuerig et al., 2018). New sources of fungicides have to keep pace with the evolution of resistant strains and emerging pathogens (Alexander and Perfect, 1997; Jian et al., 2015).

Due to the increasingly stringent regulatory requirements, crop protection approaches will have to ensure environmental conservation and sustainability (Hao et al., 2019; Kapsi et al., 2019). The use of natural products as an alternative to synthetic chemicals in the fight against different phytopathogens remains a constant need (Thuerig et al., 2018; Lorsbach et al., 2019). The primary and secondary metabolites produced by plants are important sources for developing novel environmental-friendly agrochemicals (Dayan et al., 2009). VOCs synthesized and emitted in response to pathogen infection are particularly relevant and usually have functional benefits in multiple aspects of plant defense (Ricciardi et al., 2021; Xu et al., 2021).

In this study, we analyzed the VOCs emitted when soybean plants responded to *P. sojae*. We used an odor analyzer combined with a Triple Quadrupole quality selection detector GCMS-TQ8040NX (Simadzu Company, Kyoto, Japan) and AOC-6000 multifunctional automatic sampler to establish an analysis method for VOCs induced in plant leaves. Regarding the software, the Odor Analyzer provides a complete method package and database, which was first released and used to analyze plant VOCs. One hundred and fifty components were determined with this semi-quantitative method, which is easy to operate, rapid to provide and analyze data, and suitable for the rapid screening of plant odors in culture.

Among the 150 components identified, a potent antifungal compound, 4-Ethylphenol, requires further exploration as a source or template for novel crop protection chemistry. There have been a few reports demonstrating the antimicrobial potential of 4-Ethylphenol (Xing et al., 2018). In this study, 4-Ethylphenol had a good inhibitory effect on *P. sojae* and *P. nicotianae*. Zoospore germination and mycelium growth are important for disease epidemics (Tyler et al., 1996). Our results showed that 1 mM (144.15 mg/L) 4-Ethylphenol completely inhibited *Phytophthora* spp. sporangium formation and mycelium growth. This inhibition is comparable to that of the plant-derived antifungal agent poacic acid when applied with an IC50 of 1,000 mg/L against *P. sojae* (Piotrowski et al., 2015). 4-Ethylphenol has a potent antifungal activity against three soil-borne phytopathogenic fungi, *Rhizoctonia solani*, *Fusarium graminearum*, and *Gaeumannomyces graminis* var *tritici*, and four *Fusarium oxysporum* forma specialis. Usually, these fungi are associated with *Phytophthora* spp. in the soil and cause compound infection complications, which aggravate the occurrence of plant root rot (Pemberton et al., 1998; Wen et al., 2017; Brown et al., 2021). The potent antifungal activity of 4-Ethylphenol could block the spread of these pathogens, and may play a key role in inhibiting soil-borne disease epidemics. This study provides the first report of the activity of 4-Ethylphenol against a series of *Phytophthora* spp. and fungi pathogens, demonstrates its potential as a universal broad-spectrum fungicide for soils, and justifies efforts to investigate its mechanism of action in detail.

To understand the mechanisms behind the antimicrobial action of 4-Ethylphenol, we examined the morphology of *Phytophthora* spp. mycelia treated with the VOC. Mycelium morphology could be changed after treatments with plant essential oils (Huang et al., 2019). Microscopic observation showed that *P. sojae* and *P. nicotianae* mycelia morphology was also changed after 4-Ethylphenol treatment. The leakage of intracellular materials, such as DNA and proteins, was significantly higher in treated mycelia than in the control,

confirming that 4-Ethylphenol damaged the cell structure and thus affected the normal mycelial growth.

Because it is a potential antifungal agent, plant security studies for 4-Ethylphenol application are necessary. The application of 4-Ethylphenol in a certain concentration range (0–25 mg a.i./plant) did not inhibit or harm normal soybean and tobacco growth. At the lower but effective concentration range (5–15 mg a.i./plant), it even slightly promoted soybean and tobacco growth.

We further verified that 4-Ethylphenol is effective in controlling soybean root rot and tobacco black shank in pot experiments where, to mimic the field application, 4-Ethylphenol was mixed with soil. The results showed that 4-Ethylphenol could effectively inhibit both the soil-borne pathogens without affecting the normal plant growth. Future work should explore more effective application methods and lay a foundation for creating 4-Ethylphenol field application directives.

Botanical fungicides are derived from natural products and are less likely to develop drug resistance than chemical fungicides (Shuping and Eloff, 2017). None of the natural and eco-friendly chemical alternatives currently registered and available have the full spectrum of activity and versatility of methyl bromide as pre-plant soil fumigants (Duniway, 2002; Driver et al., 2016). Based on the results described here, 4-Ethylphenol is a potent antimicrobial that regulates plant growth and has the potential to substitute traditional antifungal agents.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

QW and QX designed the experiments. TG, CL, and WG performed the experiments and analyzed the data. YW performed the GC–MS experiments and analyzed the data. TG and QW wrote the manuscript. QX and CH participated in manuscript revision or experiments. QW revised the manuscript and provided the funding for this research. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.717258/full#supplementary-material

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Invasive Alien Plants in Sub-Saharan Africa: A Review and Synthesis of Their Insecticidal Activities

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Despite the cornucopia of agricultural, economic and ecological ramifications of invasive alien plant species (IAPs) in sub-Saharan Africa, studies on their potential use as bio-insecticides have not received adequate attention compared to the burgeoning plethora of literature on their use in ethnomedicine. In the current study, we review the existing, but scattered literature on the insecticidal activity of different parts of some IAPs; specifically those invasive in sub-Saharan Africa but with published literature from Africa and elsewhere. From our literature survey, we found that 69 studies from four continents (Africa, Asia, North America and South America) reported the insecticidal activity of 23 plant species from 13 families (Asteraceae = 6 species; Solanaceae = 3 species; Apocynacee, Fabaceae and Euphorbiaceae 2 species each; Araceae, Bignoniaceae, Chenopodiaceae, Meliaceae, Mimosaceae, Myrtaceae, Papaveraceae, and Verbenaceae = 1 species each) that are invasive in, and alien to Africa. The highest number of published case studies were from India ($n = 19$) and Nigeria ($n = 15$). We found that varying concentrations of extracts or powders from different plant parts caused 50–100% mortality against a myriad of insect pests of agriculture and environmental importance. Our review discussed the prospects for exploiting IAPs as pesticidal plants in African countries especially among resource-poor small-holder farmers and locals to improve agricultural productivity and livelihoods. Finally, we highlighted safety concerns and challenges of using IAPs as bio-insecticides in Africa and formulates appropriate recommendations for future research.

Keywords: invasive alien plant species, Africa, botanical insecticide, insect pest control, resource poor farmers

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INTRODUCTION

Invasive alien plant species (IAPs) are among species whose naturalization threatens the biological biodiversity and functions of the ecosystem in their new geographic region (Richardson and Pyšek, 2012; Mostert et al., 2017; O'Connor and van Wilgen, 2020). These plants are among significant ecosystem drivers that degrades the quality of grazing, agricultural and natural lands (Richardson and van Wilgen, 2004; Davis, 2006). Due to the immense ecological and social pressures exerted by these plants, governments have announced the management of IAPs and millions of dollars are invested toward the management of these plants in South Africa and elsewhere in the world (McConnachie et al., 2010; Van Wilgen and Lange, 2011; Hoffmann and Broadhurst, 2016; Morokong et al., 2016; Hanley and Roberts, 2019).

Regardless of the efforts made toward minimizing the densities of invasion and the spread of these IAPs, follow-up treatments may be required to keep the populations of these non-native

species at a level that prevents spread and harm to human health or the environment (Marais et al., 2004; Klein, 2011; Mukwevho and Mphephu, 2020). Although manual clearing of IAPs yields temporal relief on the intensity of invasion, continuous clearing alone favors the expansion of the invasion by species that are propagated vegetatively (Radtke et al., 2013). To minimize the further spread of IAPs through plant propagules, the cut plant materials from the above- and below-ground may be further processed to be used for socio-economic and ecological benefits in sub-Saharan Africa (Shackleton et al., 2007, 2018; Ngorima and Shackleton, 2019; Mugwedi, 2020).

The potential use of IAPs in ethnomedicine and various aspects of ethnobotany in Africa have received a great deal of attention (e.g., Omokhua et al., 2016, 2018a,b) however, studies on the use and potential of invasive alien plants as pesticidal plants to manage agricultural and environmental pests is only beginning to gain recognition (e.g., Midega et al., 2016; Mkindi et al., 2017; Stevensona et al., 2017; Uyi et al., 2018a,b). Since some IAP's contain some novel secondary phytochemicals, the harvested materials may be processed to be used against microorganisms, insects and weeds and other undesired plants (Deressa et al., 2015; Amir et al., 2017; Mkindi et al., 2017; Das et al., 2018; Zerihun and Ele, 2018; Mugwedi, 2020). Like other pesticides, biopesticides may repel insect pests, disrupt their development, affect reproduction or kill live organisms on contact (Mogg et al., 2008; Litt et al., 2014; Uyi and Adetimehin, 2018). Although different scientists consider IAPs as a threat to agriculture and biodiversity, dozens of IAPs have insecticidal properties that have been rigorously screened toward major pests, pollinators and wasps (including some parasitoids) around the globe (Isman, 2008; Mkenda et al., 2015; Mkindi et al., 2017; Stevensona et al., 2017).

Due to the cost of synthetic chemicals (Dougoud et al., 2019), impacts on non-target species (Theiling and Croft, 1988; Mulè et al., 2017), target pest's genetic drift (REX consortium, 2010; Khayatnezhad and Nasehi, 2021) and ecotoxicological impacts (Pimentel, 1995; Kankam, 2021), the United Nations (UN) promotes the use of environmentally safe products, such as aqueous extracts to minimize the impact of pests on crops (Phillips and Throne, 2010; Bommarco et al., 2013; Oliveira et al., 2014). Sustainable and eco-friendly biopesticides may be easily accessible by the resource-poor small-holder farmers and locals in countries where there is greater food insecurity, particularly in Africa (Sasson, 2012). Further processing of plant propagules also curbs the further distribution of IAPs through vegetative materials, hence also benefiting the livelihoods though reducing pressures by the agricultural pests on various crops. In this paper, we review the existing, but scattered literature on the insecticidal activity of different parts of some IAPs; specifically, those that are invasive in the sub-Saharan Africa. We discuss the prospects and opportunities for using IAPs as bio-insecticides of insect pests of agricultural and environmental importance. Finally, the paper highlights the safety concerns, research gaps, the challenges of using IAPs as bio-insecticides and formulates appropriate recommendations for future research.

METHODS

The information presented in this review was obtained from journal articles that are relevant to the topic. Only literature on insecticidal (not repellence) properties of IAPs that are invasive in Africa were included. Plant like *Azadirachta indica* A. Juss (Miliaceae) that have wide usage and is already well-established for over 100 years were not considered in this review. The scientific papers analyzed were obtained from different sources such as Google Scholar, Science-Direct, PubMed, SciFinder, and Scopus. Systematically used keywords include invasive alien plants, insecticidal, pesticidal, insect pest, efficacy, mortality, with the scientific name of each plant reported to possess insecticidal properties in journal articles. We used Boolean operators (and, or, not or and not) to combine or exclude keywords in our search to obtain a more focused and productive results. The literature search was conducted between June 2019 and April 2020, and more than 120 published papers were identified. Among the excluded research papers were those that assessed the insecticidal properties of forest trees, plants that have not been declared as invasive in Africa and studies that did not include control treatments. The mean percentage of insect mortality reported here was recorded from either of the text, tables, graphs and/figures. Among the information derived from the research papers was the country in which different studies were conducted, name of the IAP's, the harvested/used plant part(s), the formulations, the target insect, developmental stages at which the formulation was applied, and the percentage mortality reported after application of the formulation. Only articles that reported data with means, sample size and a measurement of variance (standard deviation, standard error or confidence intervals) for all treatments with a clear indication of replication were considered. The scoring system of 0–4 was used to rate the insecticidal properties of IAPs against insects in Africa. The percentage mortality of 1–25, 26–50, 51–75, and 76–100% were ranked as 1, 2, 3, and 4, respectively, but the formulation that recorded zero percent mortality was ranked as 0.

RESULTS AND DISCUSSION

Impact and Distribution of Invasive Alien Plant Species in Africa

Invasive alien plant species are identified as the plants that are intentionally or accidentally introduced to the regions beyond their native ranges (Richardson and Pyšek, 2012). Naturalized alien plant species are among significant ecosystem drivers that pose major threats to the native communities (e.g., plants and arthropods) in natural and agricultural ecosystems (Van Hengstum et al., 2013; Litt et al., 2014). The increase in the intensity of invasion aggravates the degree of threat to biodiversity and ecosystem function (Valone and Weyers, 2019). The distribution and problems of the IAPs reviewed in this paper are detailed in **Tables 1A–E**. Among the common impacts of the IAPs is the degradation of grazing land, competition with native species and cultivated crops for natural resources, supporting agricultural pests between cropping seasons, presenting health hazard to humans and poisoning of livestock

(Aigbokhan et al., 2010; Alagesaboopathi and Deivanai, 2011; Park et al., 2012; Van Hengstum et al., 2013; Litt et al., 2014; Shackleton et al., 2017; Dandurand et al., 2019; O'Connor and van Wilgen, 2020). Although there is sufficient literature that documents the impacts of these plants, the global efforts on mapping the distribution of the plants in their non-native ranges is insufficient (Witt et al., 2018).

The current distribution of invasive alien plants has been recorded for various plants invading the landscapes of different countries in Africa (Henderson, 2001; Shackleton et al., 2017; Witt and Luke, 2017; Witt et al., 2018, 2019; Catarino et al., 2019), whilst other studies also predicted the future distribution of these weeds (McConnachie et al., 2010; Taylor et al., 2012; Tererai and Wood, 2014; Obiakara and Fourcade, 2018). Further, surveys on the distribution of agents associated with these IAPs contribute to the continuous update on the change of the invasion intensities (Mukwevho et al., 2018). Despite the remarkable efforts by the Centre for Agriculture and Bioscience International (CABI, sometimes also referred to as CAB International) to describe the international distribution of IAPs, insufficient records of plant distribution in other African countries result in fragmented distribution maps.

Invasive Alien Plants in Africa With Reported Insecticidal Properties

From the literature survey, we found 69 studies across the globe that reported insecticidal activities of 23 plant species that are invasive in, and alien to Africa. The identified species were from 13 plant families and comprised six species from Asteraceae, three species from Solanaceae, two species from Apocynaceae, Fabaceae and Euphorbiaceae, and one species each from Araceae, Bignoniaceae, Chenopodiaceae, Meliaceae, Mimosaceae, Myrtaceae, Papaveraceae, and Verbenaceae (**Tables 2A–I**). These reports showing the insecticidal activities of alien plants that are problematic in Africa originated from Africa, Asia, North America and South America. The highest number of published case studies were from India and Nigeria with 19 and 15, respectively, whilst countries such as Algeria, Argentina, Brazil, Colombia, Chile, China, Egypt, Ethiopia, Ghana, Kenya, Malawi, Mexico, Pakistan, Sudan, Tanzania, Togo, Tunisia, Turkey, and the United States of America have less than 6 reports each. We hypothesized that the large number of research papers from India, Nigeria and other developing countries may be due to the fact that scientists in these countries are aware of the limited availability of synthetic insecticides by the resource-poor small-holder farmers; locals in these countries are keen on identifying IAPs to control and manage insect pests of agricultural, environmental and medical importance. Due the ecotoxicological effects and high cost of synthetic insecticides, the use of plants with pesticidal properties to control insect pests in agro-ecosystems among resource-poor small-holder farmers has been historically widespread and adopted in Africa (Belmain and Stevenson, 2001; Midega et al., 2016). Despite the widespread use of these biorational methods, pest control in some ecosystems in Africa continues to rely on the use of synthetic insecticides when alternative biopesticides are unavailable (Isman, 2006, 2015; Isman and Grieneisen, 2014). Although a plethora of empirical research has demonstrated the insecticidal properties of weeds in general, our literature found evidence that some invasive alien plants in Africa possessed insecticidal properties against a range of insect pests.

Several biological assays have been conducted to ascertain the efficacy of invasive alien plants against a myriad of insect pests with varying levels of insect mortality (**Tables 2A–I**). The survey demonstrated that leaf extracts were frequently used for bioassays, compared to other parts (i.e., roots, stems, inflorescences, fruits or seeds) of the plant (**Figure 1**). A majority of studies were conducted on members of the Asteraceae which represented 25 out of 69 studies and accounted for 38% of the total studies recorded in this review (**Figure 2**). Mean mortality rank of insect pests caused by the Asteraceae ranged from 50 to 100% (**Figure 3**).

Asteraceae Species With Insecticidal Properties

Six species in the family Asteraceae were reported effective against a number of insect pest species. In a laboratory and field study conducted by Xu et al. (2009), the acetone leaf extract of *Ageratina adenophora* caused up to 73% mortality in *Brevicoryne brassicae* after a 3-day exposure. Although the use of the essential oils of *A. adenophora* has been suggested for controlling aphids, ants and weevils in stored grains, there are no reports on the insecticidal use of this plant in invaded areas in Africa. Jaya et al. (2014) observed that essential oils from *Ageratum conyzoides* leaves caused 100% mortality against *Tribolium castenum*. Moreira et al. (2007a,b) isolated compounds including (5,6,7,8,3′,4′,5′-heptamethoxyflavone, 5,6,7,8,3′-pentamethoxy-4′, 5′-methylenedioxyflavone and coumarin) from the hexane extract of *A. conyzoides* leaves and tested the efficacy of the compounds against *Rhyzopertha dominica* and *Diaphania hyalinata*. Following a 24-h exposure, varying concentrations of the isolated compounds caused between 76 and 87% mortality in adults of *R. dominica* and 100% mortality in the larvae of *D. hyalinata* (Moreira et al., 2004, 2007a,b). The leaf extracts of *A. conyzoides* have also been reported to possess strong insecticidal activities (100% mortality) against the larvae of *Acanthoscelides obtectus*, *Musca domestica* and *Epilachna vigintioctopunctata* (Calle et al., 1990; Saxena and Sharma, 2005). Liu and Liu (2014) evaluated the larvicidal activity of the essential oil of *A. conyzoides* aerial parts against *Aedes albopictus*. The authors identified the principal constituents of the essential oils of *A. conyzoides* and concluded that the oils have insecticidal and larvicidal activities. Despite the burgeoning plethora of papers on the pesticidal activity of *A. conyzoides* against a myriad of arthropod pests (see Rioba and Stevenson, 2017), studies on the indigenous use of this plant in the control and management of insect pests are scarce. The increasing reports of the use of *A. conyzoides* in ethnomedicine for the treatment of a wide range of diseases in Africa (e.g., Nwauzoma and Dappa, 2013) suggest that the locals are exploiting the potential of the plant. Whether or not the plant has found use among the locals in its invasive range in Africa remains to be documented.

In a bioassay where *Cimex lectularius* adults were exposed to 2.0 g of *Chromolaena odorata* leaf powder, 70% mortality

TABLE 1A | Published reports on the impact of some invasive alien plants in sub-Saharan Africa.

Family/Species	Growth form	Native range	Distribution ranges in Africa*	Impact of the weed	Reference (s)
Apocynaceae					
Catharanthus roseus	Shrub/herb	Madagascar	BE, BO, BF, CA, CD, ET, GAB, GU, KE, MA, MO, NA, RW, SE, SL, SA, SW, TZ, TOGO, UG, ZA, ZM	Adapts to a wider range of ecological conditions such as watercourses, rocky outcrops, grazing lands, and along plantations. The milky sap contained on the vegetative parts of the plant makes the plant to be toxic	Henderson, 2001
Nerium oleander	Shrub	Europe, Asia	EG, KE, MO, NG, SA, ZM	The plant is toxic to humans and other mammals. The modes of toxicity/poisoning include direct ingestion or of the smoked food products, and inhalation	Henderson, 2001
Araceae					
Pistia stratiotes	Aquatic	South America	AN, BE, BO, BF, BU, CA, CAR, CH, CO, CD, EG, EQ, ET, GA, GAB, GH, GU, KE, LE, LIB, MA, MO, MOR, NA, NI, NG, RW, SE, SL, SO, SA, SU, SW, TOGO, TZ, UG, ZA, ZM	The water weed covers water bodies and thereby affects the lives of organisms inhibiting the waters. The weed clogs waterways and thus prevents movement of boats, blocks irrigation canals, disrupts fishing grounds and hydro-electricity production	Henderson and Cilliers, 2002; Macdonald et al., 2003; Witt et al., 2018
Asteraceae					
Ageratina adenophora	Herb	Argentina	AL, KE, NG, SA, UG, ZM	Outcompetes the native plant and crop species, thus affecting the diversity of plants, carrying capacity of grazing lands and yields of cultured crops. The weed is not palatable to grazers and dense thickens may restrict movement of stock and machinery	Tererai and Wood (2014)

Algeria (AL); Angola (AN); Benin (BE); Burkina Faso (BF); Botswana (BO); Burundi (BU); Cameroon (CA); Central African Republic (CAR); Côte d'Ivoire (CD); Chad (CH); Congo (CO); Egypt (EG); Equatorial Guinea (EQ); Ethiopia (ET); Gambia (GA); Gabon (GAB); Ghana (GH); Guinea (GU); Kenya (KE); Lesotho (LE); Liberia (LIB); Malawi (MA); Mozambique (MO); Morocco (MOR); Namibia (NA); Nigeria (NG); Niger (NI); Rwanda (RW); South Africa (SA); Senegal (SE); Sierra Leone (SL); Somalia (SO); Sudan (SU); Swaziland (SW); Togo (TOGO); Tanzania (TZ); Uganda (UG); Zambia (ZA); Zimbabwe (ZM).

was reported after 5 days (Uyi et al., 2018a). Depending on concentrations, the leaf, stem and root powders of *C. odorata* were reported to cause between 16 and 100% mortality against adults of the *Callosobruchus maculatus* (Uyi and Igbinoba, 2016; Uyi and Obi, 2017; Uyi and Adetimehin, 2018). In Nigeria, Lawal et al. (2015) reported that the leaf extracts of *C. odorata* displayed a strong insecticidal activity by causing between 33 and 93% mortality in *Sitophilus zeamais*. In a field experiment in Ghana, Ezena et al. (2016) reported that varying concentrations of the leaf extract caused between 36 and 77% mortality in nymphs and adults of the *Brevicoryne brassicae* and *Hellula undalis* and *Plutella xylostella*. Udebuani et al. (2015) tested the efficacy of *C. odorata* leaf extract against *Periplaneta americana* by exposing the adults to different concentrations of the leaf extract and reported 12 to 69% mortality. Sukhthankar et al. (2014) investigated the insecticidal activity of different concentrations of methanolic leaf extract of *C. odorata* against the larvae of *Anopheles stephensi, Culex quinquefasciatus* and *Aedes aegypti* and found up to 100% mortality in these larvae after 24 h of exposure. Similar to *A. conyzoides*, studies documenting the indigenous use of *C. odorata* are scarce (but see Cobbinah et al., 1999). The authors conducted ethnobotanical surveys on plants used for the protection of stored cereals in Ghana and reported that cowpea treated with *C. odorata* leaf powder were free of insect infestation for 4 months and that the locals attributed this to the insecticidal or repellent activities of *C. odorata*.

Tesfu and Emana (2013) studied the insecticidal properties of different parts of *Parthenium hysterophorus* powders against *Callosobruchus chinensis* over 48 h and found that the highest dose (2/50 g seeds) of inflorescence, leaf and stem powder caused 77, 73, and 57% mortality, respectively. The leaf, stem and root extracts of *P. hysterophorus* have been reported to be effective against *Ae. aegypti*; larval mortality of 40 to 100% was recorded after exposure to the aqueous leaf extracts of *P. hysterophorus* (Kumar S. et al., 2012; Amir et al., 2017). In an investigation of the insecticidal efficacy of the leaf extract of *P. hysterophorus* against the larvae of the rice moth, *Corcyra cephalonica*, Khan and Qamar (2015a) reported 81% mortality of larvae. In another experiment, Khan and Qamar (2015b) recorded 14.4% adult mortality in *P. americana*. Reddy et al. (2018) investigated the insecticidal activity of *P. hysterophorus* against *P. xylostella* and *Aphis craccivora* in a field experiment and found that *P. hysterophorus* leaf extract showed promising toxicity (LC50 = 1140.68 mg L^{-1}) to larvae of *P. xylostella* and *A. craccivora* (LC50 = 839 mg L^{-1}) after 96 h of treatment. The authors did not report any specific mortality rates. Although several studies (see references in **Tables 2A–I**) have recommended the use of *P. hysterophorus* as a pesticidal plant in its invasive ranges in Asia and Africa, there is no evidence to show that the locals especially the resource-poor small-holder farmers are exploiting it as yet.

In Nigeria and Tanzania, the leaf and stem bark extracts of *Tithonia diversifolia* have been reported to cause 100% mortality of adult *C. maculatus* (Obembe and Kayode, 2013; Green et al., 2017). Similarly, studies on the insecticidal activity of the leaf

TABLE 1B | Published reports on the impact of some invasive alien plants in sub-Saharan Africa.

Family/Species	Growth form	Native range	Distribution ranges in Africa	Impact of the weed	Reference (s)
Asteraceae					
Ageratum conyzoides	Herb	Americas	AN, BE, BO, BF, BU, CV, CA, CAR, CO, CD, EG, EQ, ET, GA, GAB, GH, GU, KE, LIB, MA, MALI, MO, MOR, NG, RW, SE, SL, SA, SU, SW, TZ, TOGO, UG, ZA, ZM	Alternate host to a number of economically important pests, namely pathogens (e.g., Tomato Yellow Leaf Curl Tanzania Virus and the Ageratum Yellow Vein Virus) and nematodes (*Meloidogyne javanica*, *Radopholus similis* and *Helicotylenchus multicinctus*). The plant releases the allelochemicals that inhibits the seed germination and growth of other species	Witt et al., 2018
Chromolaena odorata	Shrub	Central and South America	BE, CA, CAR, CD, CO, GH, GU, KE, LIB, MO, NG, SA, TZ, TOGO, UG, ZM	Displaces native plant species and alters the fuel loads which may increase proneness to wildfires. Reduces the productivity of rangelands and may cause serious health problems to livestock and people	Muniappan et al., 2005; Witt et al., 2018; Catarino et al., 2019; Mugwedi, 2020
Parthenium hysterophorus	Herb		BO, EG, ET, KE, MO, RW, SA, SO, SW, TZ, UG, ZM	The plant is allelopathic and suppresses the natural vegetation of the invaded landscapes. Severe reduction in the productivity of rangelands and has serious health hazards (dermatitis, hay fever, and asthma) to people, livestock, and wildlife	McConnachie et al., 2010; Witt et al., 2018
Tithonia diversifolia	Shrub	Mexico and Central America,	AN, BU, CA, CAR, CO, CD, EG, ET, GU, KE, MA, MO, NG, RW, SA, SW, TZ, TOGO, UG, ZA, ZM	The plant is allelopathic and has a significant impact on native vegetation. The evergreen plant reduces species diversity and the productivity of rangelands. Intensive invasions may contribute to the local extinction of valued native species	Obiakara and Fourcade, 2018; Witt et al., 2019
Xanthium strumarium	Herb	Central and South America	BO, BU, EG, ET, KE, LE, MA, RW, SA, TZ, UG, ZA	Rapidly forms large stands, displacing other plant species. Toxic to livestock and can lead to death if eaten	Witt et al., 2018

Angola (AN); Benin (BE); Burkina Faso (BF); Botswana (BO); Burundi (BU); Cameroon (CA); Central African Republic (CAR); Côte d'Ivoire (CD); Congo (CO); Cabo Verde (CV); Egypt (EG); Equatorial Guinea (EQ); Ethiopia (ET); Gambia (GA); Gabon (GAB); Ghana (GH); Guinea (GU); Kenya (KE); Lesotho (LE); Liberia (LIB); Malawi (MA); Mali (MALI); Mozambique (MO); Morocco (MOR); Nigeria (NG); Rwanda (RW); South Africa (SA); Senegal (SE); Sierra Leone (SL); Somalia (SO); Sudan (SU); Swaziland (SW); Togo (TOGO); Tanzania (TZ); Uganda (UG); Zambia (ZA); Zimbabwe (ZM).

extract of *T. diversifolia* against *S. zeamais* showed 43% mortality in adults (Obembe and Kayode, 2013). Babarinde et al. (2008) and Adedire and Akinneye (2004) demonstrated that the leaf powder of *T. diversifolia* caused 90 and 99% mortality of *S. zeamais* and *C. maculatus*, respectively. In a field experiment, Mkenda et al. (2015) showed that the leaf extracts of *T. diversifolia* significantly reduced the population of the nymphs/larvae and adults of *Aphis fabae*, *Ootheca mutabilis*, *O. bennigseni*, *Epicauta albovittata* and *E. limbatipennis*. The authors further showed that the control offered by the leaf extracts were comparable to lambda-cyhalothrin, a commonly used synthetic pyrethroid. Although without mortality figures, Mkindi et al. (2017) reported some insecticidal activity of *T. diversifolia* leaf extract against some important pests (*Aphis fabae*, *Ootheca mutabilis* and *O. bennigsen*, *Epicauta albovittata*, *E. limbatipennis*, *Clavigralla tomentosicollis*, *C. schadabi,* and *C. hystricodes*) of beans in Tanzania and Malawi. The authors reported that *T. diversifolia* offered effective control of key pest species that was comparable in terms of harvested bean yield to a synthetic pyrethroid. The leaf extract of another species of Asteraceae, *Xanthium strumarium* caused more than 82% mortality in green peach aphid, *Myzus persicae* (Erdogan and Yildirim, 2016). In Uganda, farmers used the leaf extract and powder of *T. diversifolia* for the management of field and stored product pests (Mugisha-Kamatenesi et al., 2008; Mwine et al., 2011). *Tithonia diversifolia* is known to contain sesquiterpene lactones and diterpenoids (Chagas-Paula et al., 2012), some of which have biological activities against insects such as termites (Adoyo et al., 1997). However, there is no specific information about which compounds are responsible for its insecticidal effect. Despite the traditional use of *Xanthium strumarium* in ethnomedicine for treating a variety of diseases (Fan et al., 2019), its use by locals in the management of insect pests of agricultural and medical importance have not been documented.

