MEDITERRANEAN DIET AND CANCER: EXPERIMENTAL AND EPIDEMIOLOGICAL PERSPECTIVES

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MEDITERRANEAN DIET AND CANCER: EXPERIMENTAL AND EPIDEMIOLOGICAL PERSPECTIVES

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Editorial: Mediterranean diet and cancer: Experimental and epidemiological perspectives

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Editorial on the Research Topic
Mediterranean diet and cancer: Experimental and epidemiological perspectives

The role of diet in cancer management is gaining more attention and multiple studies suggest nutritional interventions to augment conventional anticancer therapies. Mediterranean diet is one of the healthful dietary patterns. It is composed of many types of foods and drinks rich in antioxidant and biologically active ingredients. This special issue provides new details for researchers, patients, nutritional specialists, and oncologists about the possible role of Mediterranean diet in cancer management.

Consumption of Mediterranean diet is associated with low incidence of breast cancer as shown by Azzeh et al.(a). In this case-control study, researchers concluded that consumption of a diet rich in fruits and vegetables, fish, legumes, black tea, coffee, and low dairy products can significantly reduce the risk of breast cancer [Azzeh et al.(b)]. Spices used in Mediterranean diet were reviewed by Talib et al.. The study showed that black pepper (*Piper nigrum* L.) is the most common spice used in Mediterranean diet. Giger and black cumin were the most active against cancer and apoptosis induction is the