Solanaceae Species With Insecticidal Properties

Three species in the family, Solanaceae were reported effective against a number of important field and stored product insect pests. Zapata et al. (2006) investigated the insecticidal efficacy of the leaf extract of *Cestrum parqui* against the Mediterranean fruit fly, *Ceratitis capitata* and recorded 55% mortality in the adults of this pest. Investigations on the insecticidal activity of *Solanum elaeagnifolium* showed that the leaf and seed extracts of this plant accounted for 88 and 84% mortality, respectively against the larvae of *T. castenum* in Tunisia (Ben Hamouda et al., 2015a). The leaf and seed extracts of *S. elaeagnifolium* offered effective control against the *Spodoptera littoralis* (Ben Hamouda et al., 2015b). The authors found that leaf and seed extracts, respectively caused 80 and 100% mortality in the larvae of *S. littoralis*. Ben Hamouda et al. (2015c) reported the mortality

TABLE 1C | Published reports on the impact of some invasive alien plants in sub-Saharan Africa.

Family/Species	Growth form	Native range	Distribution ranges in Africa	Impact of the weed	Reference (s)
Bignoniaceae					
Jacaranda mimosifolia	Tree	South America	AN, BO, CV, CA, CAR, EG, ET, GAB, GH, GU, KE, MA, MOR, MO, NG, RW, SA, SW, TZ, UG, ZA, ZM	The dense foliage it produces tends to shade out native plants and prevent their regeneration. Deep rooted and may thrive conditions/outcompete some species	Henderson, 2001
Chenopodiaceae					
Chenopodium ambrosioides	Herb	Mexico	BO, CA, EG, GAB, GH, KE, LE, MA, MO, NA, NG, SA, SE, TA, UG, ZA, ZM	Common weed of agricultural, pastural and natural ecosystems. Inter-seasonal host for *Erysiphe betae* (powdery mildew) of sugar beet. The plant can smother native plants and may outcompete them in the disturbed areas	Foxcroft et al., 2003
Euphorbiaceae					
Jatropha curcas	Shrub	Americas	AN, BE, BF, CV,CA, CAR, CH, CD, EG, ET, GA, GAB, GH, GU, KE, LI, MA, MALI, MO, NI, NG, RW, SA, SE, SL, SO, SU, TZ, TOGO, UG, ZA, ZM	The plant is poisonous to grazing stock of animals and may contribute to significant modifications of the ecosystems that they are invading. It cause significant shift of biodiversity.	Negussie et al., 2014
Fabaceae					
Prosopis juliflora	Tree or shrub	Caribbean	AL, BO, BF, CV, CH, EG, ER, ET, GA, GH, GU, KE, LIB, MALI, MOR, MO, NA, NG, NI, SE, SO, SA, SU, TZ, TUN, UG, ZM	Reduces grazing capacity, eliminates many species from invaded ecosystems and depletes groundwater resources. Despite some benefits in the form of firewood and edible pods, the overall net economic contribution is negative, and set to worsen as the species continues to spread	Henderson, 2007; Zachariades et al., 2011a,b; Abdulahi et al., 2017
Sesbania grandiflora	Tree	Asia	BE, CV, CH, ET, GAB, GH, MA, NG, SA, SE, SL, SO, SU, TZ	It has allelopathic effects on crop seed germination	Gillett, 1963

Algeria (AL); Angola (AN); Benin (BE); Burkina Faso (BF); Botswana (BO); Burundi (BU); Cameroon (CA); Central African Republic (CAR); Côte d'Ivoire (CD); Chad (CH); Congo (CO); Cabo Verde (CV); Egypt (EG); Equatorial Guinea (EQ); Eritrea (ER); Ethiopia (ET); Gambia (GA); Gabon (GAB); Ghana (GH); Guinea (GU); Kenya (KE); Lesotho (LE); Libya (LI); Malawi (MA); Mali (MALI); Mozambique (MO); Morocco (MOR); Namibia (NA); Nigeria (NG); Niger (NI); Rwanda (RW); South Africa (SA); Senegal (SE); Sierra Leone (SL); Somalia (SO); Sudan (SU); Swaziland (SW); Togo (TOGO); Tunisia (TUN); Tanzania (TZ); Uganda (UG); Zambia (ZA); Zimbabwe (ZM).

rate of up to 5 and 43% caused by the leaf and seed aqueous extract of *Solanum elaeagnifolium* against *M. persicae*. In an investigation into the insecticidal activity of *S. sisymbriifolium* leaf extract against *T. castenum*, Padín et al. (2013) reported 22% mortality in adult beetles. The traditional use of the leaf extract of *C. parqui, S. sisymbriifolium,* and *S. elaeagnifolium* for the control and management of insect pests in their invasive ranges in Africa have not been documented and therefore requires some ethnobotanical studies.

Apocynaceae, Euphorbiaceae, and Fabaceae Species With Insecticidal Properties

Two species each in the family, Apocynaceae, Euphorbiaceae, and Fabaceae were reported effective against some insect pests of medical, environmental and agricultural importance. Remia and Logaswamy (2010) studied the insecticidal activity of the leaf extract of *Catharanthus roseus* against *Ae. aegypti* and reported over 71% mortality in the larvae and pupae of this mosquito species. Khan and Qamar (2015a,b) investigated the efficacy of *Nerium oleander* against the larvae of a rice moth, *Corcyra cephalonica* and *P. americana* and found up to 83% mortality in the larvae of the rice moth and *P. americana*. Despite the usage of Apocynaceae species in ethnomedicine (CABI, 2020a), their use as pesticides by locals is yet to be reported.

The leaf, seed, stem bark and root extracts of *Jatropha curcas* have been found effective (i.e., with 40 to 100% mortality) against the nymphs and larvae of *P. xylostella, Helicoverpa armigera, Spodoptera frugiperda,* and *Schistocerca gregaria* (Ribeiro et al., 2012; Bashir and El Shafie, 2013; Ingle et al., 2017). Opuba et al. (2018) and Adetimehin et al. (2018) showed that 3.0 g of the leaf and stem bark powders of *J. curcas* caused 100 percent mortality in *C. maculatus* in a laboratory test. The leaf extract of *Ricinus communis* reportedly caused 100% mortality on the larvae of *P. xylostella* (Tounou et al., 2011). Investigations into the insecticidal efficacy of the leaf, seed and fruit extracts of *Prosopis juliflora* caused up to 73% mortality in adult cotton aphid, *A. gossypii* (Zerihun and Ele, 2018). Sangavi and Johnson Thangaraj Edward (2017) reported between 73 and 96% mortality in *P. xylostella* when the larvae were treated with the leaf extract of *P. juliflora* and *Sesbania grandiflora*. While the use of *R. communis* for the management of insect pests by locals is not known, *J. curcas* is used by farmers in Uganda for the control and management of both field and storage pests (Mugisha-Kamatenesi et al., 2008). The ethnopesticidal usage of *P. juliflora*

TABLE 1D | Published reports on the impact of some invasive alien plants in sub-Saharan Africa.

Family/Species	Growth form	Native range	Distribution ranges in Africa	Impact of the weed	Reference (s)
Euphorbiaceae					
Ricinus communis	Shrub		AL,AN, BE, BO, BF, BU, CV, CAR, CH, CO, EG, ET, GAB, GA, GH, GU, KE, LI, MA, MALI, MOR, MO, NA, RW, SE, SO, SA, TZ, TOGO, TUN, UG, ZA, ZM	Pollen causes respiratory allergies for animals. *R. communis* is extremely poisonous to animals and humans and pollen causes respiratory allergies in humans	Henderson, 2001; Kiran and Prasad, 2017
Meliaceae					
Melia azedarach	Tree	Asia	AN, BO, BF,CV CA, CH, CO, CD, EG, ER, ET, GH, KE, LE, MA, MALI, MO, MOR, NA, NI, NG, SE, SO, SA, SU, SW, TZ, TUN, UG, ZA, ZM	The dense monospecific stands suppress the regenerating native plants. It alters soil chemistry, and can act as respiratory irritants	Henderson, 2001, 2007
Mimosaceae					
Mimosa diplotricha	Shrub		BU, CA, CO, CD, ET, GH, GU, MA, MO, NG, RW, SA, TZ, TOGO, UG, ZM	Dry thickets are prone to fires and density of the plant restricts movement of mammals, including people. It suppresses the shaded species and thus prevents regression of other plants	Ekhator et al., 2013; Uyi, 2020
Myrtaceae					
Eucalyptus camaldulensis	Tree		AL, AN, BE, BF, BO, BU, CA, CD, CH, CO, CV, EG, EQ, ER, ET, GA, GH, KE, LE, LI, MA, MALI, MO, MOR, NA, NG, NI, RW, SA, SE, SL, SO, SU, SW, TUN, TZ, UG, ZA, ZM	The plant suppresses native plants, improves the fuel loads, depletes nutrients and excessive water use	Henderson, 2001

Algeria (AL); Angola (AN); Benin (BE); Burkina Faso (BF); Botswana (BO); Burundi (BU); Cameroon (CA); Central African Republic (CAR); Côte d'Ivoire (CD); Chad (CH); Congo (CO); Cabo Verde (CV); Egypt (EG); Equatorial Guinea (EQ); Eritrea (ER); Ethiopia (ET); Gambia (GA); Gabon (GAB); Ghana (GH); Guinea (GU); Kenya (KE); Lesotho (LE); Libya (LI); Malawi (MA); Mali (MALI); Mozambique (MO); Morocco (MOR); Namibia (NA); Nigeria (NG); Niger (NI); Rwanda (RW); South Africa (SA); Senegal (SE); Sierra Leone (SL); Somalia (SO); Sudan (SU); Swaziland (SW); Togo (TOGO); Tunisia (TUN); Tanzania (TZ); Uganda (UG); Zambia (ZA); Zimbabwe (ZM).

and *S. grandiflora* is yet to be documented and therefore warrant some ethnobotanical investigation.

Other Species With Insecticidal Properties

Insecticidal activity of at least one species from the following families: Araceae, Bignoniaceae, Chenopodiaceae, Meliaceae, Mimosaceae, Myrtaceae, Papaveraceae, and Verbenaceae was investigated. Ito et al. (2015) investigated the insecticidal activity of *Pistia stratiotes* and found that the leaf powder of this aquatic weed reduced the population of *Ae. aegypti* by 80%. Our survey also found that the leaf powder of *Jacaranda mimosifolia* caused 30% mortality in adults of *A. obtectus* (Waweru et al., 2017), while the leaf extract caused 49% mortality in adults of *T. castenum* (Padín et al., 2013). Guzzo et al. (2006) reported that the leaf and fruit extracts of *Dysphania ambrosioides* only caused low adult mortality (<5%) in *R. dominica*. The fruit extract of *Melia azedarach* has been reported to be effective in the control of several pests. For example. The fruit extract of this weed caused 44% larval mortality in *Liriomyza huidobrensis* and 100% larval mortality in *S. frugiperda* and *S. littoralis* (Hammad and McAuslane, 2010; Scapinello et al., 2014). Chiffelle et al. (2011) documented 86% mortality when the adults of the Elm leaf beetle, *Xanthogaleruca luteola* were treated with the fruit extract of *M. azedarach*. Similarly, Selvaraj and Mosses (2011) reported over 88% larval mortality in *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* when larvae were treated with the fruit extracts. Although we found no traditional usage of the Araceae, Bignoniaceae, Chenopodiaceae species as pesticidal plants, we found that in Ghana, the leaves of *M. azedarach* were used as a bioinsecticide to minimize the impact of *Ephestia cautella* on cocoa beans (CABI, 2020b).

In a laboratory experiment on the efficacy of the root extract of *Mimosa diplotricha*, Uyi et al. (2018b) reported 100% mortality in worker termites, *Macrotermes* species when exposed to different concentrations for 12 h. In a different experiment on the efficacy of the leaf and root powders of *M. diplotricha* against *C. lectularius* and *C. maculatus*, Uyi et al. (2018a, 2020) reported more than 67% mortality for both insects. Nia et al. (2015) reported 53% mortality in the nymphs and adults of *M. persicae* when the leaf extract of *Eucalyptus camaldulensis* was used to treat infestations of this pest. Khan and Qamar (2015a,b) found significant mortalities (15–76%) in *C. cephalonica* and *P. americana* when the larvae of the moth and nymphs of the cockroach were exposed to the leaf extracts of *Argemone mexicana*. We found no reports on the ethnopesticidal usage of *M. diplotricha* and *E. camaldulensis*, but for *A. mexicana*,

TABLE 1E | Published reports on the impact of some invasive alien plants in sub-Saharan Africa.

Family/Species	Growth form	Native range	Distribution ranges in Africa	Impact of the weed	Reference (s)
Papaveraceae					
Argemone mexicana	Herb	Mexico	AN, BE, BO, BF, CV, CA, CD, EG, EQ, ER, ET, GA, GH, GU, KE, LIB, MA, MALI, MO, NA, NG, NI, SE, SL, SO, SA, SU, SW, TZ, TOGO, UG, ZA, ZM	It is a toxic plant, which is also toxic to feeding animals. The allelopathic effects result on suppression of plants in the ecosystem	Van der Westhuizen and Mpedi, 2011
Solanaceae					
Cestrum parqui	Shrub	Argentina, Brazil, Bolivia, Chile, Peru, Paraguay and Uruguay	KE, SA	The plant out-competes and disrupt regeneration of native plants. Thickets along waterways blocks access by to streams. Toxic to feeding herbivores, causes skin irritation (e.g., rashes)	Henderson, 2001; Witt and Luke, 2017
Solanum elaeagnifolium	Herb	Mexico	AL, EG, LE, LI, MOR, SA, TUN, ZM	The plant acts as a vector for the Lettuce chlorosis virus between cropping seasons. Competes for natural resources with cultivated crops and reduce production on agricultural lands. The berries are toxic to livestock	EPPO, 2007
Solanum sisymbriifolium	Tree	South America	CO, NA, SA, SW	Competes with native vegetation for space and natural resources. Acts as a trap crop for the potato and tobacco cyst nematodes, though it affects their reproduction	Dandurand et al., 2019
Verbenaceae					
Lantana camara	Tree or shrub	Mexico	AN, BU, CO, CD, CV, ET, GA, GAB, GH, GU, KE, LIB, MA, MO, NA, NG, RW, SA, SE, SU, SW, TZ, UG, ZA, ZM	Displaces natural vegetation and impacting negatively on plant and arthropod biodiversity. Toxic to livestock, causing animal deaths, reduced productivity, and allelopathic effects causes loss of pasture	Henderson, 2007; Taylor et al., 2012; Shackleton et al., 2017; Witt et al., 2018

Algeria (AL); Angola (AN); Benin (BE); Burkina Faso (BF); Botswana (BO); Burundi (BU); Cameroon (CA); Côte d'Ivoire (CD); Congo (CO); Cabo Verde (CV); Egypt (EG); Equatorial Guinea (EQ); Eritrea (ER); Ethiopia (ET); Gambia (GA); Gabon (GAB); Ghana (GH); Guinea (GU); Kenya (KE); Lesotho (LE); Libya (LI); Liberia (LIB); Malawi (MA); Mali (MALI); Mozambique (MO); Morocco (MOR); Namibia (NA); Nigeria (NG); Niger (NI); Rwanda (RW); South Africa (SA); Senegal (SE); Sierra Leone (SL); Somalia (SO); Sudan (SU); Swaziland (SW); Togo (TOGO); Tunisia (TUN); Tanzania (TZ); Uganda (UG); Zambia (ZA); Zimbabwe (ZM).

von Weizsäckerl (1995) reported that the leaf extract is used in parts of India to prepare antifeedant sprays for the management of insect pests.

From the Verbenaceae family, *Lantana camara* was reported active against some mosquito species and major pests of crops due to the insecticidal potential of the plant. Remia and Logaswamy (2010) investigated the efficacy of the leaf extract of *L. camara* against *Ae. aegypti* in the laboratory and found more than 65% larval and pupal mortality. The essential oils from the leaves of *L. camara* caused between 93 and 100% in *Ae. aegypti*, *Cx. quinquefasciatus*, *An. culicifacies, An. fluvialitis* and *An. Stephensi* when adults were exposed for 24 h (Dua et al., 2010). Leaf powders and extracts of *L. camara* were also reported effective against a number of stored product pests (*S. zeamais*, *S. oryzae*, *S. granaries*, *C. chinensis*, *T. castenum*) where it caused 9–100% mortality depending on the concentration (of the extract/powder) and period of exposure (Sexana et al., 1992; Zoubiri and Baaliouamer, 2012; Rajashekar et al., 2014; Taye et al., 2014). In a laboratory experiment in China, the leaf extract of *L. camara* caused 90% mortality in the subterranean termite, *Reticulitermes flavipes*, when the workers were exposed for 24 h. The leaf extract of *L. camara* was reported to possess some insecticidal activities against some field pests (e.g., *A. fabae*, *Ootheca mutabilis*, *O. bennigseni*, *Epicauta albovittata*, *E. limbatipennis*, *Clavigralla tomentosicollis*, *C. schadabi,* and *C. hystricodes*) of beans in Tanzania and Malawi (Mkindi et al., 2017). Despite the ethnomedicinal uses of *L. camara* in Africa and the numerous studies on its pesticidal properties, there is surprisingly only one report (Mugisha-Kamatenesi et al., 2008) on the use of the plant for the management of insect pest species in the invasive range of the plant in Africa.

Prospects, Challenges, and Safety of Using IAPs as Bio-Insecticides

Prospects for Exploiting IAPs for Insect Pest Control

Due to the associated non-target effects and cost of synthetic insecticides in Africa, many resource-poor small-holder farmers on the continent rely on the use of crude plant-based materials collected from the wild and locally prepared (using the available technology or crude methods) to control and manage insect pests problems in subsistence farming, which is wide spread on the continent (Cobbinah et al., 1999; Belmain and Stevenson, 2001; Isman, 2008; Nyirenda et al., 2011; Kamanula et al., 2017). Despite the demonstrated laboratory and field efficacy of botanicals from many invasive alien plants against

TABLE 2A | Published reports on the insecticidal activities of some plant species with invasive potentials in sub-Saharan Africa.

Family	Plant species	Plant parts	Formulation/application method	Insect target	Stage of insect	Importance of insect	Mortality (%)	Country	Reference (s)
Apocynaceae	*Catharanthus roseus*	Leaf	Acetone extract/spray	Mosquito (*Aedes aegypti*)	Larvae and pupae	Pest of medical importance	>71	India	Remia and Logaswamy, 2010
	Nerium oleander	Leaf	Methanol extract/spray	Rice moth (*Corcyra cephalonica*), Cockroach (*Periplaneta americana*)	Larvae	Rice and household pests	17.4–83	India	Khan and Qamar, 2015a,b
Araceae	*Pistia stratiotes*	Leaf	Aqueous extract/spray	*Aedes aegypti* (L.)	Larvae	Vector of some parasitic diseases	80.1	Nigeria	Ito et al., 2015
Asteraceae	*Ageratina adenophora*	Leaf	Acetone extract/spray	Cabbage aphid (*Brevicoryne brassiccae*)	Adults and nymphs	Pest of cabbage and other brassicae species	73	China	Xu et al., 2009
	Ageratum conyzoides	Leaf	Essential oils/fumigant	Storage grain beetle (*Tribolium castaneum*)	Adults	Stored grains	100	India	Jaya et al., 2014
		Leaf	Hexane extract/filter paper impregnation	Lesser grain borer *Rhyzopertha dominica*	Adults	Stored grains	76- 87	Brazil	Moreira et al., 2007a,b
		Leaf	Hexane extract/filter paper impregnation	Melonworm moth, *Diaphania hyalinata*, *Tuta absoluta*	Larvae	Pest of various plants in the cucumber family	100	Brazil	Moreira et al., 2004
		Leaf	Petroleum ether extract/filter paper impregnation	*Acanthoscelides obtectus*, *Musca domestica*	Larvae	Bean weevil	100	Colombia	Calle et al., 1990
		Leaf	Petroleum ether extract/ingestion	*Epilachna vigintioctopunctata*	Larvae	Agricultural pest (eggplant)	100	India	Saxena and Sharma, 2005

TABLE 2B | Published reports on the insecticidal activities of some plant species with invasive potentials in sub-Saharan Africa.

Family	Plant species	Plant parts	Formulation/application method	Insect target	Stage of insect	Importance of insect	Mortality (%)	Country	Reference (s)
Asteraceae	*Ageratum conyzoides*	Leaf	Essential oils/addition of extract to water	Asian tiger mosquito, *Aedes albopictus*.	Larvae		–	China	Liu and Liu, 2014
	Chromolaena odorata	Leaf and root	Powder/Dust	Bed bugs (*Cimex lectularius*)	Adults	Pest of humans and animals	>70	Nigeria	Uyi et al., 2018a
		Leaf and root	Aqueous extract/filter paper impregnation	Termites (*Macrotermes* species)	Adults	Pest of crops	100	Nigeria	Uyi et al. (2018b)
		Leaf, stem and root	Powder/Dust	Cowpea beetle (*Callosobruchus maculatus*)	Adults	Pest of cowpea	16–100	Nigeria	Uyi and Igbinoba, 2016; Uyi and Obi, 2017; Uyi and Adetimehin, 2018
		Leaf extracts	Methanol extract/filter paper impregnation	Maize weevil (*Sitophilus zeamais*)	Adults	Pest of maize, and cowpea	33–93	Nigeria	Lawal et al., 2015
		Leaf extract	Aqueous extract/spray	Cabbage aphid (*Brevicoryne brassicae*), cabbage webworm (*Hellula undalis*), Diamondback moth (*Plutella xylostella*)	Adults and nymphs of aphids and larvae of moths	Pest of cabbage and other brassicae species	36–74	Ghana	Ezena et al., 2016
		Leaf extract	Aqueous extract/filter paper impregnation	Cockroach (*Periplaneta americana*)	Adults	Household pest and vector of parasitic diseases	12–69	Nigeria	Udebuani et al., 2015
		Leaf extract	Methanol extract/addition of extract to water	*Anopheles stephensi, Culex quinquefasciatus* and *Aedes aegypti*	Larvae	Vector of parasitic diseases	20–100	India	Sukhthankar et al., 2014

TABLE 2C | Published reports on the insecticidal activities of some plant species with invasive potentials in sub-Saharan Africa.

Family	Plant species	Plant parts	Formulation/application method	Insect target	Stage of insect	Importance of insect	Mortality (%)	Country	Reference (s)
Asteraceae	*Parthenium hysterophorus*	Flowers, leaf and stem	Powder/dust	Bean weevil (*Callosobruchus chinensis*)	Adults	Cowpea and chickpea	>56.6	Ethiopia	Tesfu and Emana, 2013
		Leaf and stem	Aqueous extract/addition of extract to water	*Aedes aegypti*,	Larvae	Vector of some parasitic diseases	>80	Pakistan	Amir et al., 2017
		Leaf, stem and root	Acetone and hexane extract/addition to water	*Aedes aegypti*,	Larvae	Vector of some parasitic diseases	40–100	India	Kumar S. et al., 2012
		Leaf	Methanol extract/ingestion	Rice moth (*Corcyra cephalonica*)	Larvae	Pest of rice	81	India	Khan and Qamar, 2015a
		Leaf	Methanol extract/ingestion	American cockroach (*Periplaneta americana*)	Adults	Household pest	14.4	India	Khan and Qamar, 2015b
		Leaf	Methanol extract/ingestion	*Plutella xylostella*, *Aphis craccivora*	Larvae and adults	Agricultural pests	good toxicity	India	Reddy et al., 2018
	Tithonia diversifolia	Stem bark	Aqueous extract/spray	Cowpea beetle (*Callosobruchus maculatus*)	Adults	Pest of beans	100	Nigeria	Obembe and Kayode, 2013
		Leaf	Methanol extract/fumigant	Cowpea beetle (*Callosobruchus maculatus*)	Adults	Pest of beans	100	Tanzania	Green et al., 2017
		Leaf	Aqueous extract/spray	Maize weevil (*Sitophilus zeamais*)	Adults	Pest of maize, rice	43	Nigeria	Obembe and Kayode, 2013
		Leaf	Powder/dust	Maize weevil (*Sitophilus zeamais*)	Adults	Pest of maize, rice	90	Nigeria	Babarinde et al., 2008

TABLE 2D | Published reports on the insecticidal activities of some plant species with invasive potentials in sub-Saharan Africa.

Family	Plant species	Plant parts	Formulation/application method	Insect target	Stage of insect	Importance of insect	Mortality (%)	Country	Reference (s)
Asteraceae	*Tithonia diversifolia*	Leaf	Aqueous extract and powder/spray and dust	Cowpea beetle (*Callosobruchus maculatus*)	Adults	Pest of beans	98.3	Nigeria	Adedire and Akinneye, 2004
		Leaf	Aqueous extract/spray	Aphids (*Aphis fabae*), Bean foliage beetle (*Ootheca mutabilis* and *O. bennigseni*), and flower beetle (*Epicauta albovittata* and *E. limbatipennis*)	Nymphs, larvae and adults	Pest of beans		Tanzania	Mkenda et al., 2015
		Leaf	Aqueous extract/spray	Aphids (*Aphis fabae*), bean foliage beetle (*Ootheca mutabilis* and *O. bennigseni*), flower beetle (*Epicauta albovittata* and *E. limbatipennis*) and pod suckers (*Clavigralla tomentosicollis*, *C. schadabi* and *C. hystricodes*)	Nymphs, larvae and adults	Pest of beans		Tanzania and Malawi	Mkindi et al., 2017
	Xanthium strumarium	Leaf	Ethanol extract/spray	Green peach aphid (*Myzus persicae*)	Adults	Pest of peach	>82	Turkey	Erdogan and Yildirim, 2016
Bignoniaceae	*Jacaranda mimosifolia*	Leaf	Powder/dust	*Acanthoscelides obtectus*	Adults	Pest of cowpea	>31%	Kenya	Waweru et al., 2017
		Leaf	Methanol extracts/topical	*Tribolium casteneum*	Adults	Pest of stored grains	49%	Argentina	Padín et al., 2013
Chenopodiaceae	*Dysphania ambrosioides*	Leaf and fruit	Aqueous extract/spray	Lesser grain borer (*Rhyzopertha dominica*)	Adults	Pest of stored grains	0.5–2.9	Brazil	Guzzo et al., 2006

TABLE 2E | Published reports on the insecticidal activities of some plant species with invasive potentials in sub-Saharan Africa.

Family	Plant species	Plant parts	Formulation/application method	Insect target	Stage of insect	Importance of insect	Mortality (%)	Country	Reference (s)
Euphorbiaceae	*Jatropha curcas*	Leaf, seed, bark, root	Methanol extract/leaf dip method	Diamondback moth (*Plutella xylostella*)	Larvae	Pest of cabbage and other brassicae species	40–100%	India	Ingle et al., 2017
		Leaf, root and seed coat	Methanol extract/leaf dip method	*Helicoverpa armigera*	Larvae		60%	India	Ingle et al., 2017
		Leaf		Fall army worm (*Spodoptera fragiperda*)	Larvae	Agricultural pest	3–60%	Brazil	Ribeiro et al., 2012
		Seed	Hexane extract/spray	Desert locust (*Schistocerca gregaria*)	Nymphs	Agricultural pest	20–59%	Sudan	Bashir and El Shafie, 2013
		Leaf and Stem	Powder/dust	*Callosobrucus maculatus*	Adults	Agricultural pest	100%	Nigeria	Adetimehin et al., 2018; Opuba et al., 2018
	Ricinus communis	Leaf	Aqueous extract/topical and ingestion	Diamondback moth (*Plutella xylostella*)	Larvae	Agricultural pest	100	Togo	Tounou et al., 2011
Fabaceae	*Prosopis juliflora*	Leaf	Methanol extract/spray	Cotton aphid (*Aphis Gossypii*)	Adults	Pest of cotton	73.3	Ethiopia	Zerihun and Ele, 2018
		Seed	Methanol extract/spray	Cotton aphid (*Aphis Gossypii*)	Adults	Pest of cotton	70	Ethiopia	Zerihun and Ele, 2018
		Leaf extract	Aqueous extract/spray	Diamondback moth (*Plutella xylostella*)	Larvae	Pest of cabbage and other brassicae species	96%	India	Sangavi and Johnson Thangaraj Edward, 2017

TABLE 2F | Published reports on the insecticidal activities of some species with invasive potentials in sub-Saharan Africa.

Family	Plant species	Plant parts	Formulation/application method	Insect target	Stage of insect	Importance of insect	Mortality (%)	Country	Reference (s)
Fabaceae	*Sesbania grandiflora*	Leaf	Aqueous extract/spray	Diamondback moth (*Plutella xylostella*)	Larvae	Pest of cabbage and other brassicae species	73	India	Sangavi and Johnson Thangaraj Edward, 2017
Meliaceae	*Melia azedarach*	Fruit	Aqueous extract/spray	Vegetable leaf miner (*Liriomyza huidobrensis*)	Larvae	Agricultural pest	44	USA	Hammad and McAuslane, 2010
		Fruit	Essential oil and methanol extract/ingestion	Fall armyworm (*Spodoptera frugiperda*)	Larvae	Pest of maize	100	Brazil	Scapinello et al., 2014
		Fruit	Acetone extract/leaf dipping technique	African Cotton Leafworm (*Spodoptera littoralis*)	Larvae	Pest of cotton	100	Egypt	Farag et al., 2011
		Fruit	Ethanol extract/filter paper impregnation	Elm leaf beetle (*Xanthogaleruca luteola*)	Adults	Environmental pest	86	Chile	Chiffelle et al., 2011
		Fruit	Methanol extract/addition to water	*Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti*	Larvae	Vectors of some parasitic diseases	>88	India	Selvaraj and Mosses, 2011
Mimosaceae	*Mimosa diplotricha*	Leaf	Powder/dust	Bed bugs (*Cimex lectularius*)	Adults	Pest of medical importance	>70	Nigeria	Uyi et al., 2018a
		Leaf	Powder/dust	*Macrotermes species*	Adults	Pest of crops	100	Nigeria	Uyi et al., 2018b
		Root	Powder/dust	*Callosobruchus maculatus*	Adults	Agricultural pest	67	Nigeria	Uyi et al., 2020
Myrtaceae	*Eucalyptus camaldulensis*	Leaf	Ethanol extract/leaf dipping technique	Green peach aphid (*Myzus persicae*)	Nymphs and adults	Agricultural pest	53	Algeria	Nia et al., 2015; Erdogan and Yildirim, 2016

TABLE 2G | Published reports on the insecticidal activities of some plant species with invasive potentials in sub-Saharan Africa.

Family	Plant species	Plant parts	Formulation/application method	Insect target	Stage of insect	Importance of insect	Mortality (%)	Country	Reference (s)
Papaveraceae	*Argemone mexicana*	Leaf extract,	Methanol extract/ingestion	Rice moth, (*Corcyra cephalonica*) and Cockroach (*Periplaneta americana*)	Larvae, adults	Agricultural and household pests	15.4–76	India	Khan and Qamar, 2015a,b
Solanaceae	*Cestrum parqui*	Leaf extract	Aqueous extract/leaf dipping and ingestion	Mediterranean fruit fly (*Ceratitis capitata*)	Adults	Fruits	55	Chile	Zapata et al., 2006
	Solanum elaeagnifolium	Leaf extract	Methanol extract/seed treatment	Red flour beetle (*Tribolium castaneum*)	Larvae	Pest of stored grains	88	Tunisia	Ben Hamouda et al., 2015a
		Seed extract	Methanol extract/seed treatment	Red flour beetle (*Tribolium castaneum*)	Larvae	Pest of stored grains	84	Tunisia	Ben Hamouda et al., 2015a
		Leaf extract	Methanol extract/leaf treatment and ingestion	African cotton leafworm (*Spodoptera littoralis*)	Larvae	Agricultural pest	80	Tunisia	Ben Hamouda et al., 2015b
		Seed extract	Ethanol and methanol extract/leaf treatment and ingestion	African cotton leafworm (*Spodoptera littoralis*)	Larvae	Agricultural pest	100	Tunisia	Ben Hamouda et al., 2015b
		Leaf and seed extracts	Ethanol and methanol extract/leaf treatment and ingestion	Green peach aphid (*Myzus persicae*)	Adults	Agricultural pest	5–43	Tunisia	Ben Hamouda et al., 2015c

TABLE 2H | Published reports on the insecticidal activities of some plant species with invasive potentials in sub-Saharan Africa.

Family	Plant species	Plant parts	Formulation/application method	Insect target	Stage of insect	Importance of insect	Mortality (%)	Country	Reference (s)
Solanaceae	*Solanum sisymbriifolium*	Leaf	Methanol extract/filter paper impregnation	Red flour beetle (*Tribolium castaneum*)	Adults	Pest of stored grains	22%	Argentina	Padín et al., 2013
Verbenaceae	*Lantana camara*	Leaf	Acetone extract/addition to water	Mosquito (*Aedes aegypti*)	Larvae and pupae	Vector of some parasitic diseases	>65	India	Remia and Logaswamy, 2010
		Leaf and stem	Methanol extract/fumigant	Bean weevil (*Callosobruchus chinensis*)	Adults	Pest of pulse	9- 23%	India	Sexana et al., 1992
		Leaf	Chloroform extract/filter paper impregnation	subterranean termite, *Reticulitermes flavipes*	Adults	Agricultural pest	90	China	Yuan and Hu, 2012
		Leaf	Essential oil/spray	*Aedes aegypti*, *Culex quinquefasciatus*, *Anopheles culicifacies, Anopheles fluvialitis* and *Anopheles stephensi*	Adults	Vector of parasitic diseases	93–100	India	Dua et al., 2010
		Leaf and flower	Powder/dust	Maize weevil (*Sitophilus zeamais*)	Adults	Pest of maize and rice	>45	Nigeria	Taye et al., 2014
		Leaf extracts	Acetone and methanol/direct contact application	*Sitophilus oryzae* (L.) *Callosobruchus chinensis* (Fab.) and *Tribolium castaneum*	Adults	Pests of stored grains	>92	India	Rajashekar et al., 2014

TABLE 2I | Published reports on the insecticidal activities of some invasive alien plants in Africa.

Family	Plant species	Plant parts	Formulation/application method	Insect target	Stage of insect	Importance of insect	Mortality (%)	Country	Reference (s)
Verbenaceae	*Lantana camara*	Leaf and stem	Essential oil/fumigant	Grain weevil (*Sitophilus granaries*)	Adults	Pests of stored grains	100	Algeria	Zoubiri and Baaliouamer, 2012
		Leaf extract	Aqueous extract/spray	Diamondback moth (*Plutella xylostella*)	Larvae	Pest of cabbage and other brassicae species	3.3–6.7	India	Sangavi and Johnson Thangaraj Edward, 2017
		Leaf extract	Aqueous extract/spray	Aphids (*Aphis fabae*), bean foliage beetle (*Ootheca mutabilis* and *O. bennigsen*i), flower beetle (*Epicauta albovittata* and *E. limbatipennis*) and pod suckers (*Clavigralla tomentosicollis*, *C. schadabi* and *C. hystricodes*)	Nymphs, larvae and adults	Pest of beans	NA	Tanzania and Malawi	Mkindi et al., 2017

a myriad of agricultural, medical and environmental insect pests (**Tables 2A–I**), only a few studies have documented the indigenous use of these IAPs as botanical pesticides by the locals and small-holder farmers in Africa (e.g., Cobbinah et al., 1999; Mugisha-Kamatenesi et al., 2008). Therefore, there is an urgent need to conduct ethnobotanical surveys to identify and document the IAPs used for the control and management of insect pest by locals and small-holder farmers in Africa. Although, assessing efficacy under field condition remains a serious challenge in the use of botanicals to control insect pests of crops, recent field trials on bean and cabbage pests suggest that some plant extracts are as effective as synthetic pesticides; however, botanicals tend to be much less harmful to natural enemies (Amoabeng et al., 2013; Mkenda et al., 2015). Such findings are crucial in convincing the policy makers and other relevant stakeholders to support the use of botanicals to control pests. Therefore, field studies on the insecticidal efficacy of IAPs with botanical pesticides should be prioritized and such study may receive generous funding from stakeholders in the agricultural sector because of the direct impact of such research.

Despite their efficacy against pests, botanical pesticides are often less harmful to beneficial insects and are therefore more compatible with other pest management strategies (Stevensona et al., 2017). For example, Mkenda et al. (2015) showed that *Tithonia diversifolia* (an invasive alien plant species) and other three pesticidal plant species were able to control a several of agricultural pests attacking *Phaseolus vulgaris* (common beans), but were also less harmful to beneficial insects (i.e., lady beetle and spider mites) compared to a synthetic pesticide. In similar field study, Ezena et al. (2016) investigated the insecticidal potential of three concentrations (10, 20, and 30 g/L) of the invasive *C. odorata* in the management of the major pests of cabbage (*B. brassicae and P. xylostella*) and their natural enemies in southern Ghana. The authors found that the three concentrations of *C. odorata* significantly reduced (by more than 30%) the number of *B. brassicae and P. xylostella* than tap water and conventional insecticide, lambda-cyhalothrin. The authors also found that plots sprayed with 20 g/L of *C. odorata* extract supported the highest number of insect natural enemies (*Diaretiell rapae*, *Cotesia* plutellae (Hymentoptera: Braconidae) and hoverflies compared to plots treated with lambda-cyhalothrin. Research to demonstrating compatibility of botanical pesticides with other pest management strategies is needed. Such research should also focus on determination of the underlying mechanisms that reduce the impact of pesticidal IAPs on beneficial insects and understands if this is due to selective toxicity or lower persistence. Due to their high efficacy and low toxicity to beneficial insects (e.g., Mkenda et al., 2015), there is the prospect to inform locals, small-holder farmers, and other relevant stakeholders of the potential usage of the IAPs listed in **Tables 2A–I**. This will allow for the exploitation of IAPs by harvesting and using them to control insect pests and alternately minimizing the invasion intensities and impact of IAPs in ecosystems. This will give the small-holder farmers and locals who are typically resource poor access to technologies and information to control insect pests and

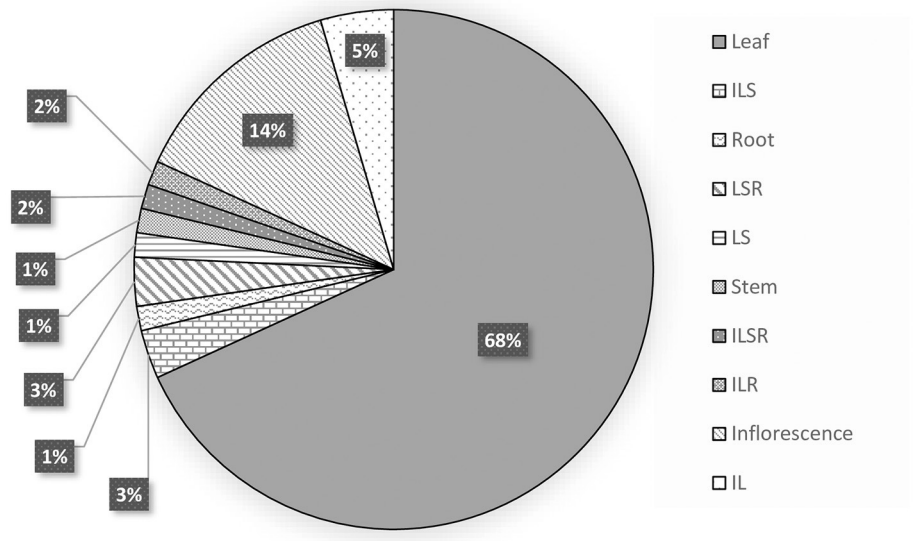

FIGURE 1 | Percentage prevalence of case studies on the parts of plant used to test for insecticidal activities of invasive alien plants. Data was obtained from 69 published case studies. IL, Inflorescence and leaf; ILS, Inflorescence; leaf and stem; ILR, Inflorescence; leaf and root; ILSR, Inflorescence; leaf, stem and root; LS, Leaf and stem; LSR, Leaf, stem, root.

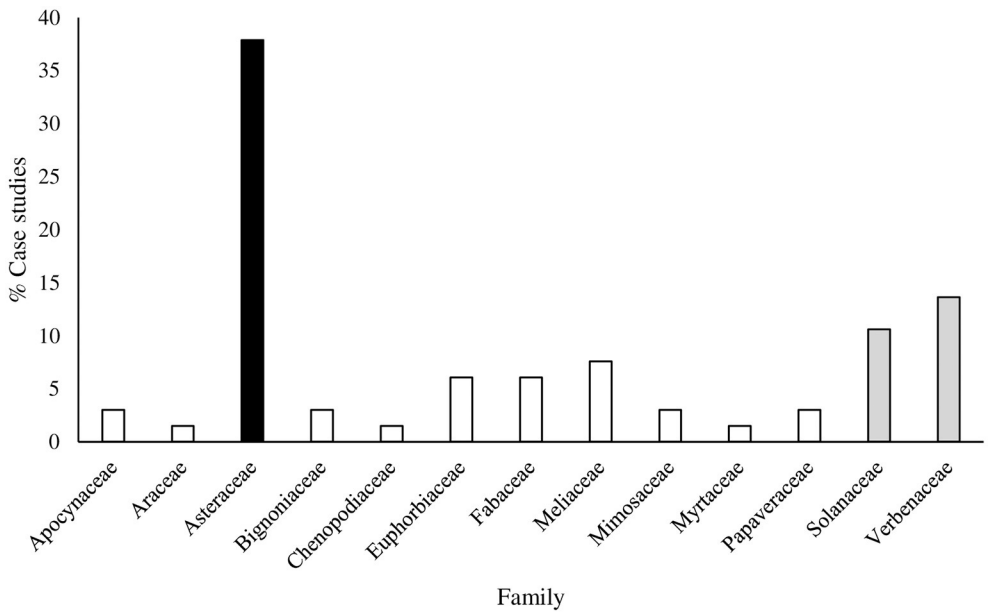

FIGURE 2 | Prevalence of case studies on different families of invasive alien plant species tested for insecticidal activities. Data was obtained from 69 published case studies.

diseases that limit crop production and successful storage of agricultural produce.

Safety and Exposure Concerns of Using Botanical Pesticides From IAPs

A key priority in the widely popular subsistence farming system in Africa is to prevent stored product insects from reducing the market and nutritional values of the harvested produce. Many small-holder farmers (peasants) in Africa use botanical pesticides, locally derived from either indigenous or IAPs to protect stored commodities (Cobbinah et al., 1999; Belmain and Stevenson, 2001; Isman, 2008; Nyirenda et al., 2011; Midega et al., 2016; Kamanula et al., 2017). The use of botanical pesticides to protect stored products may directly or indirectly expose

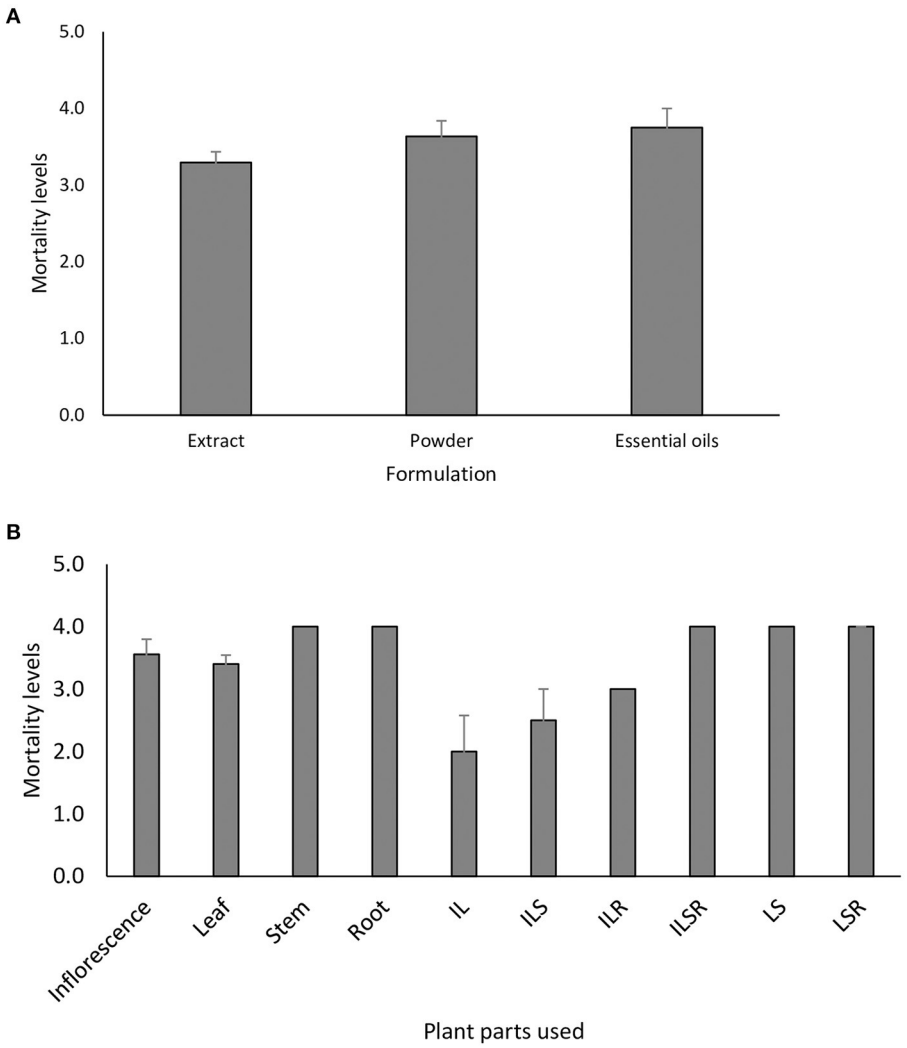

FIGURE 3 | Mean mortality rank (±se) of insect pests of agriculture and medical importance caused by **(A)** different formulations from, and **(B)** different plant parts or combinations plant parts of invasive alien plants. Data was obtained from 69 published case studies. IL, Inflorescence and leaf; ILS, Inflorescence, leaf and stem; ILR, Inflorescence, leaf and root; ILSR, Inflorescence, leaf, stem and root; LS, Leaf and stem; LSR, Leaf, stem, root.

farmers and/or consumers to potentially toxic chemicals from the plant materials used. It is important to note that naturally occurring plant chemicals are not necessarily safe. For example, some compounds (e.g., Aconitum, aconitine, nicotine, rotenone, and strychnine) of plant origin are known to be highly poisonous to mammals and fish (Kolev et al., 1996; Neuwinger, 2004).

Although the use of pesticidal plants to control pests in agro-ecosystems and other modified ecosystems is perceived to be safer than conventional pesticides, care must be exercised in the use of some of plants (especially invasive alien plants with novel biochemicals) for pest management. Invasive alien plants with potential toxicity to aquatic fauna or mammals should be restricted and discouraged. Plant scientists and entomologists should conduct special bioassays not only to show the efficacy of botanical pesticides from alien invasive plants but also to demonstrate the safety of these locally manufactured pesticides on mammals and aquatic fauna. The results of such safety and risk assessment studies should be communicated to various stakeholders including small-holder farmers who rely heavily on exploring new plant species for various purposes including to manage pests and for ethnomedicinal purpose. Although the likelihood of acute toxicity from handling plants is substantially lower than the risk from handling synthetic pesticides (Coats, 1994; Isman, 2006), the use of appropriate personal protective equipment should be encouraged when processing and handling powders and extracts from invasive alien plant materials.

Challenges of Using IAPs as Bio-Insecticides and Future Research Focus

Despite the acceptance and increasing usage of the biopesticides by the global communities, the lack of government published regulatory framework impedes the rigorous research processes

and hampers the adoption of the compounds [Gahukar, 2011; AATF (African Agricultural Technology Foundation), 2013; Ivase et al., 2017; Damalas and Koutroubas, 2018]. Like synthetic insecticides, the international and national regulations should be developed to govern the development of bio-insecticides and alternately protect the consumers and the natural ecosystems from the hazardous compounds (Chandler et al., 2011). Although the natural resources extracted from nature are generally regarded as safe to humans and the environment, risk assessment protocols and registration portfolio of bio-insecticides follow conventional insecticides (Damalas and Koutroubas, 2018; Marrone, 2019). The procedures are somewhat time-consuming and expensive for the bio-insecticide development companies. Furthermore, the costs of production of these natural compounds decelerate the commercialization processes of the products and once commercialized, the prices are inflated (Marrone, 2014; Ivase et al., 2017; Damalas and Koutroubas, 2018). The prospects of developing biopesticides include the distinct development of legislations that govern the screening and commercialization of the products (AATF, 2013; Seiber et al., 2014; Kumar and Singh, 2015; Ivase et al., 2017; Damalas and Koutroubas, 2018). Government's ability to subsidize the research on the development of compounds that are safer to use may accelerate the bio-insecticide development and commercialization processes (Marrone, 2014). Furthermore, the efficiency of bioinsecticides with limited efficacy may be integrated with compatible pest management practices to optimize the efficiency of the pest management program (Chandler et al., 2011). Further investigations on the persistence and efficiency of biopesticides derived from IAPs need to be prioritized to measure the overall cost of the benefit of the pest management products. Public and private sectors should also be encouraged to participate (i.e., technically or financially) on the development and production of this economical and environmentally friendly alternative, especially in the developing countries.

CONCLUSIONS

The diversity of invasive alien plant species (in Africa) with numerous examples of their insecticidal efficacy against important pests listed in this paper suggest that opportunity exist for using invasive alien plants in Africa as pesticides in agro-ecosystems and other managed ecosystems. This will result in small-holders spending less on synthetic insecticides, substantially reduction in crop production or pest management costs and increase productivity and quality of life. Despite the rise of research interest in plant pesticides from native plants and IAPs over the last decade in Africa (Isman and Grieneisen, 2014; Isman, 2015), surprisingly little time is invested in assessing efficacy under field conditions. The lack of meaningful chemical data (i.e., elucidation of bioactive compounds) reported alongside efficacy trials remains a major concern. Some of the published works on the effects of pesticides from native plants or IAPs are not repeatable for various reasons and adds little to our knowledge about mechanisms, efficacy or scope to use plant materials in pest management. Although the efficacy of the botanical pesticides from 23 invasive alien plant species in this study have been documented, further investigations on; (1) their efficacy under field conditions, mode of action and chemical data, (2) their compatibility with other pest management strategies, (3) the economic benefits of using pesticidal plants over synthetic products and (4) how to effectively commercialize the production of botanical insecticides from IAPs. For the first time, our review elucidates the insecticidal efficacy of the invasive alien plants in Africa and highlights the prospects for the use of these IAPs as pesticidal plants in African countries especially among resource-limited small-holder farmers and locals. It remains to be seen whether stakeholders (governments, research institutions, scientists, agriculturists, farmers, locals, extension workers, etc.) can effectively explore the safe use of botanically based insecticides (extracts, powders or other formulations) from IAPs in their regions for the control and management of insect pests in agro-ecosystems and other modified environments. This paper serves as a veritable reference for researchers and stakeholders who are interested in advancing, the science, technology or our understanding of the use of invasive alien plant to control and manage insect pests of agricultural, environmental medical importance.

AUTHOR CONTRIBUTIONS

OU and LM conceptualized the study and wrote the manuscript. OU, LM, AE, and MT interpreted the results and critically reviewed and amended the manuscript. All authors contributed to the article and approved the submitted version.

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Chemical Composition and Antifungal, Insecticidal and Repellent Activity of Essential Oils From *Origanum compactum* Benth. Used in the Mediterranean Diet

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Essential oils (EO) of *Origanum compactum* Benth. (*O. compactum*) are well known for their biological and pharmacological activities. This study aimed to assess the chemical composition, antifungal, insecticidal and repellent activities of EO of *O. compactum* used in the Mediterranean diet. Phytochemical screening was conducted using gas chromatography-mass spectrometry (GC/MS). Antifungal activity was tested by the disc diffusion method followed by a minimal inhibitory concentration (MIC) assay against *Candida albicans (C. albicans), Aspergillus flavus (A. flavus), Aspergillus niger (A. nige),* and *Fusarium oxysporum (F. oxysporum)*. Repellent potential and toxicity of EO by contact and inhalation were tested against *Callosobruchus maculatus* (*C. maculatus*). The yield of essential oil obtained by hydrodistillation of *O. compactum* was 4.41 ± 0.35%, mainly composed of Carvacrol (38%) and Thymol (31.46%). Regarding antifungal activity, the results revealed a wide antifungal spectrum of the studied EO against the tested strains, which reached 100% growth inhibition, especially against *A. niger* and *C. albicans* even at the lowest MIC values (3.125 μg/mL). Concerning insecticidal activity, the EO caused total mortality of *C. maculatus* adults at a dose of 20 μL/L air with LC_{50} value of 5.3 μL/L air. A significant reduction in the number of eggs and emergence was proportionally recorded with increasing doses up to 100% at 20 μL/L air. For repellent activity, the studied EO showed a moderate repellent activity with an average percentage of 39.16%. The outcome of this work revealed that *O. Compactum* EO could be a sustainable and environmentally friendly alternative bioinsecticide and bio-fungicide to replace the chemically synthesized forms.

Keywords: antifungal, insecticidal, essential oil, drug resistance, bioinsecticide

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INTRODUCTION

Medicinal and aromatic plants are important sources of EO that find applications in various areas of life. EO is mainly used as a food flavoring but can be successfully used for various non-food applications, as it exhibits many biological properties, including antifungal, antimicrobial, antioxidant, and insecticidal activity (Mssillou et al., 2020; Allali et al., 2021; El Moussaoui et al., 2021).

The compact flowered *Origanum compactum* Benth (*O. compactum)* is one of the most important medicinal plants in terms of ethnobotany in Morocco and southern Spain (Laghmouchi et al., 2018). *Origanum compactum* (Labiatae) is widely used traditionally against several pathologies, with a varied spectrum of use according to region, medication purposes, the parts used, and the mode of preparation (Ennabili et al., 2000; Eddouks et al., 2002). The E O from genus compactum possessed antibacterial (Bouhdid et al., 2008, 2009), antioxidant (Bouhdid et al., 2008), cytotoxic (Babili et al., 2011), antimutagenic (Mezzoug et al., 2007), antifungal (Fadel et al., 2013), and antimalarial properties (Babili et al., 2011).

Chickpea (*Cicer arietinum* L.), is one of the most nutrient-dense seed legumes available for human consumption. It is a good source of protein, vitamins, and minerals (Allali et al., 2020a). Loss of seed yield in pulse crops during storage due to various types of insects, particularly bruchids, is a major issue for farmers (Matos et al., 2020). The chickpea weevil *C. maculatus* (Coleoptera: Bruchidae) is one of the most destructive pest species against chickpea; it can lay eggs in cultivated fields, as well as in storage facilities. The larvae, which feed internally, are difficult to control with chemical insecticides. Fungi in the field and during storage also cause considerable crop losses and deterioration of seed quality (Santos et al., 2016). Contamination by fungi can have serious and dangerous consequences for human health; *A. flavus* produces aflatoxins inducing liver cancer and affecting the growth of young children (Kumar and Kalita, 2017). In addition, invasive candidiasis caused by the candida fungus is frequently associated with high mortality rates, and the emergence of resistant strains (El Moussaoui et al., 2021).

Because of its effectiveness and ease of use, the main approach currently used to control insect pests and fungi in agriculture is the application of synthetic pesticides and fungicides. However, extensive, uncontrolled, and unregulated use of these chemically synthesized products may adversely affect the environment and public health (Amzouar et al., 2016; Kumari and John, 2019; Allali et al., 2020b). On the other hand, synthetic fungicides and disinfectants generally produce chemical residues, which constitute potential environmental pollutants that are difficult to degrade (Gonzalez et al., 2009). It is thus fitting that many recent studies have focused on the search for eco-friendly substances to control pests and microbes without side effects on the environment and public health. In this context, substances of natural origin and particularly EO may represent today an eco-friendly reservoir and more sustainable solution to protect crops.

In this regard, the present work was undertaken to establish whether EO from *O. compactum* (oregano) leaves possess insecticidal and antifungal effects against species of pests and fungi attacking leguminous crops. Thus, in current study, we investigated the chemical composition of EO of *O. compactum* from Taounate, as well as their insecticidal and repellent activities against *Callosobruchus maculatus*, major pests of chickpea grains in Morocco, and the antifungal activity against some pathogenic strains of fungi implicated in the contamination of leguminous and nosocomial infections.

MATERIALS AND METHODS

Plant Material and Extraction of Essential Oils

The leaves of *O. compactum* were used to conduct this work. The whole plant was harvested in June 2020, wherein there was maximum flowering, from the Taunt region (34°30′0″N; 4°33′0″W). The botanical identification of *O. compactum* was carried out by a botanist and given a reference DO12/05005 before being deposited in the herbarium. Next, the leaves were dried in the shade in a dry and ventilated area at a temperature of 25°C for 7 days. The extraction of the EO was performed by hydrodistillation, using a Clevenger-type system according to the manufacturer's instructions. Briefly, 200 g of *O. compactum* leaves were soaked in 1.25 L of distilled water in a 2-L flask before being boiled for 3 h. The essential oils obtained were dried with anhydrous sodium sulfate and stored in a refrigerator at 4°C until use. The yield was calculated based on the dried weight of the plant using the following formula (1):

$$\mathrm{YHE} = \frac{MHE}{MD} \times 100 \quad (1)$$

Where Y_{HE} is the Yield of essential oil (%), M_{HE} is the mass of the EO (g), and M_D is the mass of dry plant matter (g).

Test Insect Collection and Rearing Conditions

The insect *C. maculatus* was collected from a sample of chickpea stored in the city of Fez, Morocco. Bruchids were reared on chickpea seeds (*Cicer aritinum*) packed in glass jars (1 L), covered internally with transparent fabric. The jars were maintained at a temperature of 25 ± 2°C, relative humidity, and a photoperiod of 14 h (light)/10 h (dark) and 65% (± 5%) relative humidity for several successive generations.

Chromatographic Analysis and Mass Spectrometry

Agilent-Technologies 6,890 N Network GC system with a flame ionization detector and HP-5MS capillary column (30 m × 0.25 mm, film thickness of 0.25 m; Little Falls, CA, United States) was used to analyze the EO. The injector and detector temperatures were set to 250 and 280°C, respectively. The temperature of the column was designed to rise at a rate of 5°C/min from 35 to 250°C, whilst the lower and upper temperatures were kept for 3 and 10 min, respectively. The carrier gas (helium) flow rate was 1.0 mL/min. Using split mode, 1.0 μL of the sample was injected (split ratio, 1:100). The gas chromatograph's manufacturer offered a built-in

data-handling program that was used for all quantifications. The composition was expressed as a proportion of the total peak area. By comparing their GC retention indices, the volatile oil constituents were detected. The mass spectra of each compound were compared to those of the NIST02 GC/MS library data and the Adams library spectra (Adams, 2007).

Antifungal Activity of Essential Oils

Fungal Strains and Culture Conditions

In this study, three filamentous fungi namely *niger, A. flavus*, *F. oxysporum*, as well as one yeast strain *C. albicans* were used for testing reasons. All fungal strains selected are pathogenic and have been associated with drug resistance (Krishnan et al., 2009; Al-Hatmi et al., 2019; El Moussaoui et al., 2021). These strains have been reported as the main producers of mycotoxins and are among the most contaminating microorganisms of dry vegetables and cereals. Spore suspensions were taken from 7-day-old cultures using tubes containing NaCl 0.9%. Afterward, the number of spores in suspension was counted before being diluted to reach an inoculum concentration of around 10^6 spores/mL (Moussa et al., 2020).

Disk Diffusion Method

Assessment of the antifungal activity of *O. compactum* EO was performed by the disc diffusion method (Balouiri et al., 2015). First, Petri plates (90 mm) containing MEA (Malt Extract Agar) medium were inoculated with 0.1 mL of previously prepared microbial culture (10^6 spores/mL). Thereafter, Wattman paper discs of 6 mm were immediately deposited on the culture media surface after being soaked with 20 μL of EO. Next, the inoculated plates were incubated at 30°C in darkness. Both, inhibition diameter and percent inhibition were determined after 48 h of incubation for *C. albicans* strain and after 7 days of incubation for fungi strains (Zhao et al., 2021).

Determination of the Minimal Inhibitory Concentration

In this work, the macro-dilution method was undertaken to evaluate the MICs of *O. Compactum* EO (Moussa et al., 2020). The EOs were immiscible in the culture medium so that their emulsification was conducted using a 0.2% agar solution in order to facilitate germ/compound interaction. To achieve this goal, in sterile hemolysis tubes containing sterile malt extract, broth serial dilutions were made with increasing concentrations up to a final volume of 5 mL in each tube. Consequently, the concentrations of *O. Compactum* EO obtained in the tubes ranged from 100 to 0.09 μg/mL. Next, 100 μL of the media control of each fungal strain was aseptically transferred into each prepared tube except for the media control. Fluconazole FLU (5 mg/mL) was used as a positive control under the same conditions. Finally, the tubes were incubated at 30°C with a rotary shaker for 48 h for yeast and 7 days for fungi. The MIC values of samples correspond to the lowest concentration at which no visible growth was observed in the liquid medium (Bouddine et al., 2012).

Insecticidal Activity of Essential Oils

Toxicity of Essential Oils by Contact Test

Contact toxicity bioassays were performed as described elsewhere (Dutra et al., 2016; Matos et al., 2020) with slight modification. For each EO concentration, 100 g of chickpeas were infested by 5 pairs of insects aged 0–48 h, packed in plastic containers (250 mL) duly closed by a perforated lid, and covered with a thin transparent cloth. Next, EOs were added to the grains using an automatic pipette and then shaken for 2 min. After 48 h of confinement, adult mortality was assessed as reported elsewhere (Dutra et al., 2016). Based on the results obtained in preliminary tests, treatments at different concentrations (1, 5, 10, and 20 μL/100 g) were performed. Parallelly 100 g of chickpeas infested with five pairs of insects without oils were used as control. Dead insects were counted daily until the end of the experiment. Three replicates were performed to measure insecticidal activity, and expressed as a percentage of the average mortality of *C. maculatus* adults before being transformed into corrected mortality by Abbott's formula (2):

$$Pc = 100 \times \frac{P0 - Pt}{100 - Pt} \quad (2)$$

Where Pc is the corrected percentage of mortality (%), Po is the observed mortality in the trial, and Pt is the observed mortality in the control.

Eggs laid by females were counted after 12 days from the start of experiment, whilst the emerged individuals were counted after 30 days. The reduction percentage in the number of eggs and adults emerged in each concentration of essential oil was calculated using the following formula (3):

$$PR = \frac{\mathrm{NC} - \mathrm{NT}}{\mathrm{NC}} \times 100 \quad (3)$$

Where PR is the egg-laying or reduction percent of emerged insects (%), NC is the number of eggs or insects hatched in the control and NT is the number of eggs or insects hatched in the treatment.

Toxicity of Essential Oils Tested by Inhalation

In the current work, the toxicity of EO was tested by inhalation against *C. maculatus*. To achieve this objective, in 1-L glass jars, small masses of cotton were suspended with a thread attached to the inside of the lid. Doses of 1, 5, 15, and 20 μL/L air of *O. compactum* OE were deposited into the cotton using a micropipette. Afterward, ten *C. maculates* bruchids (male and female) aged between 0 and 48 h were placed in each jar with a perfectly tight seal. For each dose, three replicates were performed. The comparison was made with a control sample (cotton without test solutions).

The Abbott formula (4) was used to calculate the observed mortality rate:

$$Pc = 100 \times \frac{P0 - Pt}{100 - Pt} \quad (4)$$

Where Pc is the corrected mortality percent (%); Po is the observed mortality in the trial, and Pt is the observed mortality in the control.

Repellent Activity of Essential Oils

The repellent effect of the essential oil of *O. compactum* against adults of *C. maculatus* was evaluated using the preferential area

method on filter paper described by McDonald et al. (1970). Briefly, 9 cm diameter filter paper discs were used for this purpose. These discs were cut into two halves, each with an area of 31.80 cm^2. For one of the two halves, a volume of 0.5 mL of each EO concentration previously prepared in acetone (1, 5, 10, and 20 μL/mL) was uniformly spread to reach doses of 0.016, 0.079, 0.157, and 0.315 $\mu L/cm^2$ per disk, while the other half received only 0.5 mL of acetone (control). Afterward, the Petri dishes were closed with Parafilm for 30 min. Next, the number of bruchids presented on the half of the disc treated with essential oil was counted against the number of the untreated part. Three replicates for each experiment were done under the same environmental conditions as the insect rearing (**Figure 1**).

The percentage of repulsion (PR) was calculated according to the following formula (5) (Zandi-Sohani et al., 2013):

$$PR = \frac{NC - NT}{NC + NT} \times 100 \quad (5)$$

Where PR is the percentage of repulsion (%), NC is the number of insects in the control area, and NT is the number of insects in the treatment area.

The result of the repulsive effects of the essential oil was interpreted according to the classification of McDonald et al., 1970.

Statistical Analysis

The bioassay experiment followed a randomized design with three replicates for each treatment. Results were expressed as mean ± SE. SPSS for Windows® statistical software (version 21.0) was used to perform the analyses. Data were assessed by one-way analysis of variance (ANOVA) to determine significant values. Fisher's minimum significant difference (LSD) test was used as a *post-hoc* test for multiple comparison purposes at $\alpha = 0.05$. The LC50 and LC95 lethal concentrations with their confidence intervals were determined by the probit method (Finney, 1971).

RESULTS AND DISCUSSION

Essential Oil Extraction

The EO yield recovered from the Hydrodistillation of leaves of *O. Compactum* was 4.41 + 0.35%. This EO yield was slightly lower than that obtained by Rezouki et al. (2020). Several factors can influence the yield of EO from aromatic and medicinal plants. According to Baranauskienė et al. (2013), the EO production of fresh plants was lower than that of dried plants. This can be explained by increases in the biosynthesis of terpenes and their derivatives after the harvest. This biosynthesis is stimulated by plant water stress and ultimately leads to an increase in the yields of EO. When the plant dies, biosynthetic activity stops, and evaporative losses of EO are no longer compensated, resulting in a drop in distillation yields (Bencheikh et al., 2015). Similarly, Ghasemi et al. (2013) confirmed that drying methods can negatively or positively influence oil content depending on drying time and temperature.

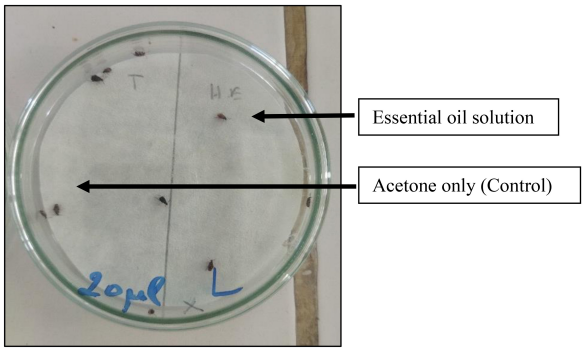

FIGURE 1 | Repellent test of *Origanum compactum* essential oil against *C. maculatus*.

TABLE 1 | Phytochemical compounds identified in *O. compactum* EO.

Peak	RT	Compound name	RI	Area (%)
1	4.542	Beta-Myrcene	114	0.55
2	4.936	o-Cymene	212	9.07
3	6.341	Borneol L	562	0.60
4	5.285	Gamma-Terpinene	299	11.11
5	5.659	L-Linalool	392	2.41
6	4.852	Alpha-terpinene	191	0.84
7	6.430	4-Terpineol	584	0.90
8	6.538	Alpha terpineol	611	0.58
9	7.317	Thymol	805	31.46
10	9.461	(-) Caryophylleneoxide	1,339	1.21
11	7.413	Carvacrol	829	38.73
12	8.361	Caryophyllene	1,065	1.47
13	6.984	Pulegone	722	1.07
Monoterpenes				97.32
Sesquiterpenes				2.68
Total				100

RT, Retention time; RI, Retention index.

Gas Chromatography-Mass Spectrometry Analysis

Thirteen compounds were identified in the studied EO by the GC-MS analysis (**Table 1** and **Figure 2**). Carvacrol (about 38.73%), thymol (31.46%), gamma-Terpinene (11.11%), and o-cymene (9.07%) were the major constituents of the EO. The chemical composition of the studied oil was close to that reported by several studies conducted in Morocco. The EO of Moroccan *O. compactum* was characterized by its high content of thymol and carvacrol, which agreed with previous works (Mohammed et al., 2020; Rezouki et al., 2020; Zeroual et al., 2021). The literature has indicated that the yield and chemical composition of EO vary according to the harvesting period, the extraction method, and the drying of the plant, so that our results are in agreement with previous works (Rezouki et al., 2021).

Antifungal Activity

Fungi are frequently implicated in the contamination of leguminous crops during harvest or storage, particularly the

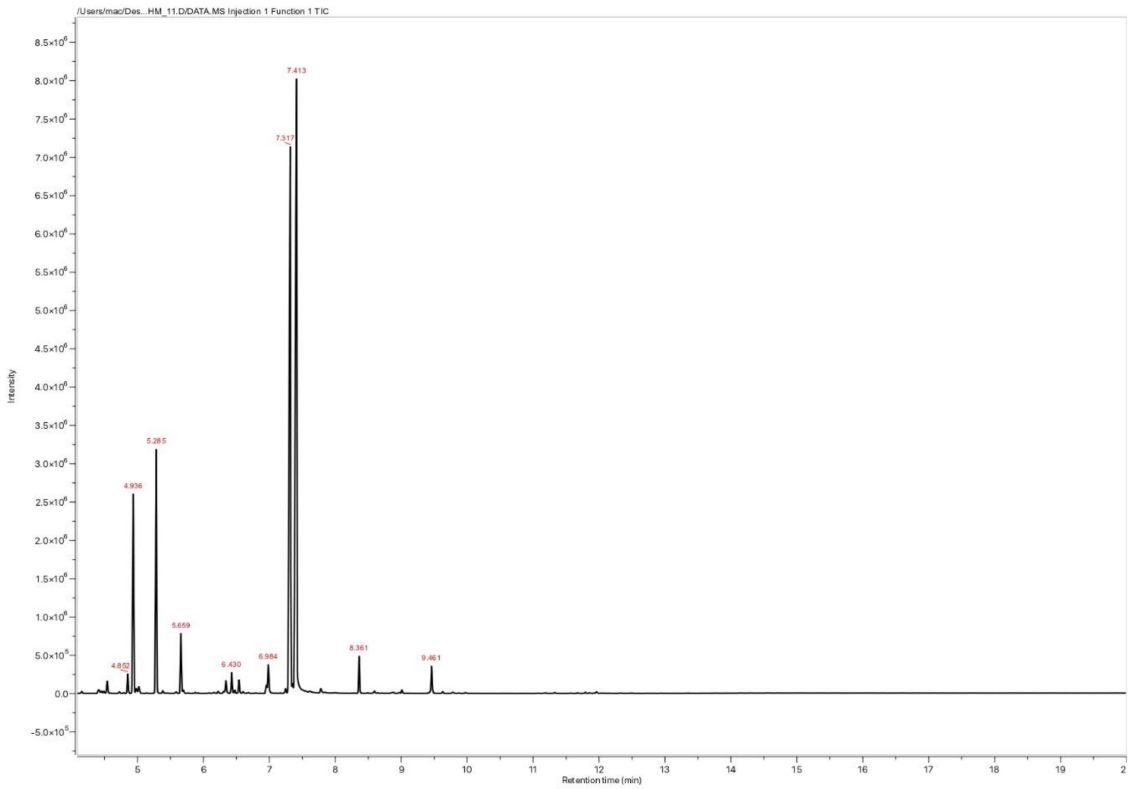

FIGURE 2 | GC-MS chromatographic profile of *O. compactum* EO.

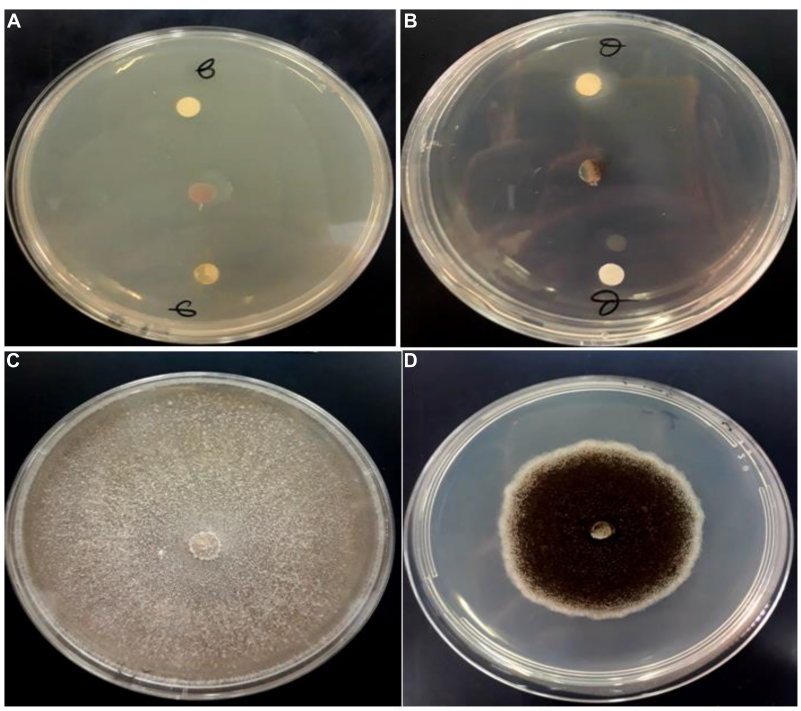

FIGURE 3 | Antifungal activity of *O. Compactum* EO tested by disc diffusion method against *F. oxysporum* **(A)** and *A. niger* **(B)**. **(C,D)** Are untreated fungi for *F. oxysporum* and *A. niger,* respectively (negative controls).

TABLE 2 | MIC results of *O. compactum* Benth. EO against fungal strains.

Fungal strains	Minimal inhibitory concentration (μg/mL)	
	Essential oil	Fluconazole
A. niger	3.125	128
A. flavus	6.25	256
F. oxysporum	12.5	160
C. albicans	3.125	400

genera *Aspergillus* and *Fusarium* (Sud et al., 2005). These mycotoxin-producing filamentous molds along with *C. albicans* are pathogenic and responsible for many fungal infections in hospitalized patients worldwide (El Moussaoui et al., 2021). **Figure 3** describes the growth inhibition of the studied fungal strains treated by *Origanum compactum* EOs. The results revealed a high antifungal potential of the studied EOs against the tested strains, marked by a maximum growth inhibition rate (100%).

In addition, the fungicidal effect of *O. compactum* EO was achieved by very low MIC values (**Table 2**). From this table, it can be seen that *A. niger* and *C. albicans* were the more sensitive to the oil since they were inhibited by the lowest concentration of the EO (3.125 μg/mL). In contrast, higher MICs 6.25 and 12.5 μg/mL were required to inhibit the growth of *A. flavus* and *F. oxysporum,* respectively. On the other hand, EO showed lower MIC values against tested fungal strains when compared to the Fluconazole standard. Our study reported lower MIC values against tested fungal strains when compared to previous work investigating *O. compactum* against *A. niger*, *A. alternata*, *B. cinerea*, *P. digitatum*, *P. italicum*, *V. dahlea,* and *P. expansum,* which were inhibited by MICs ranging from 300 to 450 μg/mL (Mohammed et al., 2020). Another recent study revealed that the MIC values of *Origanum majorana* L.against human pathogenic fungi, including *Candida* species, ranged from 58 to 468 μg/mL (Hajlaoui et al., 2016).

The chemical composition of EOs is closely related to their antifungal effect. Potential individual or synergetic effects between the major and minor compounds may occur.

In this respect, some works revealed that *Botrytis cinerea in vitro* mycelial growth and spore germination were strongly inhibited by carvacrol and thymol, the main compound of our Oregano essential oil (Zhao et al., 2021). In addition, other recent research has found that the high content of thymol, carvacrol, γ-terpinene, and *p*-cymene is roughly correlated with *in vitro* and *in vivo* biological activities (Bouyahya et al., 2017). For a better understanding, previous literature has investigated the mechanism of action of EO in fungi. Indeed, the antifungal effect of oregano might be attributed in part to EO terpenes and phenolic compounds involved in cell membrane damage, leakage of cellular materials, inhibition of electron transport, and ATPase in the mitochondria, which ultimately lead to the death of the microorganism (Lagrouh et al., 2017). More comprehensive research revealed that EO of oregano significantly reduced the production of the phospholipase enzyme in *C. albicans* (Brondani et al., 2018).

Insecticidal Activity of Essential Oils

In this experiment, different doses of *Origanum compactum* EO (0; 1; 5; 10; and 20 μL/L air volume) were used to evaluate their

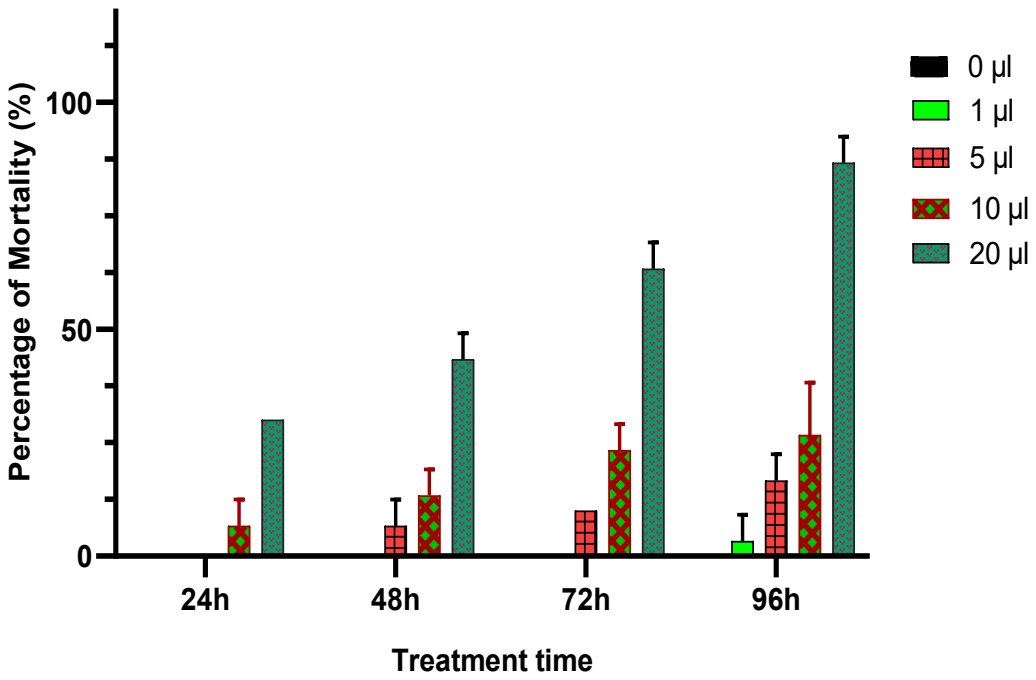

FIGURE 4 | Percentage of mortality (means ± *SD*) *of C. maculatus* adults exposed to an inhalation test of different doses of *O. compactum* EO.

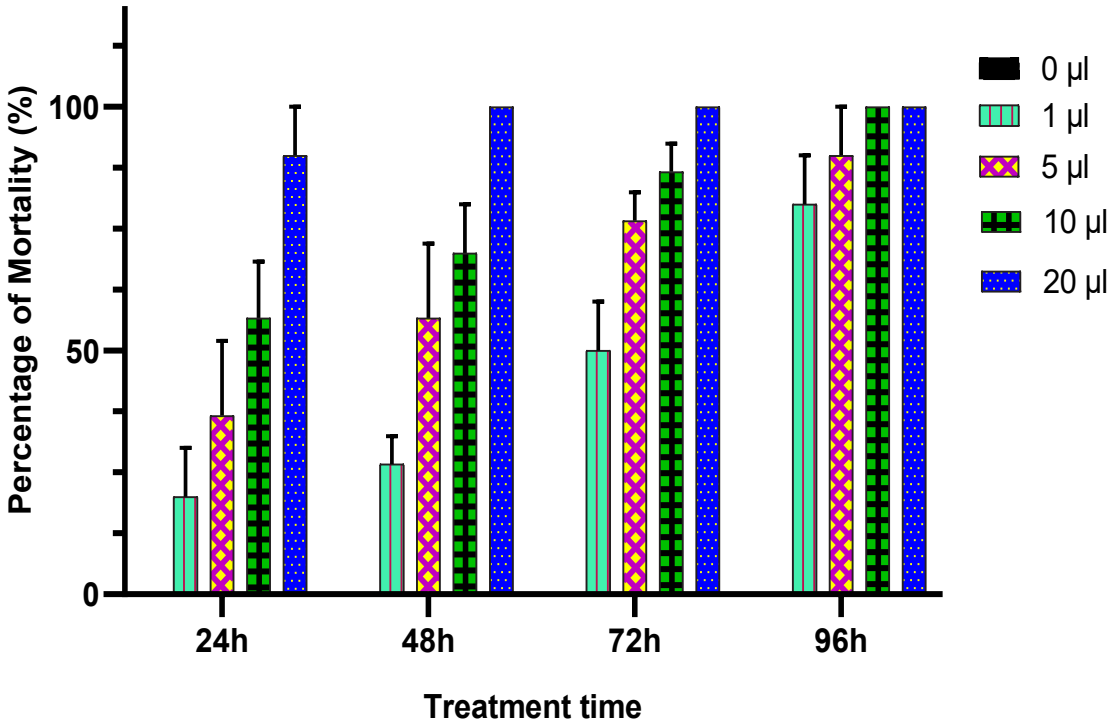

FIGURE 5 | Percentage of mortality (means ± *SD*) of *C. maculatus* adults exposed to a contact test with different doses of *Origanum compactum* EO.

toxicity against *C. maculatus* through inhalation. Mortality of adults was noted every 24 h for 4 days, and the results obtained are shown in **Figure 4**.

According to the results obtained (**Figure 4**), the oregano EO showed a significant insecticidal effect on the longevity of treated adults. The mortality of *C. maculates* adults increased with increasing doses and duration of exposure to EO. Significant mortality (86.21%) was observed in chickpea bruchid adults treated with a dose of 10 µL/L of oregano EO after 96 h of exposure, which showed the powerful insecticidal effect of the oil.

In this test, the EO of *O. compactum* at different doses was applied in direct contact with *C. maculatus* in order to evaluate their toxicity against this pest. The obtained results are listed in **Figure 5**. Generally, the mortality of *C. maculatus* adults increased when a high dose of the essential oil was applied, and/or when the duration of contact with it approached 96 h. Indeed, at the lowest concentration (1 µL/100 g), the EO of *O. compactum* tested by direct contact caused 80% mortality of *C. maculatus* adults after 96 h of exposure, while at the same concentration tested by inhalation, it caused only 20.69% mortality. At the

TABLE 3 | LC_{50} and LC_{95} values calculated based on the mortality of *C. maculatus* adults by the inhalation test after 24 h of exposure to *O. compactum* EO.

Bioassays	LC_{50}	LC_{95}	X^2
Inhalation test	33.61 (24.58;62.83)	211.23 (10.93;1900.8)	1.21
Contact test	5.53*	75.67*	17.47

X^2, Chi-square.
**Confidence intervals are too wide, they do not lend themselves to calculation.*

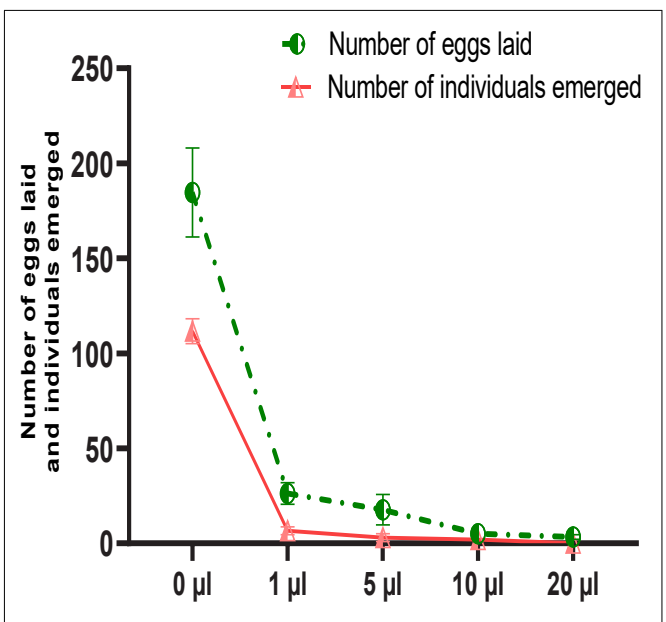

FIGURE 6 | Female fecundity and emergence of new individuals (mean values ± *SD*) after a direct contact test with different doses of EO.

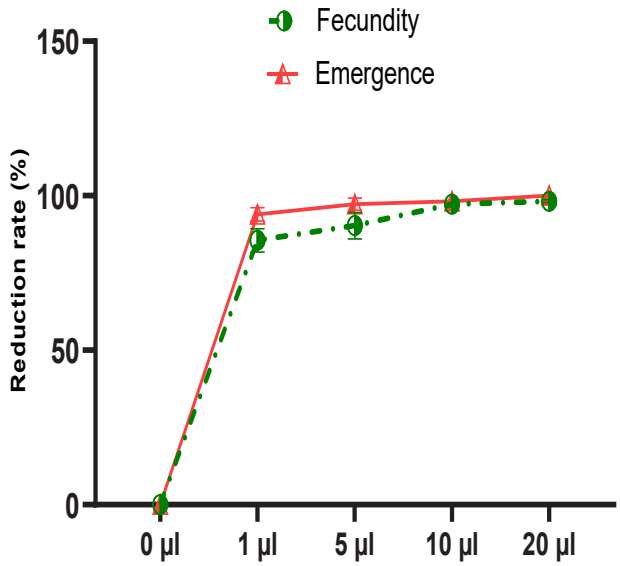

FIGURE 7 | Fecundity and emergence reduction rates after a direct contact test with different doses of EO.

highest concentration (20 µL/100 g), the tested oils showed significantly higher action when compared to the control and caused 100% mortality with the contact test and 63.33% with the inhalation test after 72 h of exposure. Statistical analysis showed that the LC_{50} and LC_{95} values obtained with the inhalation test (33.61 µL/L air) were higher than those observed with the contact test (5.53 µL/L of air) (**Table 3**).

Despite the significant reduction in mortality of *C. maculatus* adults, no oil concentration completely prevented oviposition in females. **Figure 6** shows that the number of eggs laid is inversely proportional to the concentration of the EO tested. Thus, at the lowest concentration, the average number of eggs laid per female was 26.33 ± 5.68 representing a respective reduction of 85.7% in egg-laying when compared to the control (**Figure 7**). At the highest concentration, the average number of eggs laid per female decreased sharply to 3.33 ± 1.15 corresponding to a 98.2% of reduction in oviposition. The number of eggs laid per female of *C. maculatus* in the control jar was 184.67 ± 23.43. For emergence, a significant reduction rate of 100% was observed at the highest dose tested (20 µL/100 g).

REPELLENT ACTIVITY

The obtained findings figured out that repellent activity was moderate at different doses with a maximum repulsion rate of 56.67 ± 15.26% after 60 min at a dose of 0.315 µL/cm^2, corresponding to the highest average repulsion rate (39.16%) calculated according to McDonald et al. (1970) (**Table 4**).

According to the results obtained, *Origanum compactum* EO were effective in the protection of legume seeds. This EO reduced significantly the life span of *C. maculatus* adult bruchids, even at the lowest doses used. This high efficiency resulted in a low value of the LC_{50}, 5.53 µL/100 g (contact test) that might be induced by the action of major compounds of these EO (Allali et al., 2021).

Our results showed that the toxicity of EO of *Origanum compactum* increased with increasing doses to reach the maximum at the highest concentrations used. It is therefore appropriate that our results are in agreement with those reported previously (Pavela et al., 2016), which demonstrated that EO of *O. compactum* applied by fumigation on *Tetranychus urticae* adults caused mortality of more than 50%.

Several authors have observed the acaricidal/insecticidal effect of other oregano species. The essential oil of *O. syriacum* is effective by fumigation on *T. cinnabarinus* (Tunc and Şahinkaya, 1998). Aqueous extracts of *O. majorana* were also found to be effective against *T. Urticae* (Pavela et al., 2016). According to Koschier (2008), carvacrol-rich oregano oils show significant activity against several insects, mites, and plant pathogens. For comparison purposes, species among genus *Origanum* have shown significant efficacy against several pests of stored products. For example, *Origanum acutidens* oil rich in carvacrol (87.0%), showed a mortality of 68.3 and 36.7% against two adult insects, *Sitophilus granarius* and *Tribolium confusum*, respectively (Kordali et al., 2008). Moreover, the EO of Oregano has demonstrated strong insecticidal activity against the larvae of *Spodoptera littoralis* with an $LC_{50} \leq 0.05$ mL/larva (Pavela, 2015).

Regarding the mode of action of EO on insect pests, a recently published study reported that EO applied by contact on *Sitophilus granarius* insects, pests of cereal seeds, can affect a variety of biological processes in the insects (Renoz et al., 2021). According to these authors, Mentha arvensis oils induced significant physiological changes in exposed insects,

TABLE 4 | Results of repellent activity of EO from *O. compactum* against *C. maculatus*.

	Doses of EO (µL/cm^2)				Probability (P)	Average %PR	Class
	0.016	0.079	0.157	0.315			
30 min	13.33 ± 11.55	13.33 ± 11.55	40 ± 20	46.67 ± 11.55	0.03*	28.33	Moderatelyrepellent (II)
60 min	13.33 ± 5.77	33.33 ± 11.55	53.33 ± 11.55	56.67 ± 15.26	0.005**	39.16	Moderatelyrepellent (II)
120 min	0 ± 0	26.67 ± 11.55	46.67 ± 11.55	53.33 ± 11.55	0.0007**	31.67	Moderatelyrepellent (II)

PR, percentage of repulsion.
*Each number is the mean standard error of three replicates. The symbol * indicates that the difference between the values in the same row are significant, whilst the symbol ** means highly significant ($p > 0.05$) using the LSD test. Repulsion class: Class 0—0–0.1%; Class I—0.1–20%; Class II—20.1–40%; Class III—40.1–60%; Class IV—60.1–80%; Class V—80.1–100%.*

particularly on vital functions related to the muscular and neurological systems, cellular respiration, protein synthesis, and detoxification.

CONCLUSION

In this present work, chemical composition, antifungal, insecticidal, and repellent actions of EO from *O. compactum* were investigated. In summary, the EO was discovered to be rich in carvacrol and thymol components, which have been remained the primary contributors to pharmacological activities. Consequently, the plant can be a promising source of natural agents with various applications and benefits in health, food, and agriculture. For safety reasons, there is a need to better understand the effect of sublethal dosages of EO on non-target organisms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the institutional ethical committee of care and use of the laboratory animals at the Faculty of Sciences Dhar El Mehraz, Sidi Mohamed Ben Abdallah Fes University, Morocco, reviewed and approved the present study # 04/2019/LBEAS.

AUTHOR CONTRIBUTIONS

AAi and EY: writing—original draft preparation. RS and AE: formal analysis. MB, AMS, AAl, HKA, H-AN, and NA: writing—reviewing and editing. LO and FM: supervision and data validation. All authors contributed to the article and approved the submitted version.

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Plant Extracts as Potential Acaricides for the Management of Red Spider Mite, *Oligonychus coffeae* Nietner (Acarina: Tetranychidae), in the Tea Ecosystem: An Eco-Friendly Strategy

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The effects of the application of aqueous extracts of a selection of five traditional plants (*Murraya paniculata*, *Cassia tora*, *Amphineuron opulentum*, *Tithonia diversifolia,* and *Cassia alata*) were compared with that of synthetic acaricide in reducing the population of red spider mite (*Oligonychus coffeae*), a major tea pest, alongside their impact on natural enemies and green leaf yield. Analysis of large-scale field trials showed that all the five plants extract treatments resulted in similar yield; this was analogous to the application of synthetic acaricide. A reduction in the pest population was observed to be on par with the synthetic acaricide, with a higher number of natural enemies treated using the pesticide-plant-treated plot in comparison to the synthetic acaricide-treated plot, which indicated pesticidal plants had a lower impact on natural enemies. A phytotoxicity study on tea leaves indicated that aqueous extracts of selected plants are non-phytotoxic and do not impart any taint to the prepared tea samples. Therefore, the present investigation outlines how plant extracts used as a botanical pesticide display toxicity against red spider mite on tea plants without harming the beneficial insects, increasing the yield and avoiding any lethal consequence for the tea plants or consumers.

Keywords: pesticidal plants, tea, red spider mite, phytotoxic effect, natural enemies

INTRODUCTION

Tea, *Camellia sinensis* (L.) O. Kuntze, is a perennial plantation crop and requires warm humid weather for ample growth and production. Such climate conditions also house a diverse range of insect pests and diseases that attack this crop, which turns them into a limiting factor for the production of tea (Hazarika et al., 2009; Majumder et al., 2012). The red spider mite, *Oligonychus coffeae* Nietner (Acarina: Tetranychidae), is among the foremost tea pests in India (Somchoudhury et al., 1995; Babu, 2010; Barua et al., 2016), and it causes the loss of up to 35–40% of the crop (Sundararaju and Sundara Babu, 1999; Hazarika et al., 2009). The tea plant's mature leaves are attacked by the veins and the mid-rib; finally, the whole leaf is affected. In cases of severe infestation, the tender foliage may also become damaged (Rau, 1965; Jeppson et al., 1975). The red spider mite feeds on the leaf epidermis by constantly puncturing it using their chelicerae (Jeppson et al., 1975; Babu, 2010). This pest remains active throughout the year, and unhindered infestation leads to

100% crop loss if appropriate management strategies are not suitably adopted. In tea-growing areas, planters use different synthetic acaricides to maintain this pest under the economic threshold level (ETL) of 4% (Gurusubramanian et al., 2008; Babu, 2010).

Continuous and non-judicial application of these chemical acaricides leads to certain undesirable issues, such as water pollution, degradation of valuable soil microbes, a decline in biological control agents, resurgence, development of resistance in pest, secondary pest outbreaks, and pesticide residues in manufactured tea (Cranham, 1966; Mobed et al., 1992; Roy et al., 2008, 2012; Hazarika et al., 2009; Babu, 2010). To overcome these problems, tea growers have attempted to find alternative forms of crop protection, which is essential to sustainable tea production. To develop ecologically safe pesticides, identification of toxins or antifeedants from plants is nowadays being highlighted as a prospective process (Wheller et al., 2001; Babu et al., 2004, 2008; Babu, 2010).

Botanicals play an important role in organic tea production and are considered substitute plant protection products, keeping the red spider mite population below the ETL between tea harvests (Babu et al., 2011). The continuous application of plant extracts of the same species for an extended period of time may decrease responses amongst herbivores (Liu et al., 2005). Alteration of plant species or mixing the different plant species used for plant extracts for pest control is therefore essential (Chen et al., 1995), as pest resistance is less likely to occur when using mixtures (Feng and Isman, 1995). If the plant materials are locally available, utilization of crude or raw plant extracts containing a blend of bioactive components is a simpler and more cost-effective approach. Aqueous extracts of different locally available plants in India (*viz. Clerodendron infortunatum, Acorus calamus, Aegle marmelos*, *Xanthium strumarium*, *Terminalia chebula, Duranta repens,* and neem kernel) have been evaluated against the red spider mite in tea, and these show varying degrees of control under field conditions (Babu et al., 2008; Roy et al., 2014, 2016). To ensure a broad choice of protectants, the selection of plants should not be a constrain to a limited choice of plants, and further exploration for other prospective plants is encouraged. It is much more important to conduct various types of bioassays during the screening of a botanical pesticides (Akhtar and Isman, 2004).

In this study, an investigation was undertaken to ascertain the bioefficacy of water extracts of a selected group of plants that commonly exist in the locality of tea-growing areas in northeast India against red spider mites. These plant species were selected because of their ample abundance around tea gardens, bushland, and roadsides, their familiarity to tea planters, and the substantial amount of accessible information that exists on their effectiveness and safety of use (Mollah and Islam, 2005, 2008; Roy et al., 2010; Deka et al., 2017; Green et al., 2017).

Analyses of the selected plant extracts and their effects on the eggs, oviposition deterrence, and adult mortality of red spider mites and *Chrysoperla carnea*, *Oxyopes javanus,* and *Stethorus gilvifrons,* the major predators of red spider mites (Das et al., 2010; Perumalsamy et al., 2010; Babu et al., 2011), were carried out under both laboratory and field conditions. This paper evaluates whether aqueous extracts of selected pesticidal plants have prospective use as biopesticides for the tea plant, and it also highlights the effects of using pesticidal plants.

MATERIALS AND METHODS

Insect Rearing

Different stages of red spider mites were collected from the Tea Research Association, North Bengal Regional Research and Development Centre (NBRRDC) experimental plot (88° 55′ 0″ East, 26° 54′ 0″ North longitude) West Bengal, India. Organic tea is cultivated without the application of synthetic pesticides, chemical fertilizers, and growth regulators. After field collection, these mites were transferred on to 1-year-old potted tea plants (clone TV 1) that were kept in a greenhouse at 26 ± 3°C, 78 ± 4% RH, and an 18L: 7D photoperiod, and they were used for raising the susceptible population without any type of exposure to pesticides. From this stock, red spider mite adults were transferred onto fresh tea leaves (6 cm^2) placed on moistened cotton pads (ca. 1.5 cm thick) in plastic trays (42 × 30 × 6.5 cm), which served as rearing chambers. Rearing chambers were kept under controlled conditions (26 ± 3°C, 78 ± 4% RH, and an 18L: 7D photoperiod) in a humidity chamber (Biojenik). Withered and dried leaves were regularly replaced. By adopting this technique, more than 30 generations of susceptible mite populations were maintained and utilized for carrying out the bioassays (**Figures 1A,B**).

Preparation of Plant Extracts

Fresh and full-grown leaves of *Murraya paniculata* (L.) Jack (Sapindales: Rutaceae), *Cassia tora* (L.) (Fabales: Fabaceae), *Amphineuron opulentum* (Kaulf.) Holt., (Polypodiales: Thelypteridaceae) *Tithonia diversifolia* (Hemsl.) A. Gray (Asterales: Asteraceae), and *Cassia alata* (L.) (Fabales: Fabaceae) were collected (**Figures 2A–E**) locally from nearby areas of NBRRDC, West Bengal. The plant materials (leaves, flower, and succulent stems) were shopped into fine pieces and allowed to dry in the shade. The dried materials were ground using an electric dicer, and they were later passed through a 30 mesh sieve and finally reserved in a 2 kg capability airtight glass jar. The aqueous extract of each selected plant was prepared by following the method of Nagappan (2012). To prepare different concentrations, *viz.,* 2, 4, 6, 8, and 10 gm/l, different quantities of the powder, *viz.,* 20, 40, 60, 80, and 100 gm, were weighed individually into plastic containers containing 1 L of water and then kept for 24 h. The water extracts of each concentration were filtered using a muslin cloth, labeled, and organized for application. All of the spray fluids including control were mixed with 0.1% Teepol AG as an emulsifier.

Acaricidal Activity of Pesticidal Plant Extracts (Leaf Disc Experiment)

Tea leaves collected from the NBRRDC experimental plot were cut into discs 2 cm in diameter. The leaf discs were positioned on soaked cotton set aside in a petri dish, and throughout the study, the wetness in the cotton was prolonged by wetting it using water regularly. A total of 20 red spider mites that were 24 h old

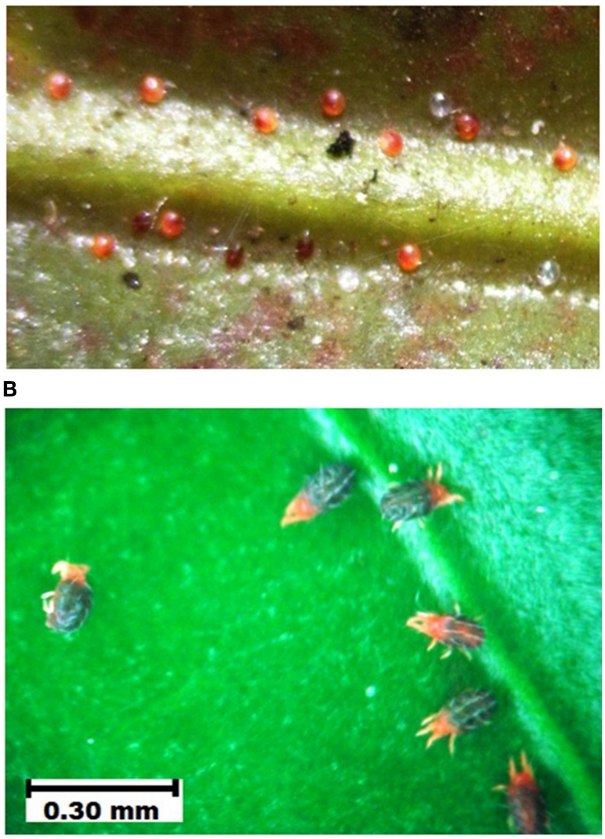

FIGURE 1 | Tea red spider mites (**A**: eggs, **B**: adults).

were consigned from the stock culture onto each leaf disc. Using a glass atomizer, the spray fluid of the prepared plant extract concentrations were sprayed onto each leaf disc. To ensure the fine droplets fell onto the leaf disc, the distance between the leaf disc and the atomizer was set as constant (1 ft). A control (distilled water mixed with teepol) and Fenpyroximate 5EC (Mitigate) (dose: 0.5 ml/L) were sprayed along with the different concentrations of extracts. The experiment was replicated 30 times. Following the treatments, observations were recorded after 24, 48, and 72 h, and the total numbers of live mites were recorded with a compound microscope at a specified 10X magnification. The percentage mortality of mites was analyzed statistically through analysis of variance to calculate the critical difference (CD at $p = 0.05$; Snedecor and Cochran, 1989). After significant effects were identified, differences between means were considered significant at a 95% confidence interval based on Tukey's *post-hoc* Honestly Significant Difference (HSD) test to separate the means.

Ovicidal Action of Pesticidal Plant Extracts

To evaluate the ovicidal action of the selected plant extracts, 15 adult female mites were introduced on a full-grown tea leaf (leaf number four or five from the apex of the tea bush) to lay eggs, and they were kept in a petri dish overnight. To maintain the moisture conditions in the leaves, water-soaked cotton was padded in around the leaves. After hatching the eggs, the mites were removed using a fine camel-hair brush. In each leaf, exactly 30 eggs were chosen for the bioassay; the remaining eggs were carefully detached using a fine needle. For the bioassay, we used 30 eggs per experiment where these were assessed with different concentrations of the pesticidal plant extracts, *viz.*, 2, 4, 6, 8, and 10 gm/l (w/v) sprayed with a fabricated atomizer (made with glass, size: 100 ml and pressure: 30 psi). A control (distilled water mixed with teepol) and Fenpyroximate 5EC (dose: 0.5 ml/L) were sprayed along with the different concentrations of extracts. The experiment was repeated 30 times. After oviposition, the hatchability of the egg was recorded for both control and experimental batches for a period of 12 days. After 12 days, if the eggs did not hatch, the eggs were considered non-feasible (Sarmah et al., 1999). The egg mortality (%) was analyzed statistically through analysis of variance to calculate the critical difference (CD at $P = 0.05$; Snedecor and Cochran, 1989).

Ovipositional Deterrent and Repellent Activity of Pesticidal Plant Extracts

The ovipositional deterrent and repellent effects of each of the pesticidal plant extracts on adult mites were tested using the choice test method (Roy et al., 2016). The adaxial surface of each leaf disc (2 cm diameter) was positioned facing upward in a petri dish. Using a camel-hair brush, 50% of the leaf disc (either side from the midrib) was painted with each of the tested concentrations from each of the plant extracts (2, 4, 6, 8, and 10 gm/l) and another 50% of the leaf disc was painted with 0.1% soap water (considered as control) and allowed to dry. After drying, 20 gravid red spider mite females were positioned on the center of each leaf disc. After 24 h, the mites left on the treated area were considered as repelled, and the eggs laid on both halves of the leaf disc were recorded up to 4 days after treatment. The experiment was replicated 30 times. Using the following formula, the Discrimination Quotient (DQ) was calculated (Roobakkumar et al., 2010):

$$DQ = [(C - T)/(C + T)]$$

[C: eggs (in number) laid on the control area, T: eggs (in number) laid on the treated area. DQ shows a range (0–1), which is an indication of a conclusion showing the consequence of any treatments on the insect's ovipositional behavior].

Large Scale Field Study of Pesticidal Plant Extracts

To evaluate the effectiveness of each selected plant extract against red spider mites in tea plants, this study was conducted at field sites for two consecutive seasons in tea gardens representing two different geographical locations, *viz.*, Zone A: Mission Hill Tea Garden, Darjeeling (27.1° 94′ 5″ N, 87° 17′ 43″ E longitude) and Zone B: the experimental plot at the NBRRDC, Dooars (26.1° 55′ 0″ N, 88.1° 56′ 0″ E longitude) West Bengal, India. The Mission Hill Tea Garden location was at an elevation of 235 m above MSL with a mean maximum temperature of 30^0

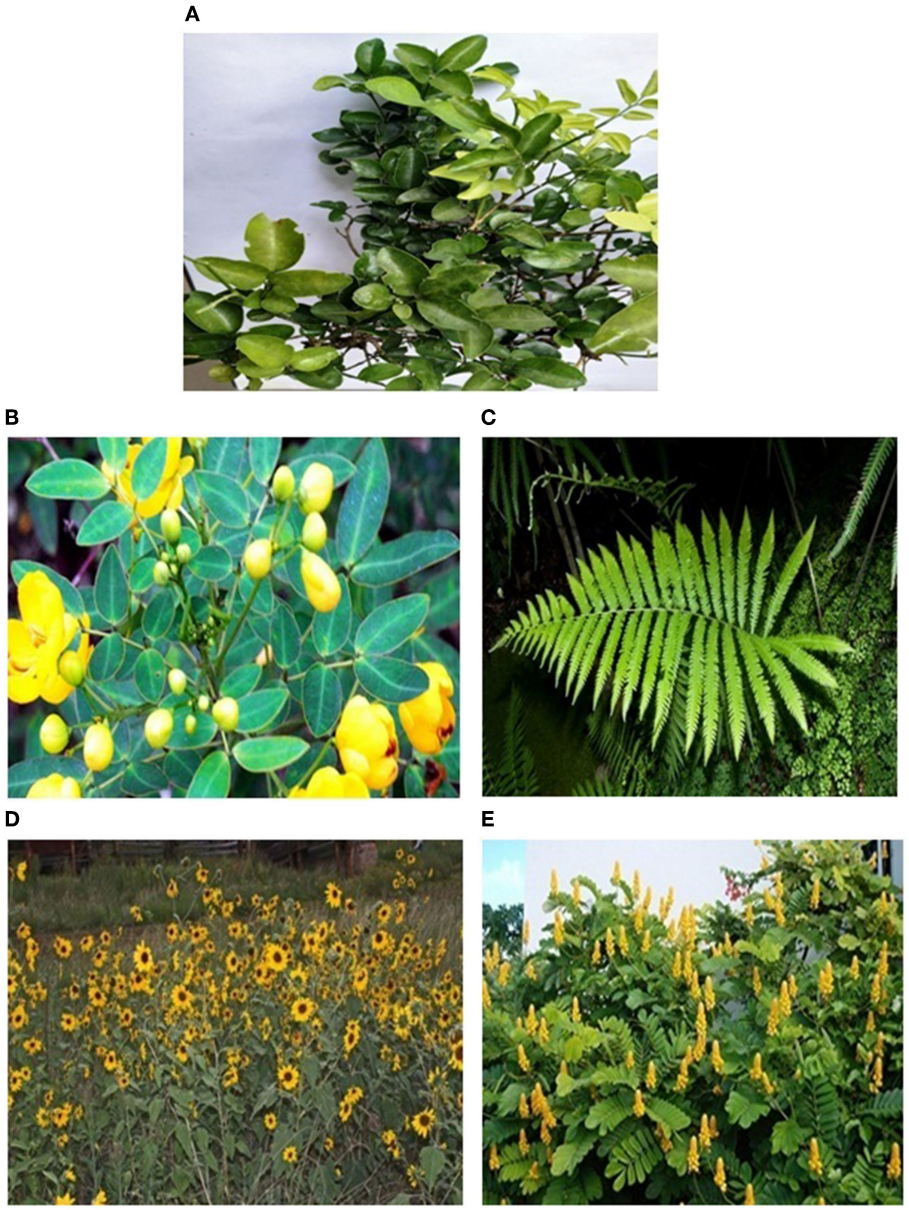

FIGURE 2 | Pesticidal plants evaluated against tea red spider mite. **(A)** *M. paniculata,* **(B)** *C. tora*, **(C)** *A. opulentum*, **(D)** *T. diversifolia*, and **(E)** *C. alata* (source: North Bengal Regional R and D Centre, Tea Research Association, West Bengal, India).

and mean minimum temperature of 6°C, with a mean annual rainfall of 1,300 mm; in the North Bengal Regional R&D Centre experimental plot, the location was at an elevation of 214 m above MSL with a mean maximum temperature of 36° and mean minimum temperature of 10°C and a mean annual rainfall of 1,200 mm. The tea gardens where field trials took place were more than 50 years old. Sections of gardens were planted with mixed clones (mostly TV1, Teenali 17, S3A3, TV25, and TV26). Each experiment was conducted in a randomized block design (RBD) that was replicated for five blocks (Anderson and McLean, 1974). Including the untreated control, each treated plot consisted of 100 bushes that were separated by three buffer rows (8 m spacing). A preliminary study using different doses (*viz.,* 2, 4, 6, 8, and 10 g/l w/v) of plant extract against red spider mite shows that 10 g/l showed better efficacy against this pest. Therefore, a dose of 10 g/l (40 kg/ha; 400 L of spray fluid is required to cover a one-hectare area of the tea plantation) was considered for further field study. In all the trials, the synthetic acaricide Fenpyroximate 5EC, which was applied as per the central insecticide board's (CIB) instructions (@ 200 ml/ha), was used as a positive control, and water was used as a negative control.

Observations of the red spider mite population were recorded from both surfaces (adaxial and abaxial) of randomly collected tea leaves from each treated and control plot (Babu et al., 2008). Before spraying, 100 leaves were randomly collected from each treatment, and the red spider mite population there was counted for pretreatment assessment. Using a hand-operated knapsack sprayer (droplet diameter: 1.6 mm, hollow cone NMD 60450 nozzle, discharge rate: 450 ml/min at 40 psi pressure, and droplet size: 140 mL), spraying was carried out, the bushes were drenched for better coverage and control. Prior to being re-filled with another formulation for application, the spraying machine was cleaned thoroughly with soap and water. Similar to the pre-treatment assessment, post-treatment assessments were also carried out at 7-day intervals up to the fourth week, and, accordingly, the decrease in mites (mean) during each treatment was analyzed with the following formula:

$$\% \text{ decrease of red spider mite} = \left(\frac{(X - Y)}{X} \times 100\right)$$

Here, X indicates the pre-treatment population count and Y the post-treatment population count.

All the data were angularly transformed prior to the statistical analysis. The differences among the population density of mites before and after each treatment were assessed through Tukey's *post-hoc* Honestly Significant Difference (HSD) test to separate the means at a 95% confidence interval, and the critical difference (CD, $p = 0.05$) was analyzed accordingly (Snedecor and Cochran, 1989).

Sampling for the Presence of Non-target Beneficial Insects

To find out the effects of pesticidal plant extracts on non-target beneficial insects, a direct spray test was conducted by following the methodology of Leatemia and Isman (2004). For the bioassay, the study was conducted for two consecutive seasons under field conditions in tea gardens representing two different geographical locations, *viz.*, Zone A: Mission Hill Tea Garden, Darjeeling and Zone B: the experimental plot at the NBRRDC, Dooars West Bengal, India. Each of the plant extracts were sprayed (dose: 4 kg/ha) with Fenpyroximate 5EC (@ 200 ml/ha) and a control (distilled water mixed with teepol). The populations of non-target beneficial organisms like *C. carnea*, *O. javanus,* and *S. gilvifrons* were recorded on day 0 (pre-spray) and day 21 (post-spray; a total of two rounds of spraying was carried out; the first spray on day 0 and the second spray on day 7). Visual observations were made from 30 randomly selected bushes per replication to estimate the population of *C. carnea* nymphs and *O. javanus* adults. A total of 50 leaves were collected at random per replication, and these were observed under a binocular microscope to estimate the larval population of *S. gilvifrons*.

Phytotoxicity, Tainting, and Organoleptic Tests

To evaluate the phytotoxic effect of each plant extract (at X, 2X, and 4X concentrations) on tea leaves, a field experiment was carried out. Four treatments were used: (1) 10 gm/l, (2) 20 gm/l, (3) 40 gm/l of water, and (4) a control (water spray), and three replications were maintained at 84 square meters per replication. Similar to the experiment large scale field study, spraying was carried out, and observations were recorded on day 0 (pre-treatment) and days 3, 7, and 14 (post-treatment) on yellowing, stunting, necrosis, epinasty, hyponasty etc. The injury levels were graded with the following Phytotoxicity Rating Scale (PRS):

Leaf injury	PRS
0–00	0
1–10%	1
11–20%	2
21–30%	3
31–40%	4
41–50%	5
51–60%	6
61–70%	7
71–80%	8
81–90%	9
91–100%	10

To find out whether the pesticidal plant extracts resulted in any taint (odor and foreign taste) to the black tea, on days 7 and 14 after spraying, tea shoots were harvested and processed in a mini CTC (crush, tear, curl) machine. The prepared samples of tea were assessed by a professional tea taster to check whether there was a taint or not. For the organoleptic test, liquor strength and leaf infusions were considered and scored (1–2: poor quality, 3–5: moderate quality, 6–8: good quality, and 10: very good quality; Roy et al., 2014).

Effect of Pesticidal Plant Extracts on Green Leaf Yield

In order to find out the effects of aqueous plant extracts on reducing the mite infestation, the yield (green leaf) was also recorded during the bioassay carried out in the field conditions by maintaining a standard plucking round of 7-day intervals, and the yield (green tea leaf) was recorded for the first six rounds of plucking. The average yield was expressed in kg/plot. The yield recorded at each plucking was converted into made tea for one hectare as described by Ponmurugan and Baby (2007) using the formula

green leaf (kg.) $\times$no. of bushes/ha $\times$ Conversion Factor (0.225).

RESULTS

Acaricidal Activity of Pesticidal Plant Extracts (Leaf Disc Experiment)

Findings of the leaf disc experiment indicated that, amongst the five plant extracts, *T. diversifolia* and *C. alata* @ 10 gm/l concentrations were more effective than the other three plants even after 24 h of treatment, showing maximum mortality (**Table 1**). After 72 h, both plants offered more than 80% mortality at 10 g/l concentration, which was similar to the

TABLE 1 | Acaricidal activity of the pesticidal plant extracts (leaf disc experiment).

Treatment	Concentration	Percent mortality after (mean ± SE)*		
		24 h	48 h	72 h
M. paniculata	2 gm/l	11.3 ± 1.10^{b}	17.2 ± 2.32^{b}	19.7 ± 3.11^{b}
	4 gm/l	12.0 ± 2.15^{b}	16.2 ± 2.16^{b}	22.6 ± 2.15^{c}
	6 gm/l	22.0 ± 2.23^{c}	27.4 ± 3.25^{c}	29.5 ± 3.21^{c}
	8 gm/l	38.2 ± 3.14^{d}	42.5 ± 4.15^{e}	49.0 ± 4.15^{e}
	10 gm/l	47.4 ± 5.14^{e}	48.0 ± 4.25^{e}	67.0 ± 5.15
C. tora	2 gm/l	9.0 ± 1.12^{b}	16.5 ± 1.56^{b}	19.0 ± 2.14^{b}
	4 gm/l	12.8 ± 2.12^{b}	17.8 ± 2.14^{b}	19.0 ± 3.14^{b}
	6 gm/l	23.6 ± 3.21^{c}	27.7 ± 3.25^{c}	29.4 ± 3.26^{c}
	8 gm/l	32.5 ± 3.25^{d}	36.8 ± 3.24^{d}	39.5 ± 3.36^{d}
	10 gm/l	44.7 ± 4.15^{e}	48.3 ± 4.15^{e}	52.5 ± 5.45^{f}
A. opulentum	2 gm/l	9.2 ± 1.12^{b}	12.2 ± 2.15^{b}	16.0 ± 2.56^{b}
	4 gm/l	11.4 ± 1.12^{b}	15.2 ± 2.13^{b}	19.4 ± 3.15^{b}
	6 gm/l	34.2 ± 3.21^{d}	42.0 ± 4.56^{e}	48.2 ± 5.15^{e}
	8 gm/l	43.5 ± 5.14^{e}	49.0 ± 5.51^{e}	56.6 ± 6.15^{f}
	10 gm/l	48.0 ± 6.15^{e}	59.0 ± 6.15^{f}	70.0 ± 7.45^{g}
T. diversifolia	2 gm/l	42.4 ± 4.16^{e}	48.7 ± 5.24^{e}	55.5 ± 4.56
	4 gm/l	46.4 ± 4.16^{e}	52.5 ± 4.56^{f}	62.7 ± 6.15^{g}
	6 gm/l	69.7 ± 6.24^{g}	70.0 ± 7.45^{g}	72.6 ± 7.18^{h}
	8 gm/l	72.6 ± 7.15^{h}	75.0 ± 7.21^{h}	79.6 ± 8.84^{h}
	10 gm/l	75.4 ± 8.15^{h}	79.0 ± 8.45^{h}	82.0 ± 8.78^{i}
C. alata	2 gm/l	40.7 ± 4.45^{d}	45.4 ± 4.16^{e}	50.0 ± 4.16
	4 gm/l	45.5 ± 6.18^{e}	49.0 ± 4.15^{e}	52.0 ± 5.65
	6 gm/l	67.4 ± 7.45^{g}	69.0 ± 6.78^{g}	75.7 ± 6.54^{h}
	8 gm/l	69.6 ± 8.52^{g}	72.5 ± 8.15^{h}	75.6 ± 8.48^{h}
	10 gm/l	75.5 ± 9.15^{h}	78.7 ± 9.18^{h}	81.0 ± 8.53^{i}
Fenpyroximate 5EC	0.5 ml/L	78.4 ± 3.33^{h}	81.6 ± 3.45^{i}	86.5 ± 3.67^{i}
Control (water + 0.1% soap)-		0.0 ± 0.00^{a}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}
F-value		8.4	9.5	10.2
ANOVA *P*-value		<0.0001	<0.0001	<0.0001
CD ($P = 0.05$)		8.27	8.34	9.23
C.V.%		10.12	10.14	10.83

All the spray fluids were mixed with Teepol (0.1%).

**Values represent the mean of 30 observations ± SE (20 mites/observation); Values in the same column with different superscript are significantly different from each other at $p < 0.05$ by Tukey's post-hoc Honestly Significant Difference (HSD) test.*

synthetic acaricide Fenpyroximate 5EC, while in the other three plants, 52–70% mortality of the red spider mite was recorded.

Ovicidal Action of Pesticidal Plant Extracts

Ovicidal action of the extracts was reliant on concentrations, i.e., percent mortality increased with a rising concentration of each plant extract. In comparison to the control, the aqueous extract of all of the selected plants affected the hatchability of the eggs. The experimental findings showed that the highest mortality was recorded against *T. diversifolia* at the highest concentration (10 gm/l) among the tested concentrations of the selected plant extract to the level of 66.7%, followed by 58.4, 57.4, and 48.5% egg mortality, which was recorded in highest concentrations against *C. alata*, *A. opulentum,* and *M. paniculata*, respectively. This is comparable to the market sample Fenpyroximate 5EC, where around 70% egg mortality was recorded. The lowest mortality against the highest concentrations was recorded against *C. tora* (34.15%) (**Table 2**).

Ovipositional Deterrent and Repellent Activity of Pesticidal Plant Extracts

The number of eggs laid was considerably lower on the treated side of the leaf disc where plant extracts (2, 4, 6, 8, and 10 gm/l) were sprayed than on the control side. Regarding the number of eggs laid, adult mites showed discrimination in terms of their egg laying when the leaves were treated with the pesticidal plant extracts. In this experiment, the DQ value was high (range 0.432–0.819) in all tested plants at superior concentrations, i.e., 10 g/extract. The DQ value was highest (0.819) in *T. diversifolia,* followed by *C. alata, A. opulentum, M. paniculata,* and *C. tora,* respectively (**Table 3**).

TABLE 2 | Ovicidal action of pesticidal plant extracts on eggs of the tea red spider mite.

Treatments	Concentration	Percent egg mortality (mean ± SE)*
M. paniculata	2 gm/l	10.13 ± 2.21
	4 gm/l	12.90 ± 2.11
	6 gm/l	13.82 ± 2.35
	8 gm/l	34.21 ± 5.14
	10 gm/l	48.51 ± 6.15
C. tora	2 gm/l	6.67 ± 1.13
	4 gm/l	7.14 ± 1.61
	6 gm/l	10.13 ± 2.21
	8 gm/l	25.11 ± 4.11
	10 gm/l	34.15 ± 6.21
A. opulentum	2 gm/l	13.3 ± 2.31
	4 gm/l	23.22 ± 5.15
	6 gm/l	26.32 ± 6.62
	8 gm/l	53.24 ± 7.24
	10 gm/l	57.45 ± 8.15
T. diversifolia	2 gm/l	15.32 ± 3.51
	4 gm/l	25.41 ± 5.14
	6 gm/l	34.15 ± 6.21
	8 gm/l	45.25 ± 6.15
	10 gm/l	66.71 ± 8.15
C. alata	2 gm/l	14.11 ±3.22
	4 gm/l	22.18 ± 3.26
	6 gm/l	34.15 ± 6.21
	8 gm/l	46.5 ± 6.15
	10 gm/l	58.4 ± 5.14
Fenpyroximate 5EC	0.5 ml/L	70.00 ± 3.33
Control (water + 0.1% soap)	–	0.0 ± 0.00
CD at ($P = 0.05$)		7.17
CV (%)		8.01

*All the spray fluids were mixed with Teepol (0.1%). *Values represent the mean of 30 observations ± SE (30 eggs/observation).*

Large-Scale Field Study

The field evaluation results of each selected plant extract against red spider mite at Zone A (Mission Hill Tea Garden, Darjeeling) and Zone B (NBRRDC experimental plot, Dooars) during season I and season II are presented in **Table 4**.

In Zone A, during season I, pre-treatment observation of the red spider mite population ranged from 23.72 to 25.02%. Results indicated that all the treatments were found to be significantly superior to untreated checks in minimizing the population of mites (**Table 4**). The mean percent reductions in the red spider mite population in the cases of *A. opulentum* (67.03%) and *T. diversifolia* (69.77%) at doses of 4 kg /ha were similar to Fenpyroximate 5EC (68.16%). The reduction in the mean red spider mite population was slightly inferior in plots treated with *M. paniculata* (50.85%), *C. tora* (59.40%), and *C. alata* (53.46%) compared to plots treated with Fenpyroximate 5EC.

During season II also, both *A. opulentum* (63.06%) and *T. diversifolia* (64.93%) minimized the mite population in a manner similar to the Fenpyroximate 5EC (62.84%), which were significantly superior when compared to *M. paniculata* (48.97%), *C. tora* (57.48%), and *C. alata* (51.17%; **Table 4**).

In Zone B, during season I, the incidence of red spider mite infestation was significantly reduced in plots treated with various insecticides as compared to untreated control (**Table 4**). Among all the tested pesticidal plants, *T. diversifolia* (71.66%) and *A. opulentum* (71.03%) significantly reduce the number of red spider mites followed by *C. tora* (62.10%), *C. alata* (54.74%), and *M. paniculata* (52.80%). However, there was no significant difference in mean percent control of red spider mite among *C. tora, A. opulentum, T. diversifolia,* and Fenpyroximate 5% EC after 21 days. Similar to season I, during season II, the plots treated with pesticidal plant extracts were found to be the most effective treatments for the management of red spider mites. While comparing the overall mean data recorded during the study period, the mean infestation incidence was significantly on par in plots treated *T. diversifolia* and *A. opulentum* at 4 kg/ha and Fenpyroximate 5% EC at 200 ml/ha (**Table 4**). Treatments using *C. tora, C. alata,* and *M. paniculata* extracts were found to be inferior to treatments using Fenpyroximate 5% EC at 200 ml/ha but superior to untreated checks in reducing the mean percent reduction of the red spider mite.

Effects on Non-target Beneficial Insects

The population level of non-target beneficial organisms was recorded on day 0 (pre-treatment) and day 21 after two rounds of spray (post-treatment). The most common insect predators in the tea plant ecosystem, *viz., C. carnea*, *O. javanus,* and *S. gilvifrons,* were recorded during the trial period. The present results indicate that the tested pesticidal plants did not have any adverse effect on these three non-target beneficial organisms (**Figures 3A,B**). The numbers of beneficial insects in tea plants treated with plants extracts were similar to that noticed in the control. In both the seasons, among all the treatments, the synthetic acaricide (Fenpyroximate 5EC) significantly reduced numbers of non-target beneficial insects compared to all other treatments ($P < 0.05$; **Figures 3A,B**).

Phytotoxic Effect, Tainting, and Organoleptic Test

The phytotoxicity study was carried out separately after the application of aqueous extracts of selected plants at 10, 20, and 40 gm/l water. Observations recorded on phytotoxicity symptoms indicate that none of the concentrations showed any type of phytotoxic effect on tea leaves. There was no visible injury on the tip or the surface of the tea leaves, nor was there wilting, necrosis, vein clearing, hyponasty, or epinasty. Likewise, after the application of pesticidal plant extracts, tea shoots (two leaves and a bud) were plucked on days 1, 3, 5, 7, 10, and 14, and manufactured samples were tested by professional tea tasters which revealed that the made tea did not show any taint and scored 6.5–7 in organoleptic test, which represented excellent color, liquor, quality, and potency.

TABLE 3 | The percentage of female red spider mites that remained on the leaves where introduced and the numbers of eggs oviposited on the control (untreated) or treated tea leaves with pesticidal plant extracts in choice tests.

Treatments	Concentration (gm/L)	% of adults moved on to leaves after 24 h			Avg. no of eggs/female after 72h			
		Control (mean ± SD)	Treated (mean ± SD)	*p*-value	Treated (mean ± SD)	Control (mean ± SD)	*p*-value	DQ value#
M. paniculata	2	51 ± 3.12	49 ± 3.11	0.6416	3.36 ± 0.56	3.21 ± 0.87	0.5684	0.032
	4	61 ± 3.43	39 ± 3.47	<0.0001*	2.98 ± 0.23	3.98 ± 0.45	<0.0001*	0.106
	6	64 ± 3.56	36 ± 3.67	<0.0001*	2.05 ± 0.45	4.45 ± 0.67	<0.0001*	0.201
	8	73 ± 3.55	27 ± 4.34	<0.0001*	1.98 ± 0.11	4.98 ± 0.78	<0.0001*	0.401
	10	83 ± 4.25	17 ± 2.11	<0.0001*	1.01 ± 0.12	5.67 ± 0.89	<0.0001*	0.512
C. tora	2	49 ± 2.23	51 ± 4.23	0.7856	2.76 ± 0.23	2.32 ± 0.34	0.5387	0.023
	4	61 ± 3.19	39 ± 3.45	<0.0001*	2.45 ± 0.12	2.56 ± 0.36	<0.0001*	0.101
	6	64 ± 4.16	36 ± 3.31	<0.0001*	1.95 ± 0.11	3.54 ± 0.56	<0.0001*	0.198
	8	73 ± 4.23	27 ± 2.11	<0.0001*	1.43 ± 0.11	3.78 ± 0.45	<0.0001*	0.345
	10	82 ± 5.11	18 ± 2.18	<0.0001*	0.95 ± 0.01	4.56 ± 0.68	<0.0001*	0.432
A. opulentum	2	52 ± 3.56	48 ± 2.22	0.6534	4.00 ± 0.56	4.34 ± 0.34	0.5138	0.042
	4	62 ± 3.67	38 ± 2.12	<0.0001*	3.96 ± 0.43	4.74 ± 0.45	<0.0001*	0.135
	6	64 ± 4.55	36 ± 3.11	<0.0001*	3.01 ± 0.23	5.21 ± 0.32	<0.0001*	0.224
	8	74 ± 4.78	26 ± 2.45	<0.0001*	2.29 ± 0.21	5.72 ± 0.57	<0.0001*	0.427
	10	84 ± 4.54	16 ± 1.12	<0.0001*	1.43 ± 0.07	6.07 ± 0.67	<0.0001*	0.618
T. diversifolia	2	56 ± 3.11	44 ± 3.11	0.0023	6.00 ± 0.67	6.32 ± 0.45	0.5467	0.061
	4	66 ± 3.29	34 ± 3.10	<0.0001*	5.58 ± 0.56	6.47 ± 0.56	<0.0001*	0.336
	6	70 ± 3.45	30 ± 4.12	<0.0001*	5.02 ± 0.54	7.34 ± 0.67	<0.0001*	0.423
	8	78 ± 4.18	22 ± 2.15	<0.0001*	4.12 ± 0.34	7.89 ± 0.78	<0.0001*	0.628
	10	90 ± 5.12	10 ± 1.13	<0.0001*	3.41 ± 0.32	8.98 ± 0.89	<0.0001*	0.819
C. alata	2	54 ± 2.45	46 ± 2.12	<0.0001*	5.01 ± 0.45	5.23 ± 0.32	0.5678	0.051
	4	64 ± 3.21	36 ± 2.45	<0.0001*	4.48 ± 0.43	5.44 ± 0.54	<0.0001*	0.225
	6	68 ± 4.56	32 ± 3.34	<0.0001*	4.01 ± 0.23	6.32 ± 0.67	<0.0001*	0.312
	8	76 ± 4.78	24 ± 2.23	<0.0001*	3.11 ± 0.21	6.56 ± 0.76	<0.0001*	0.512
	10	88 ± 5.12	12 ± 2.12	<0.0001*	2.12 ± 0.04	7.89 ± 0.89	<0.0001*	0.712

All the spray fluids were mixed with Teepol (0.1%).

**Means are significantly different between treated and untreated (control) by t-test (mean ± SD, $p \leq 0.05$).*

#DQ, discrimination quotient.

Effect of Pesticidal Plant Extracts on Green Leaf Yield

The average yield recorded from the first six pluckings (at 7-day intervals) from both the experimental locations during the trial period is presented in **Table 4**. In spite of there being more insect pest populations on tea plants being treated with selected pesticides than with the synthetic pesticides, harvested yields acquired from the pesticidal plant treatments were shown to be as good as those from the synthetic pesticides. The yield (green leaf in kg/plot) was significantly low in the untreated control, confirming the better efficacy of the different tested plant extracts (**Table 4**). This is most prominent with the use of *T. diversifolia* and *A. opulentum* where the yields were statistically comparable in zone A (2,184 and 2,179 kg/ha, respectively) and zone B (2,106 and 2,102 kg/ha, respectively) with Fenpyroximate 5EC (2,195 kg/ha in zone A and 2,112 kg/ha in zone B) and was statistically higher when compared to the control (2,038 kg/ha in zone A and 1,951 kg/ha in zone B). The synthetic pesticides (Fenpyroximate 5EC) showed the highest increase in yield in both locations. In the case of other pesticidal plants, *viz., M. paniculata, C. tora,* and *C. alata,* the yields were slightly lower (not statistically significant) than with *T. diversifolia, A. opulentum,* and Fenpyroximate 5EC in both the locations. In comparison to the yields recorded in the control plot, the pesticidal plant treatments showed an ability to considerably enhance the percentage of the yield.

DISCUSSION

Earlier works on the pesticidal plant species that are being used to control different tea pests have reported bioactivities against insects, bacteria, fungi, and parasites (Sarmah et al., 1999; Singh, 2000; Weinzierl, 2000; Raja et al., 2003; Isman, 2006; Roy et al., 2010, 2011; Roy and Mukhopadhyay, 2012; Vasanthakumar et al., 2012; Deka et al., 2017). Conversely, none of these works have dealt with the studies on the impact on field crop performance or tri-trophic interactions.

The findings obtained from the leaf disc experiment show that all of the selected plant extracts showed acaricidal activity where 52–82% mortality of adult red spider mites were recorded with the highest dose (10 g/l) after 72 h (**Table 1**). Similar properties

TABLE 4 | Efficacy of pesticidal plant extracts on red spider mite in field conditions and effect on yield.

Treatment details	Dose/ha	Zone A					Zone B				
		Season I		Season II			Season I		Season II		
		Pre-spray population (mites per leaf)	Mean % reduction of mite population after week 4	Pre-spray population	Mean % reduction of mite population	*Made tea (kg/ha/year)	Pre-spray population	Mean % reduction of mite population	Pre-spray population	Mean % reduction of mite population	*Made tea (kg/ha/year)
M. paniculata	4 kg	23.79 (28.85)	50.85^{c} (45.04)	16.60 (23.79)	48.97^{c} (44.78)	2,138^{c} ± 7.11	24.47 (31.30)	52.80^{c} (46.18)	18.18 (24.92)	46.89^{c} (42.72)	2,055^{c}± 6.33
C. tora	4 kg	24.34 (29.21)	59.40^{b} (50.06)	16.84 (23.98)	57.48^{b} (47.74)	2,159 ^{b} ± 6.23	24.67 (30.42)	62.10^{b} (51.66)	18.04 (24.86)	57.04^{b} (48.64)	2,091^{b}± 5.31
A. opulentum	4 kg	23.72 (28.80)	67.03^{a} (54.74)	17.31 (24.32)	63.06^{a} (52.30)	2,179^{a} ± 5.21	25.21 (31.78)	71.03^{a} (55.98)	17.18 (24.20)	65.01^{a} (53.54)	2,102^{a}± 6.36
T. diversifolia	4 kg	25.02 (29.66)	69.77^{a} (56.46)	17.09 (24.58)	64.93^{a} (53.45)	2,184^{a} ± 8.22	24.18 (31.95)	71.66^{a} (57.68)	18.18 (24.96)	66.77^{a} (54.61)	2,106^{a}± 7.23
C. alata	4 kg	24.29 (29.18)	53.46^{bc} (46.56)	17.66 (24.38)	51.17^{bc} (44.06)	2,117^{d} ± 9.21	25.47 (30.30)	54.74^{c} (47.30)	15.27 (22.76)	51.53^{bc} (44.27)	2,046^{c}± 7.34
Fenpyroximate 5EC	200 ml	24.73 (29.47)	68.16^{a} (54.55)	17.39 (24.64)	62.84^{a} (52.06)	2,195^{a} ± 7.11	24.71 (32.46)	68.66^{ab} (55.63)	16.46 (23.68)	64.21^{ab} (52.90)	2,112^{a}± 7.01
Control	–	24.42 (29.26)	0.00^{d} (0.00)	17.74 (25.05)	0.00^{d} (0.00)	2,038^{e} ± 6.12	27.52 (31.98)	0.00^{d} (0.00)	17.07 (24.11)	0.00 (0.00)	1,951^{d}± 6.01
CD	–	N/A	4.28	N/A	4.09	0.23	N/A	4.19	N/A	4.77	0.25

All the spray fluids were mixed with Teepol (0.1%).

Figures in parenthesis are angular transformed values; Values in the same column with different superscripts are significantly different with each other at $p < 0.05$ by Tukey's post-hoc Honestly Significant Difference (HSD) test.

**Mean of 10 replications obtained from six rounds of leaf plucking.*

of indigenous plant extracts *Linostoma decandrum, Ageratum houstonianum, A. haustonianum Bidens pilosa, Allamanda catharitica, B. pilosa, Crassocephalum crepidioides, Casuarina equisetifolia, Gliricidia sepium, Conyza bonariensis, Lantana camara,* and *Ocimum basilicum*, which were recorded by Bora et al. (1998) and Radhakrishnan and Prabhakaran (2014). Pesticidal plants as insecticides (botanical insecticides) are gaining importance since numerous plants show insecticidal properties. Currently, botanical insecticides comprise only 1% of the world insecticide trade, however, its annual sales growth in near about 15% is utterly promising (Wink, 1993). In the house and backyard sector, the impact of botanical insecticides is most prominent (Isman, 1995). Plant products that have prospective use as insecticidal compounds have gained astonishing importance in recent years (Nattudurai et al., 2015). Therefore, based on the finding of our laboratory studies, large-scale field trials were carried out using the extracts of the same plants.

The aqueous extracts of different plants were sprayed on the eggs of red spider mite, and all the pesticidal plants influenced the hatchability of the eggs and showed the ovicidal activity of the extracts and at higher concentrations, all of which was an effect similar to that of synthetic acaricides. Chemical substances present in the host plants possibly block the micropyle region of the egg, preventing the gaseous exchanges that finally destroy the embryo in the egg. Raja et al. (2003) screened nine different plants using different solvent extracts against *Spodoptera litura* as ovicidal and ovipositional deterrents, and they noticed efficacy irrespective of the solvents and concentrations used for the extraction. The curtailed blastokinesis and anomalous rupture of additional embryonic membranes in the embryo, or irregular diffusion of extracts through the egg chorion to remarkable parts of eggs at unusual times of the perceptive time, could also be related to an explanation of the inconsistency of morphological possessions (Slama, 1974). Vasanthakumar et al. (2012) studied the acaricidal action of the leaf extracts of *Gliricidia maculate, Vitex negundo, Wedelia chinensis, Pongamia glabra,* and *Morinda tinctoria* on red spider mites and reported that the aqueous extracts of *P. glabra* and *M. tinctoria* showed utmost ovicidal activity, ovipositional anticipation, and 100% adult mortality. The current findings also showed similar results with reference to the ovicidal action of the selected pesticidal plant extracts against the red spider mites.

Along with the ovicidal and adulticidal properties, the aqueous extracts of five selected pesticidal plants also indicated a repellency action against adult red spider mites. This indicates the selected pesticidal plants have repellent effects that depress the feeding behavior and movement of the mites. Roy et al. (2016) and Handique et al. (2017) also reported similar activity when they sprayed leaf extracts of *D. repens, Nyctanthes arbor-tristis, Phlogacanthus thyrsiformis,* and *Sapindus mukorossi* against red spider mites. Similar to the red spider mites on tea, the repellent properties of different plant extracts were studied and established against different pests, *viz., A. aegypti* (Yang et al., 2004), *Culex tritaeniorhynchus* (Karunamoorthi et al., 2008), and whitefly, *Bemisia tabaci* (Al-mazra'awi and Ateyyat, 2009). The aqueous extracts of *M. paniculata*, *C. tora*, *A. opulentum*, *T. diversifolia,*

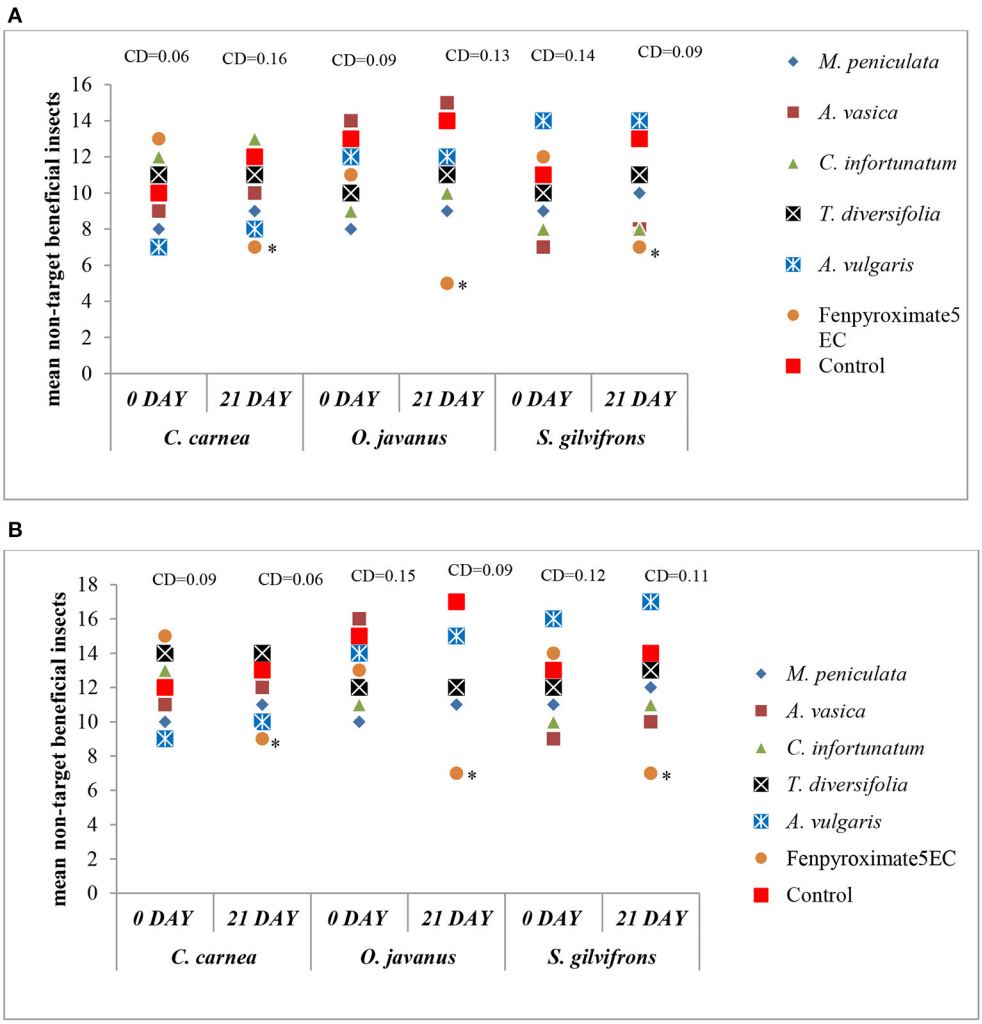

FIGURE 3 | Effect of aqueous extracts of selected plants on non-target beneficial organisms before treatment (0 day) and after 21 days. **(A)** during season-I and **(B)** during season-II. *Values are significantly different between day 1 and 21 by *t*-test ($p < 0.05$).

and *C. alata* also exerted ovipositional deterrent effects by preventing the red spider mites from laying eggs on the treated leaves. The ovipositional deterrence property of ethanol extract of *N. arbor-tristis* was also evaluated and confirmed against *Helicoverpa armigera* (Chauhan et al., 2008). Our findings also agree with those of Roobakkumar et al. (2010) and Handique et al. (2017) in terms of the use of plant extracts against the red spider mites.

In lower-economic countries, synthetic pesticides are frequently used, which leads to several harmful impacts on the ecosystems as well as human health (Ecobichon, 2001). In these countries, the application of pesticidal plant extracts as biocontrol options has been argued for extensively as sustainable options suitable for smallholder farmers (Isman, 2006; Sola et al., 2014), and our experimental findings also confirmed this and indicated that application of extracts of pesticidal plant extract can control pests effectively. It has also been demonstrated that pesticidal plants can minimize the population of red spider mites and can support yield increase comparable to those where synthetic pesticides were used. The relatively regular application of pesticidal plant extracts highlights their use as synthetic pesticide substitutes; they are active compounds that break down rapidly and they show little persistence (Casida, 1980). This means that, during the preparations of the water extracts of these plants, the labor inputs might increase, however, for the commercialization of these products, integrating photostabilizers and sticking agents may perhaps lengthen their effectiveness on crops and hence reduce the frequency of application. This kind of substitution for synthetic pesticides is commonly accepted by the small tea growers due to the high cost of synthetic pesticides; the use of pesticidal plants minimally requires labor costs for harvesting and processing. A cost-effective analysis of the application of pesticidal plants shows that these of more cost-benefit than synthetic pesticides (Amoabeng et al., 2014;

Mkenda et al., 2015). By contrast, the lower persistence of these plants indicates that the consumer's health is at a minimum risk because of the reduced exposure to the plant's bioactive compounds that decompose into harmless natural products, unlike with the synthetic pesticides, which stick to the plants for few days or remain in the soil for weeks or months or even years. It indicates that leaves can be harvested without the risk of leftover residue as exposure to UV light and microorganisms present in that environment helps to quicken the breakdown of naturally occurring compounds (Isman, 2000; Angioni et al., 2005; Caboni et al., 2006). The findings of this research indicated another important transaction when comparing pesticidal plant and synthetic plant protection and their impact on non-target beneficial organisms. Toxicity and the perseverance of synthetic pesticides as anticipated their effect on predators, parasitoids, and pollinators is generally extremely high (Potts et al., 2010; Stanley and Preetha, 2016). In fact, our findings indicated that synthetic pesticides regularly used in the tea ecosystem for the control of red spider mites resulted in the non-existence of predators/parasitoids observed over the cropping season. In general, it leads to the occurrence of pest resurgence after the application of synthetic pesticides enabling populations of pest species to spread out in the non-existence of beneficial insects (Roubos et al., 2014; Welch and Harwood, 2014). On the other hand, pesticidal plant extracts showed less of an impact on predators/parasitoids. A marginal difference was noticed (statistically not significant) amongst the pesticidal plant extracts and the control (water spray) in terms of non-target beneficial insects as compared to the reductions noticed in the synthetic treatment.

It is due to the lower persistence of the pesticidal plant extracts and the various modes of accomplishment where the pesticidal plant treatments may perhaps act against pests as anti-feedants and repellents through toxicity post-ingestion (Tembo et al., 2018). The lower toxic effect and the persistence of the pesticidal plant extracts are supported by their condensed effects on beneficial insects. Compared to the synthetic pesticide, pests are less affected by the pesticidal plants extracts; however, it would facilitate the natural pest directive. As a result, controlling pest populations is additionally effective as regulation of pests is more effective when the ratio between the pests and predators in numbers is less (Arditi and Ginzburg, 1989; Rusch et al., 2010). Even though all the selected plant extracts could reduce a similar or lower number of pests in comparison to the synthetic pesticide, crop yields were repeatedly comparable with that of the synthetic chemicals. This may be due to the increased decline in the pest population through natural enemies, and perhaps the bush can tolerate a definite amount of damage and can physiologically compensate to maintain overall yield (Rubia et al., 1996; Brown, 2005). We may see forms of crop protection via the direct control of fungal or bacterial pathogens or indirect physiological support by acting as a topical green fertilizer, foliar feed, biostimulants, or the phytotonic effects of the plant extracts (Jama et al., 2000; Shaaban, 2001; Soylu et al., 2010; Marei et al., 2012; Rasoul et al., 2012; Pretali et al., 2016).

Interpretation of the phytotoxicity on leaves of tea plants indicated that the selected plants are non-phytotoxic to tea, and the teas made from the leaves sprayed with these extracts when evaluated by professional tea tasters revealed that prepared tea had no taint. Thus, the present study suggests that plant extracts used as a pesticide showed toxicity against red spider mites on tea plants without harming the beneficial insects, increasing the yield without being accompanied by toxic consequences for the part of the tea plants and consumers.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

BD performed laboratory and field trials, analyzed and interpreted the data of the work, and prepared the original manuscript. AB designed and guided the laboratory as well as the field trials and reviewed and edited the writing. CB collected plant materials, prepared the extracts, and assisted in field trials. SS assisted in conducting of laboratory and field trials. All authors read and approved the final manuscript.

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Bio-Insecticidal Nanoemulsions of Essential Oil and Lipid-Soluble Fractions of *Pogostemon cablin*

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The present study aimed to develop nanoemulsions (NEs) of essential oil (EO) and lipid-soluble extract (HE) of *Pogostemon cablin* leaves using biosurfactant, saponin. Hydro-distilled EO and fat-soluble HE were analyzed using GC-MS, which revealed 38.7 ± 2.7% and 37.5 ± 2.1% patchoulol, respectively. EO and HE were formulated with saponin to prepare corresponding coarse emulsions (CEs); furthermore, high-speed homogenization for 2 min was followed by ultrasonication for 3 min with constant frequency of 50 kHz. of the CEs resulted in respective NEs. NEs were characterized for the physico-chemical properties such as emulsion intrinsic stability, particle size distribution, polydispersity index (PDI), and transmission electron microscopy (TEM) for morphology and accurate nanodroplet diameters. CEs and NEs were investigated for insecticidal efficacy against adults of *Tetranychus urticae* and larvae of *Spodoptera litura*. Stable NEs of EO and HE at 500 μg mL^{-1} concentration exhibited corresponding average particle size of 51.7 and 89.9 nm, while TEM image revealed spherical-shaped droplets with the average droplet diameters of 15.3 and 29.4 nm, respectively. NEs of EO and HE displayed highest efficacy in contact toxicity (LC_{50} 43.2 and 58.4 μg mL^{-1}) after 48 h and fumigant toxicity (LC_{50} 9.3 and 13.6 μg mL^{-1}) after 24 h against *T. urticae*. In addition, NEs of EO showed considerable antifeedant and feeding deterrent action (AI 99.21 ± 0.74 and FI 99.73 ± 1.24) against *S. litura* larvae.

Keywords: biopolymer, volatile oil, ultrasonication, acaricidal, patchouli, *Tetranychus urticae*

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INTRODUCTION

The present concept of green crop protection tools emphasized the exploitation of bioactive volatile and non-volatile phyto-constituents, which serve as potential sources of new molecules with a complex mechanism of action (Pavela and Benelli, 2016). In spite of huge versatility of the natural compounds, common constraints exist for their delivery systems, which are related to limited aqueous solubility and stability. Nanotechnological interventions are represented as one of the promising solutions of the problem (Nenaah et al., 2015; Campolo et al., 2017). Further increasing interests have been focused in recent studies regarding the development of NEs for encapsulation of volatile bioactive compounds/EOs for their promising application in agriculture (Khot et al., 2012). EOs have been recognized as eco-benign promising crop protection tool (Kundu et al., 2021); thus, nano-formulations of EOs are being designed, developed, and evaluated to manage many economically important pests (Heydari et al., 2020).

NEs provide a structural framework where bioactive ingredients are dispersed in the aqueous medium and stabilized with the help of surfactant particles. The stable oil-in-water NEs usually comprised of lipophilic active ingredient(s), surfactant, and water (Noori et al., 2018). It appears mostly transparent or slightly translucent in texture with the particle diameter within 100 nm (Balasubramani et al., 2017), since Sugumar et al. (2014) considered NEs having droplet size ranging between 20 and 200 nm. High-pressure homogenization has been used to prepare emulsions with reduced diameter; however, the process could only be sustained on high consumption of energy. Micro-fluidization is another energy-intensive technique, which shears the emulsion droplet size through molecular collision under microfluidic compartment (McClements, 2004). Related studies on the preparation of NEs using various techniques have indicated ultrasonic energy as competitive or even relatively superior employing rotor–stator dispersing to achieve uniform nanodroplets (Kentish et al., 2008).

Nano-sizing of lipophilic components including EOs helps to form kinetically stable emulsion with improved dispersibility in aqueous medium and higher degree of aqueous diffusion (Hashem et al., 2018). Furthermore, bioavailability improved as NEs with the increased surface area dimensions could easily penetrate the cell wall and reach the specific target binding sites; therefore, nano-emulsification reduced the application rate of active ingredients (Acevedo-Fani et al., 2015). However, comprehensive investigations are truly imperative to ascertain proper encapsulation and stability of nanodroplets. Thus, emulsifiers play a crucial role, and the critical micelle concentrations (CMC) of emulsifiers usually determine the kinetic stability of the emulsion (Kumar et al., 2019a). Naturally occurring green emulsifiers such as biosurfactants, amphiphilic proteins, and polysaccharides have been exploited to prepare nanoemulsions. Biosurfactants like saponins are preferred as these are required in small quantities to develop stable nanodroplets of lipophilic compounds (McClements and Gumus, 2016). Besides, steroidal saponins have been reported to possess insecticidal properties (Dolma et al., 2021).

Pogostemon species are perennial herbaceous plants, which belong to Lamiaceae family, are native to Philippines, and are widely distributed across warm and humid tropical climate of South Asian countries including India (Kusuma et al., 2018). Volatile EO of *P. cablin* is primarily constituted with sesquiterpenes, namely, patchoulene and patchouli alcohol (Sundaresan et al., 2012). Investigations on biological properties of the *Pogostemon* EO and phytochemicals revealed multidimensional pharmacological functions against a panel of targets (Hu et al., 2018; Roshan et al., 2022). Significant antifeedant activity of the EO has also been recorded against cosmopolitan pests (Huang et al., 2014). Furthermore, the oil was reported to exhibit LD_{50} 0.2 μg/adult against *Tribolium castaneum* (Feng et al., 2019) and LD_{50} 8.0 μg/insect against *Choristoneura rosaceana* (Machial et al., 2010). Based on the assumptions of higher efficacy of EO and extracts of *Pogostemon*, patchouli alcohol appeared as the key component responsible for broad-spectrum activities (Lima et al., 2013).

Hence, the hypothesis has been built with the proof of concept to utilize the EO and lipid-soluble fractions of *P. cablin* for the preparation of NEs with improved efficacy against acarid and insect. With these backgrounds, the present research was designed to profile volatile chemical constituents of EO and lipid-soluble fractions of *P. cablin* for the development of NE-based delivery system in an attempt to achieve potential bio-insecticide.

MATERIALS AND METHODS

Plant Materials

Fresh leaves of *P. cablin* (5.0 kg) were collected from farmer's field, Hirisave village (12.9172° N and 76.4563° E) near Hassan district of Karnataka, India, during the month of April 2019. The voucher specimen (PC-2019-KHV-01) was authenticated from ICAR-National Bureau of Plant Genetic Resources, New Delhi, India. Fresh leaves were cleaned, gently washed with water, and used for isolation of EO. Shade dried leaves were powdered and used for extraction.

Distillation of EO

Fresh leaves of *P. cablin* (1.0 kg) were hydro-distilled in a Clevenger's apparatus (Borosil Glass Works Ltd., Mumbai, India) for continuous 12 h according to the method reported by Kundu et al. (2016). Pale yellowish-colored EO was collected from the apparatus. Furthermore, EO was partitioned with diethyl ether (3 × 50 mL) followed by passing through anhydrous sodium sulfate (20 g) using a glass funnel and stored. The yield of EO (%) was calculated as 1.43% (v/w).

Extraction

Coarsely powdered leaves (1.0 kg) of *P. cablin* were submerged with 2.5-L hexane (Merck® India Ltd, Mumbai, India) and sonicated for 2 h at 35°C using bath sonicator (PCI Analytics Ltd, Mumbai, India) following the method reported by Dutta et al. (2021). The extraction was repeated thrice with the same sample followed by filtration and concentrated to dryness under reduced pressure in a rotary evaporator (Heidolph, Germany) below 40°C to afford the crude HE (109.7 g).

GC-MS Analysis

Pogostemon EO and HE were analyzed in a 5590C GC-MS (Agilent Technologies®, USA) using a stationary phase column (30 m × 0.25 μm, 0.25 μm, Agilent Technologies®, USA) which was equipped to a mass spectrometer. Samples (1 μL, each) were injected through auto-injector under split-less mode. Helium was used as carrier gas with the flow rate of 1 mL min^{-1} and pressure of 10 psi. Then, oven condition was programed where temperature started at 30°C held for 1 min., then increased at the rate of 3°C min^{-1} to reach 60°C, and then held for 5 min. Hereafter, temperature was increased with the rate of 2°C min^{-1} to reach 150°C and with the hold time of 5 min. Next, temperature was again raised at the rate of 5°C min^{-1} to reach 220°C with the hold time of 5 min. At last, temperature increased to 280°C at the rate of 10°C min^{-1}. Both the samples were analyzed with the runtime of 90 min. The MS acquisition parameters were programed with the ion source temperature of

170°C, electron ionization of 70 eV, transfer line temperature of 280°C, solvent delay of 3 min., and E.M. voltage of 1,419 V. The ionization energy (70 eV) was fixed with scanning rate of 1 s with the mass range of 50–550 amu. Volatile aromatic constituents were identified by matching their mass spectra, fragmentation pattern, reference standard, and retention index (literature and experimental) using Adams (2007), NIST, and WILEY libraries (Kumar et al., 2021).

Critical Micelle Concentration

Saponin ($C_{36}H_{54}O_{11}$, sapogenin content 20–35%) sourced from *Quillaja* sp. (Merck® India Ltd. (Mumbai, India) was used as biosurfactant. CMC of saponin was determined from electrical conductance (EC) of different concentrations. For that, 100 to 12,000 μg mL^{-1} of saponin was prepared in aqueous medium and EC values were determined by a probe-type waterproof EC meter (HI 98304, Hanna, New Delhi, India). At first, the EC meter probe was calibrated with the ready-made KCl solution of known strength. Then, the prepared saponin aqueous solutions were measured by stirring and maintaining temperature equilibrium at 25 ± 1°C. The probe was washed thoroughly with de-ionized water after each measurement, starting from lower to higher concentrations of saponin.

Nanoemulsions (NEs) Preparation

At first, primary coarse emulsions (CEs) of EO and HE of *P. cablin* were prepared separately with the double concentration of pre-determined CMC values of saponin (0.5%). To prepare the CEs, EO (0.5g) and HE (0.5g) were separately mixed with the surfactant, and saponin (0.25 g) with minimum amount of deionized water, and finally, the volume was made up to 50 mL and vortexed for 5 min to obtain CEs (1%) of EO and HE. The freshly prepared CEs were thoroughly dispersed individually in high-speed homogenizer (IKA Ultra-Turrax T25, India) for 2 min. Thereafter, emulsion dilution technique was used to prepare nanoemulsions (NEs) from the CEs (Ghosh et al., 2013). Both the CEs (1%) were diluted serially with 0.5% aqueous solution of saponin to prepare secondary emulsions of lower concentrations (31.25–500 μg mL^{-1}) of EO and HE. The diluted secondary emulsions were then subjected to ultrasonication using a probe ultrasonicator (MISONIX, Ultrasonic Liquid Processors, USA) for 3 min. at the amplitude of 50 kHz. to obtain NEs.

Stability of NEs

NEs of EO and HE were subjected to centrifugation at 10,000 rpm for 20 min. for checking any phase separation. Furthermore, the stability of the prepared emulsions was observed at room temperature at different time intervals and at accelerated storage condition (54 ± 1°C) for 14 days.

Particle Size of NEs

Average particle diameter, distribution, zeta potential, and polydispersity index (PDI) of the NEs and CEs of EO and HE were determined using a Zetasizer (Microtrac, Germany) following the principles of dynamic light scattering. Samples were measured with the help of a probe attached with the instrument with a laser light source. Microtrac FLEX data analysis program was used for the measurement of average droplet size. All the measurements were replicated thrice for each concentration.

Transmission Electron Microscopy

NEs were visualized using TEM (JEM 1011, JEOL, Japan) operated at an acceleration voltage of 80 kV to determine the morphology and droplet size at all the concentrations. The Cu-coated grid (200 mesh) of TEM was impregnated with each concentration of NE and kept for 15 min. for partial drying. The grids were further stained with 2% uranyl acetate and allowed to dry again for 3 h, and micrographs were acquired at the magnification of 80,000x at 100 nm under TEM.

Acaricidal Assays

Culturing of *T. urticae*

Adults of *Tetranychus urticae* were collected from tomato ecosystem which had not been exposed to any acaricide before. The acarids were reared on surface of the mulberry (*Morus alba*) leaves and kept on wet sponge in the laboratory at 27 ± 1°C, 65 ± 5% RH, and 13:11 h under L:D photoperiod till three generations before conducting the bioassay to obtain pure culture. Mites took 8–10 days during summer and 10–16 days during winter to complete one generation.

Leaf Dip Assay

CEs and NEs were tested against adults of *T. urticae* following leaf dip method. Test samples at the strength of 31.25–500 μg mL^{-1} were used for acaricidal assay. Mulberry (*Morus alba*) leaves were cleaned and treated separately with different concentration of the samples. Treated leaves were allowed to dry for 2 h, and 25 adults of *T. urticae* were transferred to the treated leaves. Saponin solution was used as negative control. All the Petri plates were incubated at ambient laboratory conditions under insect culture chamber maintaining 27 ± 1°C. Observations were taken after 24- and 48-h exposure. Mortality (%) was calculated, and probit analysis and LC_{50} values (μg mL^{-1}) were determined using statistical software.

Fumigation Assay

CEs and NEs were tested for fumigant toxicity against adults of *T. urticae*. Using a stereomicroscope, 25 adults were transferred on the cleaned mulberry leaf holding on the dorsal part of the hysterosoma using a handling brush. The leaves were kept inside the glass jars. Each treatment (2.0 mL) of each sample was socked in cotton balls and hung with the help of lid inside the jar. Test concentrations of each sample were kept at 100–5.0 μg mL^{-1}. Each treatment was replicated five times along with negative control, and the dead adults were counted after 24 h. The treated adults were considered dead if appendages did not respond even after touching with the brush. Mortality (%) was recorded, and further, LC_{50} values (μg mL^{-1}) were determined.

Insecticidal Activity Against *S. litura*

Insect Culture

Eggs of *S. litura* were collected from tomato plants and incubated under laboratory conditions with high RH of 80

± 5%. When eggs turned into dark color, matured (3–4 days), and were inoculated for hatching, freshly hatched larvae were fed on castor (*Ricinus communis*) leaf bouquets till larvae entered into pupation. Then, sex was identified, separated in different jars for emergence, and released for oviposition in jar, which consists of 15–20% honey solution dipped in a cotton wad for food and zig-zag folded paper strip for egg laying. Field collected cultures were reared in laboratory for 2–3 generations to obtain pure culture and used for testing.

Larvicidal Assay

Larvicidal activity of the CEs and NEs based on EO and HE was studied using potter tower spray at five different test concentrations (500–31.25 μg mL^{-1}). Fresh castor leaf disk was kept in each Petri dish, and five third instar larvae of *S. litura* were released. Each test concentration (1.0 mL) was sprayed on the third instar larvae of *S. litura* kept in Petri dish. Each treatment was replicated thrice. Petri dishes were kept under laboratory ambient conditions, and observations were taken after 24 and 48 h. Mortality (%) was calculated, and lethal concentration in terms of LC50 values (μg mL^{-1}) was determined.

Antifeedant Assay

CEs and NEs were evaluated using non-choice and choice leaf disk method (Sengonca et al., 2006). Briefly, fresh castor leaves were collected from field and cleaned thoroughly. Leaves were cut evenly maintaining disk desired size (3 × 3 cm^2), were dipped in various test concentrations (500–31.25 μg mL^{-1}) separately, and were allowed to air dry at room temperature for 3 h. Additionally, leaf disks dipped in saponin solution were used as negative control. In each Petri dish, one layer of wet filter paper was placed to avoid drying of the leaf disks, if any. One third instar larva was introduced into each treated plate and placed in an incubator at 27 ± 1°C with 65 ± 5% RH and a 14:10 (L: D) photoperiod. Each treatment was replicated eight times. Observation of the larvae was taken after 24-h exposure to determine the effect of on their feeding behavior. Larvae were found to be sterile and could not feed the leaves; however, most of the treated plates, close to complete consumption of leaves, were observed in control. Feeding of treated and control leaves was measured after 24 h. using a Leaf Area Meter (ADC Bioscientific Ltd., India), and the antifeedant index (AI) was determined by the following equation AI% = [(1 – T/C) × 100], where T is the average area of treated leaf consumed and C is the average leaf area consumed without treatment. The Feeding Index (FI) was calculated as [(C – T)/(C + T)] × 100.

Statistical Analysis

Data were measured using the Statistical Package for the Social Sciences (SPSS, Version 14.0, IBM, NY, USA). The results were expressed as mean±standard deviation (SD), and differences between variables were tested using one-way ANOVA. Statistically significant level was determined at p-value < 0.05.

TABLE 1 | Chemical composition of volatile organic components of EO and HE of *P. cablin* leaves.

[a]Compounds	[b]RI^{exp}	[c]RI^{lit}	[d]RA (%)		[e]Identification
			EO	HE	
α-Pinene	928	932	0.2 ± 0.0	–	RI, MS
β-Pinene	969	974	0.3 ± 0.1	–	RI, MS
dl-Limonene	1,018	1,024	0.2 ± 0.0	–	RI, MS
Nonanal	1,103	1,105	–	3.0 ± 0.4	RI, MS
Tridecane	1,302	1,308	–	0.6 ± 0.1	RI, MS
trans-β-Caryophyllene	1,409	1,412	3.7 ± 0.5	4.3 ± 0.5	RI, MS
γ-Elemene	1,430	1,436	3.3 ± 0.4	0.5 ± 0.1	RI, MS
α-Guaiene	1,436	1,440	17.7 ± 1.2	12.4 ± 0.9	RI, MS
Aromadendrene	1,438	1,439	0.5 ± 0.1	-	RI, MS
β-Patchoulene	1,441	1,443	3.6 ± 0.5	0.2 ± 0.0	RI, MS
α-Patchoulene	1,452	1,457	2.6 ± 0.3	3.6 ± 0.5	RI, MS
Seychellene	1,458	1,460	-	2.7 ± 0.3	RI, MS
α-Selinene	1,474	1,475	2.5 ± 0.2	0.1 ± 0.0	RI, MS
γ-Gurjunene	1,478	1,479	0.1 ± 0.0	-	RI, MS
β-Selinene	1,481	1,487	0.2 ± 0.0	-	RI, MS
β-Guaiene	1,482	1,490	3.2 ± 0.4	2.5 ± 0.3	RI, MS
α-Bulnesene	1,509	1,505	18.1 ± 1.2	7.8 ± 0.7	RI, MS
Globulol	171	1,575	0.1 ± 0.0	–	RI, MS
Caryophyllene oxide	1,575	1,578	0.4 ± 0.1	–	RI, MS
Viridiflorol	1,607	1,612	0.5 ± 0.2	–	RI, MS
Cubenol	1,634	1,642	0.2 ± 0.0	–	RI, MS
Patchouli alcohol	1,677	1,680	38.7 ± 2.7	37.5 ± 2.1	std, RI, MS
(Z,Z)-Farnesol	1,721	1,718	0.2 ± 0.0	–	RI, MS
Leden oxide (I)	1,876	1,890	0.6 ± 0.1	–	RI, MS
Hexadecanoic acid	1,958	1,964	–	2.1 ± 0.2	RI, MS
Octadecenoic acid	2,147	2,140	–	6.4 ± 0.5	RI, MS
Octadecanoic acid	2,186	2,188	–	2.4 ± 0.3	RI, MS
Docosane	2,213	2,208	–	2.3 ± 0.3	RI, MS
Tetracosane	2,395	2,402	–	2.1 ± 0.3	RI, MS
Squalene	2,837	2,829	–	1.3 ± 0.2	RI, MS
Non-acosane	2,891	2,900	–	0.8 ± 0.2	RI, MS
Tricontane	2,989	3,000	–	0.9 ± 0.2	RI, MS
Total identified (%)			96.9	93.5	
Classified on functional groups					
Monoterpene hydrocarbons (%)			0.7	–	
Sesquiterpene hydrocarbons (%)			55.5	34.1	
Oxygenated sesquiterpenes (%)			40.6	38.7	
Aldehydes			–	3.0	
Long chain fatty acids			–	10.9	
Long chain hydrocarbons			–	5.0	

[a]Compounds are listed in order of their elution from a HP-5MS column. [b]Retention index on HP-5MS column, experimentally determined using homologous series of C_8-C_{30} alkanes. [c]Retention index taken from Adams (2007), NIST (2012) and WILEY libraries. [d]Relative area % values are expressed as means ± SD. [e]Identification methods: std, based on comparison with reference standard; RI, based on comparison of calculated RI with those reported in Adams and NIST; MS, based on comparison with WILEY and NIST 12 MS databases.

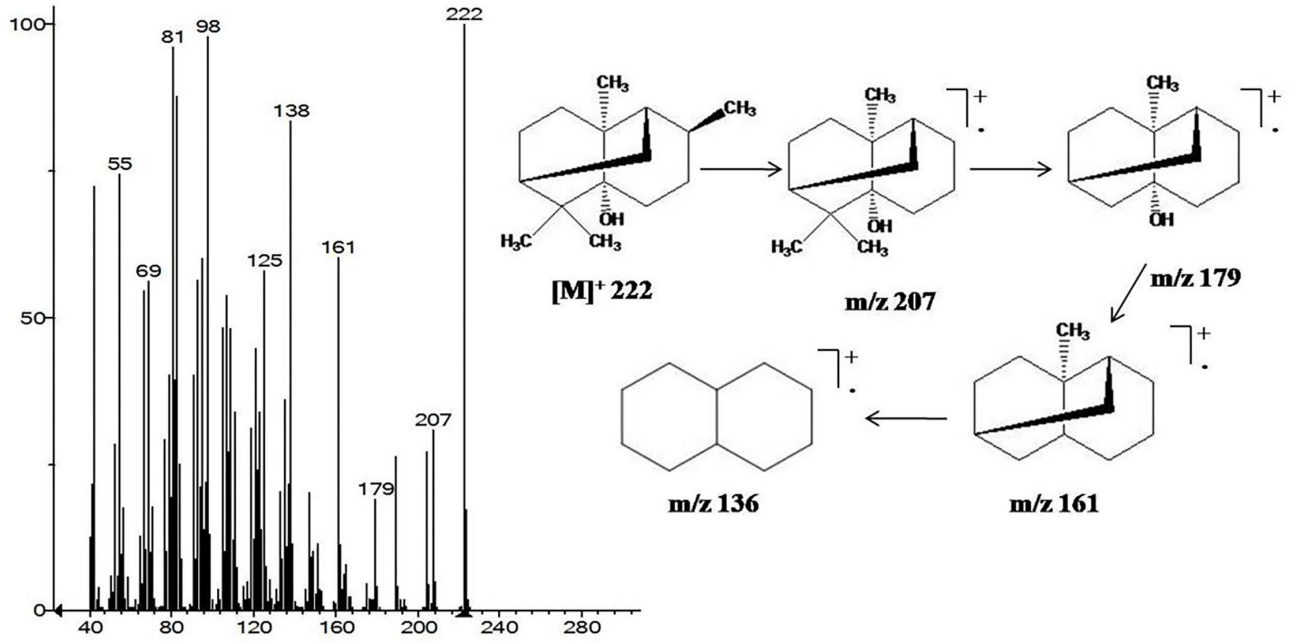

FIGURE 1 | GC-MS fragmentation pattern of the most abundant patchouli alcohol characterized in EO and HE of *P. cablin.*

RESULTS

Volatile Composition of EO and HE

Volatile constituents of EO of *P. cablin* leaves were identified in GC-MS which showed several peaks, corresponding to twenty-four mono- and sesquiterpenoids, representing 96.9% of the oil (**Table 1**). Sesquiterpene hydrocarbons (55.5%) were most abundant followed by oxygenated sesquiterpenes (40.6%). Patchouli alcohol (38.7 ± 2.7%) was found as the major oxygenated sesquiterpene followed by α-bulnesene (18.1 ± 1.2%) and α-guaiene (17.7 ± 1.2%). Other major sesquiterpenes of the oil were *trans*-β-caryophyllene (3.7 ± 0.5%), β-patchoulene (3.6 ± 0.5%), β-elemene (3.3 ± 0.4%), β-guaiene (3.2%), α-patchoulene (2.6 ± 0.3%), and α-salinene (2.5 ± 0.2%). Only three monoterpene hydrocarbons such as α-pinene (0.2 ± 0.0%), β-pinene (0.3 ± 0.1%), and *dl*-limonene (0.2 ± 0.0%) were identified accounting only 0.7% of the EO.

Mass spectrum of patchouli alcohol showed molecular ion $[M]^+$ peak at m/z 222, which was further broken to give daughter ion peaks at m/z 207, 179, and 161 after sequentially losing methyl and hydroxyl moieties. Other peaks at m/z 138, 125, 98, 81, and 69 were also originated due to subsequent cleavage of hydrocarbons (**Figure 1**). Similarly, α-bulnesene was characterized from its characteristic $[M]^+$ peak at m/z 204 and further fragmented to daughter ion peaks at m/z 189, 175, 161, 147, 135, 121, 107, 93, and 79 with removal of methylene and methyl groups (**Figure 1**).

Nineteen aromatic compositions, representing 93.5% of the non-polar fat-soluble HE, have been identified in GC-MS and mentioned as per their elution in HP-5MS stationary phase (**Table 1**). However, total sesquiterpene hydrocarbon content of hexane soluble fraction was 34.1%, while oxygenated sesquiterpene was 38.7%. Most abundant patchouli alcohol (37.5 ± 2.1%) was identified as the sole oxygenated sesquiterpene. Besides, long-chain fatty acids and long-chain hydrocarbons were found in the HE, representing 10.9 and 5.0%, respectively. Among fatty acids, octadecenoic acid (6.4 ± 0.5%) was identified as the major compound followed by octadecanoic acid (2.4 ± 0.3%) and hexadecanoic acid (2.1 ± 0.2%). Similarly, long-chain hydrocarbons, mainly docosane (2.3 ± 0.3%), tetracosane (2.1 ± 0.3%), squalene (1.3 ± 0.2%), triacontane (0.9 ± 0.2%), and non-acosane (0.8 ± 0.2%), were identified.

Characterizations and Stability of NEs

Based on the recorded electrical conductance of various concentrations of saponin, sharp change was observed at 0.25% (**Figure 2**); thus, double concentration, 0.5%, has been selected for the final preparation of CEs and NEs. Primary CEs with the 1% strength of EO (w/w) and HE were prepared separately and diluted to get various test concentrations (500–31.25 $\mu g\ mL^{-1}$). Subsequently, ultrasonication-assisted nano-emulsification of CEs of EO and HE in aqueous medium resulted in the preparation of respective NEs (**Figure 3**). Formation of cavitations in the liquid due to ultrasonic wave helped to utilize the energy for shearing of coarse droplets in nano size range which was further stabilized by the surfactant particles. The prepared NEs (31.25 to 125 $\mu g\ mL^{-1}$) were found transparent; however, slight turbidity was recorded at 250–500 $\mu g\ mL^{-1}$ concentrations. Here, non-toxic biosurfactant,

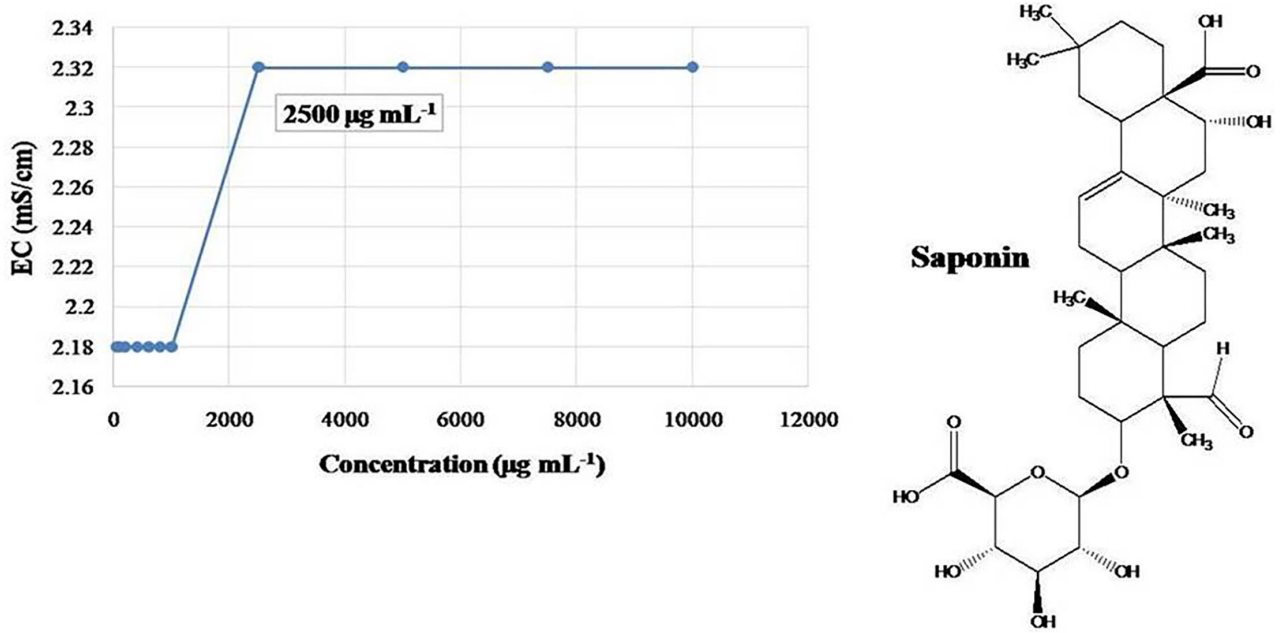

FIGURE 2 | Critical micelle concentration (CMC) of biosurfactant, saponin as measured by electrical conductance (EC).

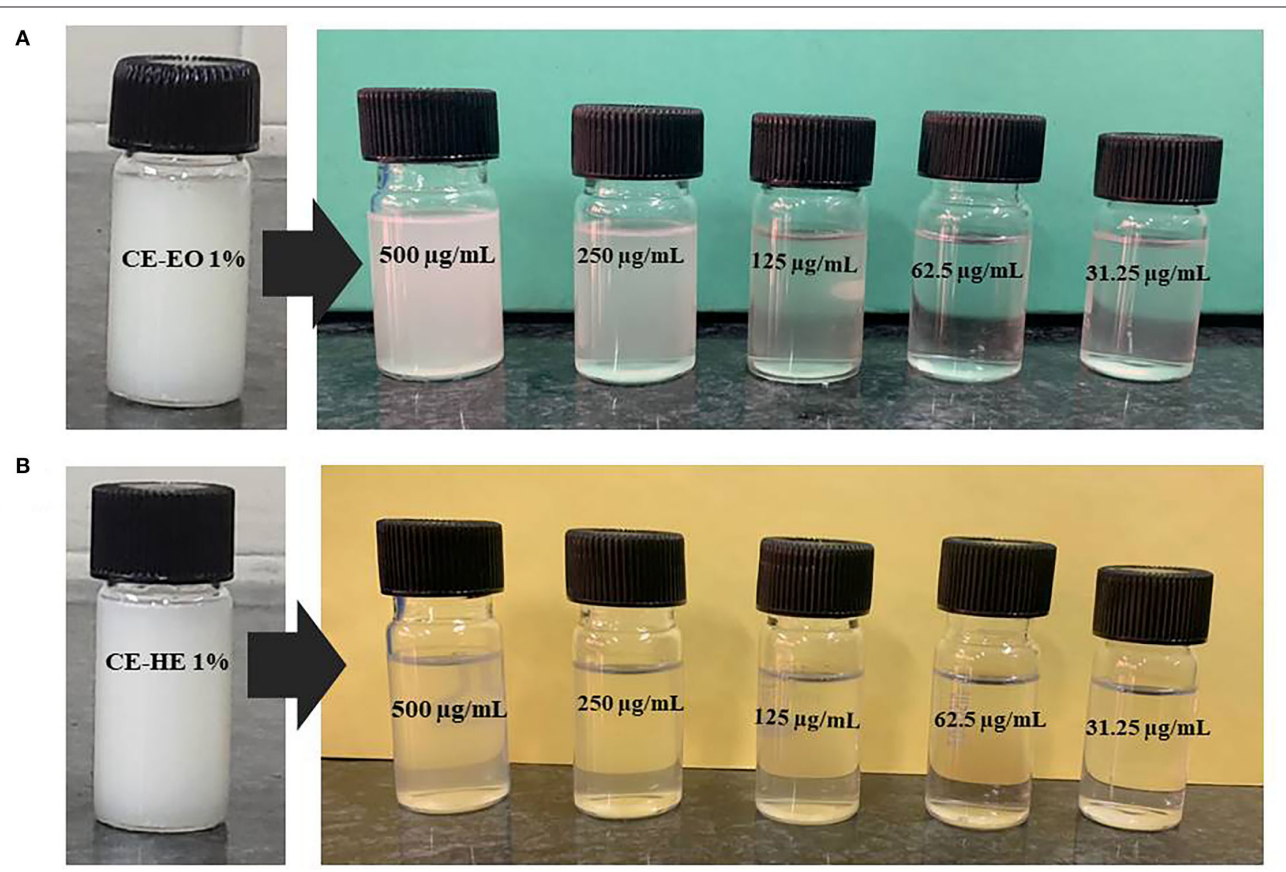

FIGURE 3 | Visual appearance of CEs and different concentrations of NEs of **(A)** EO and **(B)** HE of *P. cablin.*

TABLE 2 | Physico-chemical characterizations of NEs loaded with EO and HE of *P. cablin* leaves.

Concentrations (μg mL^{-1})	Mean droplet diameter (Z-average) (nm)*	Zeta potential (mV)	Polydispersity index (PDI)	P^{H}
NE-EO				
500	71.68 ± 0.84	−29.21 ± 0.49	0.49 ± 0.02	4.58
250	62.25 ± 0.30	−27.42 ± 0.12	0.62 ± 0.02	4.95
125	58.43 ± 076	−23.49 ± 1.52	0.55 ± 0.03	5.28
62.5	57.82 ± 0.67	−23.03 ± 0.91	0.52 ± 0.03	5.99
31.25	46.19 ± 0.75	−22.62 ± 0.12	0.69 ± 0.06	6.40
NE-HE				
500	89.87 ± 0.62	−29.06 ±0.21	0.51 ± 0.03	4.18
250	67.93 ± 0.64	−29.18 ± 2.34	0.57 ± 0.05	4.59
125	61.40 ± 0.90	−27.28 ± 1.07	0.52 ± 0.02	4.84
62.5	56.67 ± 0.76	−20.55 ± 0.58	0.67 ± 0.03	5.62
31.25	49.13 ± 0.5	−26.50 ± 0.48	0.65 ± 0.03	6.01

**Mean diameter of droplets are expressed in mean ± SE (n = 3).*

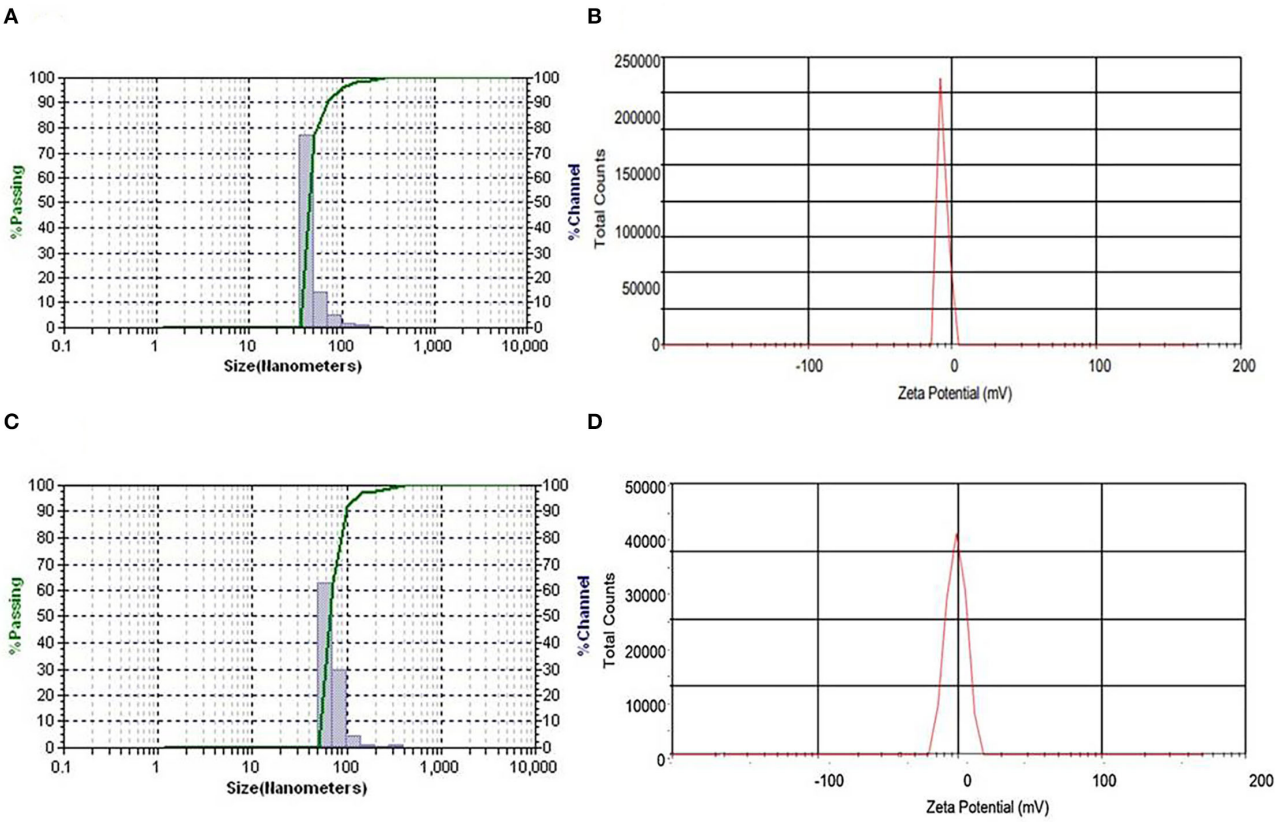

FIGURE 4 | (A) Particle size distribution and **(B)** zeta potential of 500 μg mL^{-1} concentration of NEs of EO. **(C)** Particle size distribution and **(D)** zeta potential of 500 μg mL^{-1} concentration of NEs of HE.

saponin, was effectively used to stabilize the developed NEs at 0.5% concentration.

The properties of serially diluted NEs of EO and HE are shown in **Table 2**. The average droplet diameter at 500 μg mL^{-1} concentration of NE of EO as prepared by homogenization followed by ultrasonication was 71.68 ± 0.84 nm with the polydispersity index (PDI) of 0.49 ± 0.02, signifying narrow dimension of particle size distribution (**Figure 4A**). Likewise, average droplet diameter at the same concentration of NE-HE was 89.87 nm (**Figure 4C**) with corresponding PDI 0.51 ± 0.03. There was a clear indication that with decrease in concentration of EO and HE in the NEs, mean droplet size decreased. However,

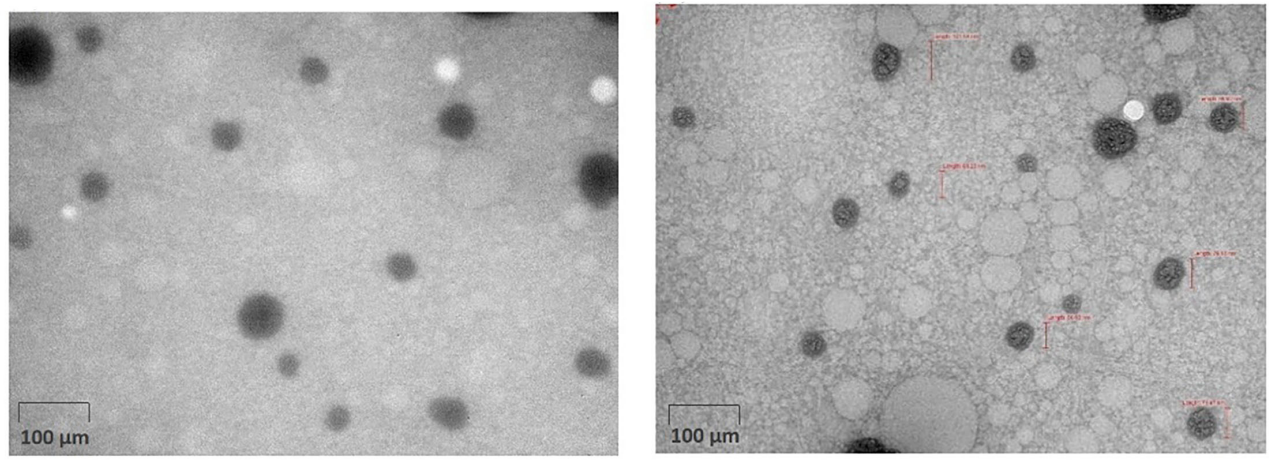

FIGURE 5 | TEM image of 500 µg mL^{-1} concentration of **(A)** NE of EO **(B)** NE of HE.

TABLE 3 | Contact toxicity of CEs and NEs against the adults of *T. urticae* after 24 and 48 h of treatment.

Emulsions	Exposure time (h)	LC_{50}(µg mL^{-1})[a]	95% Confidence limit µg mL^{-1})		Slope ± SE[b]	Intercept±SE[c]	(χ^2)[d]	df
			Lower	Upper				
CE-EO	24	223.6	188.5	293.1	0.85 ± 0.32	−3.39 ± 1.35	29.0	25
	48	134.9	105.3	154.3	1.21 ± 0.13	−1.56 ± 0.97	57.2	34
CE-HE	24	235.6	194.2	301.1	1.03 ± 0.77	−1.86 ± 1.11	25.3	18
	48	168.7	113.5	224.0	0.56 ± 0.09	−3.23 ± 1.32	18.4	12
NE-EO	24	89.7	61.2	113.2	0.95 ± 0.38	−2.60 ± 0.52	32.2	27
	48	43.2	29.6	75.1	0.87 ± 0.25	−4.17 ± 1.92	13.0	9
NE-HE	24	97.2	63.6	144.5	0.44 ± 0.17	−3.27 ± 1.18	39.1	28
	48	58.4	24.7	72.9	1.35 ± 0.70	−1.03 ± 0.69	18.6	22

[a]*LC_{50} Concentration (µg mL^{-1}) at which 50% mortality observed.* [b]*Slope at the response of regression equation ± standard error.* [c]*Intercept of the regression equation ± SE.* [d]*χ^2, Chi-squared values at different df and probability level (0.05).*

no significant relationship was observed between concentration and PDI as a narrow spectrum was maintained for PDI both in case of NEs of EO (0.49–0.69) and HE (0.51–0.67), suggesting uniform size distribution of the droplets irrespective of change in concentration. As 0.5% saponin concentration was maintained throughout the study for all the samples, there was always less chance of much variation in size distribution as the micelles remained same. However, with increased loading of EO and HE, the micelles got swelled by entrapment of EO and HE. Therefore, the mean droplet diameter was found to be more at higher loading concentrations. In the present study, zeta potential of the prepared NEs of EO and HE at 500 µg mL^{-1} was found to be −29.21 ± 0.49 mV and −29.06 ±0.21 mV (**Figures 4B,D**) at the native P^H of 4.58 and 4.18, respectively. Thus, the absolute droplet charges were found very less. For all the samples, zeta potential was found to be higher than −20 mV, suggesting formation of stable NEs.

Morphology and size of the droplets of NEs were visualized under TEM. **Figure 5** displayed TEM images of the droplets of NEs of EO and HE. It was quite evident from the TEM images displaying spherical-shaped nanodroplets. The average diameter of the NE of EO droplets was 15.32 nm, which is relatively three times smaller than the average diameter determined obtained from particle size analyzer. Similarly, the average diameter of the NE of lipid-soluble HE droplets was 29.41 nm. However, droplet diameter varied within the range of 12.78 to 38.97 nm. The variation in droplet size could be attributed to the fact that TEM analyses of the droplets in the dry state gave accurate size based on the real morphology of the droplets, whereas average hydrodynamic diameter of the droplets was obtained from the particle size analyzer which was the average size of hydrated micelles. Furthermore, NEs were found to be stable with no phase separation even after 14 days of storage under accelerated storage condition at 54 ± 1°C and at room temperature. The average droplet diameters at 500 µg mL^{-1} concentrations of NEs of EO and HE after 14 days of accelerated storage were 91.22 ± 1.29 nm and 99.41 ± 0.72 nm, respectively. Furthermore, no aggregation of droplets

was observed upon TEM analysis, indicating kinetic stability of the emulsions.

Acaricidal Action on *T. urticae*

Acaricidal activity of CEs and NEs of EO and HE of *P. cablin* against adults of *T. urticae* revealed significant mortality after 24 and 48 h of the treatment (**Table 3**). Both the CEs were effective after 48 h with the LC_{50} values <170 μg mL^{-1}. Of *T. urticae* were found dead along the midrib of the treated leaves. CE-EO was comparatively more toxic than CE-HE. The magnitude of acaricidal efficacy was more after 48 h for the emulsions. Contact toxicity by leaf dip method with respect to time of application might contribute immensely to the actual acaricidal action. As hypotheses, NE-EO and NE-HE were very potent with higher effectiveness, LC_{50} values <100 μg mL^{-1}. NE-EO exhibited highest efficacy with the LC_{50} 89.7 μg mL^{-1} and 43.2 μg mL^{-1} after 24- and 48-h exposure, respectively. Likewise, NE-HE was relatively high toxic with their corresponding LC_{50} values of 97.2 μg mL^{-1} and 58.4 μg mL^{-1} after 24- and 48-h exposure.

Fumigant toxicity of the emulsions showed promising action at the sublethal concentrations after 24-h exposure (**Table 4**). Adults were found dead outside the border of the treated leaves. Among the tested emulsions, NE-EO (LC_{50} 9.3 μg mL^{-1}) displayed maximum fumigant action, while similar trend of fumigant action was observed for NE-HE (LC_{50} 13.6 μg mL^{-1}). NEs of the oil and non-polar fractions were equally effective in fumigant toxicity, even more potent than the contact toxicity assay.

Insecticidal Action Against *S. litura*

Larvicidal assay of NEs of EO and HE against third instar larvae revealed moderate action. CEs were found less effective with the LC_{50} values >400 μg mL^{-1}. However, NEs-EO were effective, performing LC_{50} values 125.8 and 145.9 μg mL^{-1} after 48 and 24 h, respectively (**Table 5**). Similar findings were noticed for the NEs-HE, which exhibited LC_{50} values of 167.5 μg mL^{-1} and 190.6 μg mL^{-1} after 48- and 24-h exposure, respectively.

Antifeedant activity of CEs and NEs of EO and HE demonstrated sufficient antifeedant activity at all the test concentrations in both no-choice and choice assays against larvae of *S. litura* (**Figure 6**). At the highest concentration of 500 μg mL^{-1}, CE-EO and CE-HE showed the maximum AI value of 89.75 ± 2.12 and 87.55 ± 2.45, respectively, while NE-EO and NE-HE at the same concentration possessed AI value of 99.21 ± 0.74 and 98.75 ± 1.02, respectively (**Table 6**). Indeed, antifeedant activity of the CEs has been improved nearly 10% at the higher test concentrations and over 20% at the lower concentrations. On the contrary, NE-EO and NE-HE exhibited FI values of 99.73 ± 1.24 and 97.34 ± 1.0, respectively.

TABLE 4 | Fumigant activity of CEs and NEs against the adults of *T. urticae* after 24 h of treatment.

Emulsions	LC_{50} (μg mL^{-1})[a]	95% Confidence limit μg mL^{-1})		Slope ± SE[b]	Intercept ± SE[c]	(χ^2)[d]	df
		Lower	Upper				
CE-EO	35.8	21.7	47.9	2.28 ± 0.29	−2.18 ± 0.69	34.6	29
CE-HE	52.4	27.8	72.1	3.94 ± 0.35	−3.25 ± 0.68	22.2	16
NE-EO	13.7	11.6	23.5	4.12 ± 0.35	−2.28 ± 0.65	56.1	30
NE-HE	19.4	12.9	31.6	2.71 ± 0.34	−1.44 ± 0.72	28.3	23

[a]LC_{50} Concentration (μg mL^{-1}) at which 50% mortality observed. [b]Slope at the response of regression equation ± standard error. [c]Intercept of the regression equation ± SE. [d]χ^2, Chi-squared values at different df and probability level (0.05).

TABLE 5 | Contact toxicity of CEs and NEs against third instar larvae of *S. litura* using leaf dip assay after 24 and 48 h.

Emulsions	Exposure time (h)	LC_{50} (μgmL^{-1})[a]	95% Confidence limit μg mL^{-1})		Slope ± SE[b]	Intercept ± SE[c]	(χ^2)[d]	df
			Lower	Upper				
CE-EO	24	413.2	395.6	449.5	3.33 ± 0.22	−4.29 ± 0.18	23.7	13
	48	420.5	301.7	442.3	1.48 ± 0.13	−2.69 ± 0.29	39.4	21
CE-HE	24	569.7	423.5	599.8	2.61 ± 0.18	−3.14 ± 0.23	18.8	15
	48	509.1	483.1	524.4	2.25 ± 0.23	−1.25 ± 0.27	19.2	10
NE-EO	24	145.9	137.2	159.7	2.18 ± 0.22	−2.73 ± 0.22	36.5	26
	48	125.8	117.2	144.3	1.17 ± 0.19	−2.28 ± 0.25	44.8	16
NE-HE	24	190.6	172.9	205.3	1.42 ± 0.20	−1.64 ± 0.30	52.3	26
	48	167.5	147.0	191.6	2.22 ± 0.16	−3.39 ± 0.27	17.2	13

[a]LC_{50} Concentration (μg mL^{-1}) at which 50% mortality observed. [b]Slope at the response of regression equation ± standard error. [c]Intercept of the regression equation ± SE. [d]χ^2, Chi-squared values at different df and probability level (0.05).

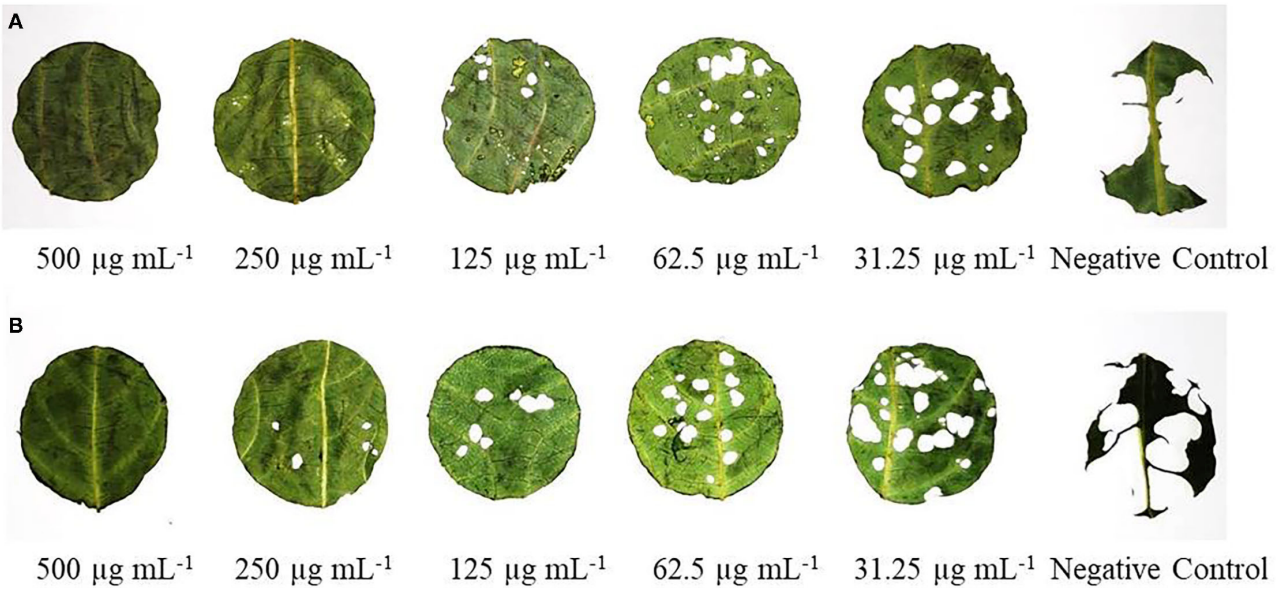

FIGURE 6 | Visual display of antifeedant action of NEs of **(A)** EO and **(B)** HE against *S. litura* third instar larvae after 24 h.

TABLE 6 | Evaluation[a] of CEs and NEs of EO and HE of *P. cablin* for determination of antifeedant index (AI) and feeding index (FI) after 24 h.

Conc. (µgmL^{-1})	CE-EO		CE-HE		NE-EO		NE-HE	
	[b]AI (%)	[c]FI (%)	AI (%)	FI (%)	AI (%)	FI (%)	AI (%)	FI (%)
500	89.75 ± 1.12^{e}	81.73 ± 1.04^{e}	87.55 ± 2.45^{e}	77.86 ± 2.38^{e}	99.21 ± 0.14^{e}	99.73 ± 1.24^{d}	98.75 ± 1.02^{de}	97.34 ± 1.05^{de}
250	85.26 ± 2.45^{d}	74.29 ± 3.74^{d}	77.75 ± 1.15^{d}	63.60 ± 2.45^{d}	97.01 ± 1.10^{d}	97.86 ± 1.33^{d}	98.11 ± 1.68^{d}	96.31 ± 2.10^{d}
125	70.78 ± 1.39^{c}	54.77 ± 2.22^{c}	65.43 ± 2.50^{c}	48.62 ± 3.57^{d}	86.72 ± 2.21^{c}	76.56 ± 2.88^{c}	84.81 ± 2.30^{c}	73.63 ± 3.48^{c}
62.5	55.63 ± 1.08^{b}	38.53 ± 0.89^{b}	52.72 ± 4.22^{b}	35.79 ± 2.10^{d}	80.08 ± 1.62^{b}	66.80 ± 3.06^{b}	76.08 ± 2.45^{b}	61.39 ± 1.15^{b}
31.25	35.66 ± 3.06^{a}	21.30 ± 1.33^{a}	33.91 ± 3.56^{a}	20.42 ± 3.16^{d}	72.36 ± 1.74^{a}	56.69 ± 1.22^{a}	70.29 ± 1.10^{a}	54.20 ± 0.74^{a}

[a]*Data are presented as means ± SD;* [b]*Antifeedant index (AI) is calculated as AI = (1 − T/C) × 100,* [c]*Feeding index (FI) is calculated as FI = (C–T)/(C + T) × 100.*

DISCUSSION

Comprehensive information on EO of *P. cablin* displayed abundance of either patchoulene or patchouli alcohol (Sundaresan et al., 2012). However, α-guaiene has been reported as the major constituent of *Pogostemon* EO (Tsai et al., 2007). Recently, forty-seven volatile constituents consisting of twenty sesquiterpenes were reported from the EO, which mentioned abundance of curzerene followed by *epi*-cadinol and acetophenone (Kumar et al., 2019b). Exceptionally, aciphyllene and acetophenone are often identified in higher content in the commercially available EO, which was further authenticated to be found as an adulterant, the major constituent of *P. heyneanus* (Murugan et al., 2010). Another literature report, from South Indian sample of *P. cablin,* suggested high content of acetophenone, β-pinene, and *(E)*-nerolidol in the EO (Anjana and Thoppil, 2013). Such variations in chemical compositions of EO could be attributed due to the distillation techniques, associated temperature on extraction, agroclimatic factors along with genetic variations of the planting materials, etc. (Kundu et al., 2013b; Dutta et al., 2020). A recent report suggested quality control and regulation aspects of patchouli EO are highly dependent on the variable composition of patchoulol and other sesquiterpenes (Pandey et al., 2022).

Saponin was used as emulsifier to develop the NEs which certainly contributed in positive manner to enhance the efficacy of the developed emulsions. It is the first report on the use of natural polymer for the preparation of NEs of *Pogostemon* bioactives, though most of the previous studies have been reported on the use of various non-ionic surfactants for the fabrication of NEs of EOs (Balasubramani et al., 2017; Campolo et al., 2017). The wide availability and relatively lower cost of the non-ionic surfactants could be responsible for its higher use. Contrastingly, xanthan gum along with subcritical water has been used to develop oil-in-water NEs (Ahmadi and Jafarizadeh-Malmiri, 2021a). They also suggested the application of natural gums and/or saponin using subcritical water for the preparation of EO-based NEs (Ahmadi and Jafarizadeh-Malmiri, 2021b). Thus, the use of green biopolymer has an edge over the excessive use of conventional emulsifiers in sustainable agriculture. In

addition, saponin-like biopolymeric emulsifiers may enhance the biofunctional properties of the component.

In the present study, stable NEs were developed with saponin employing ultrasonication. Low energy subcritical water-based green method has been reported recently for the preparation of thyme oil-in-water NEs (Ahmadi and Jafarizadeh-Malmiri, 2020). The developed NEs exhibited better stability over a period of 14 days. Zeta potential of any emulsion has been recommended as the key indicator for its stability, which is related to electrostatic repulsion among the nanodroplets (Zainol et al., 2012). The stability of the NEs will be maintained with the limited or no coagulation of droplets, and therefore, the colloidal system should retain its droplet diameter in nano size range (<100 nm), resulting in better Brownian motion of the particles in the system (Heydari et al., 2020).

Previously, a homogenization method consisting of multiple adjuvants has been reported for the preparation of NEs of *Pogostemon* EO with non-ionic surfactant, propylene glycol as co-surfactant, and lecithin as emulsifier at 5% concentration; however, concomitant data on the physico-chemical properties have not been generated (Adhavan et al., 2017). Likewise, another study also reported the use of Tween 80 and/or Triton X-100 to stabilize geranium EO maintaining the oil surfactant ratio ranging from 5:1 to 1:5, suggesting better stability of the emulsion with higher amount of surfactant (Jesser et al., 2020). In the present context, NEs were prepared using ultrasound-assisted nano-emulsification of EO and lipid-soluble HE of *P. cablin* leaves with only biosurfactant, saponin at 0.5% concentration, without any use of co-surfactant, and/or co-emulsifier. In addition, better droplet size and emulsion stability of these NEs have been ascertained with rigorous physico-chemical characterizations and process validations.

A preliminary investigation on NEs of *P. cablin* oil (18%) with polyoxyethylene suggested excellent formicidal action on *Atta opaciceps* and *Atta sexdens* (Rocha et al., 2018). Furthermore, *P. cablin* oil-based tincture, candle, and crystal cake have been found effective as mosquito repellent (Das et al., 2016). Recently, patchouli oil has been nano-encapsulated on chitosan and evaluated for enhanced shelf life of maize seeds (Roshan et al., 2022). Recent past, the EO has been reported very effective in repellent action and fumigant toxicity against *T. cinnabarinus* (Cheng et al., 2020). Furthermore, better performance of NEs has been reported in literature against various insects (Badawy et al., 2018; Abdelgaleil et al., 2019). Current study revealed tremendous performance of the NEs over CEs against *T. urticae* both in fumigant and contact toxicity assays. However, required concentration of the emulsions was found to be very less to cause lethal action in fumigant assay, causing suffocation to death due to volatile nature of the chemical constituents. Nevertheless, acaricidal action in contact toxicity has been found satisfactory.

Plant volatiles have been found effective against many agriculturally important pests (Kundu et al., 2013a; Ahluwalia et al., 2014; Keerthiraj et al., 2021). Comprehensive studies have been reported on insecticidal activity of *P. cablin* oil. In the present study, NEs were found to be highly effective to cause larval mortality. Comparative assessment of the insecticidal activity of CEs and NEs revealed higher effectiveness of NEs due to nano sizing of the delivery system with higher surface area and better penetration through insect cell wall. Strong antifeedant and larvicidal action of the plant have been reported in literature against noctuid insects (Huang et al., 2014). NE-EO and NE-HE displayed considerable action with respect to AI and FI against *S. litura* larvae even at the lowest concentration of 31.25 $\mu g\ mL^{-1}$. The salient finding of the antifeedant action certainly corroborates the literature report describing more than 80% feeding deterrent action of the oil (Huang et al., 2014). Major chemical components such as patchouli alcohol, α-bulnesene, and α-guaiene in both EO and HE could be responsible for higher efficacy. Though it is unclear whether the most abundant constituent, patchouli alcohol, is only responsible for the acaricidal and insecticidal action, further mechanism of action needs to be studied.

CONCLUSIONS

In summary, patchouli alcohol has been identified as the major constituent of EO and HE of *P. cablin* leaves. Stable NEs have been prepared with only 0.5% saponin either for EO or HE. The NEs of EO and HE were stable even after 30 days at room temperature with satisfactory qualitative characteristics. The results of acaricidal and antifeedant activities have demonstrated promising effect of NEs of EO and HE against *T. urticae* and *S. litura* following a dose-dependent trend, though NEs of EO have been performed slightly better than HE. As far as our literature survey could ascertain, this is the first report on potential activity of NEs of *P. cablin* against *T. urticae*. The output of the study has been well justified with the generation of key information for the development and utilization of NEs. However, future research regarding basic and fundamental studies on the mechanism of action of the compositional terpenoids is still needed for the development of next-generation bio-acaricides.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KM conducted the experiments. AK conceptualized the research work, analyzed the data, and wrote the manuscript. AD performed the analysis. SS supervised the experiments. BN analyzed the data and edited the manuscript. All authors contributed to the article and approved the submitted version.

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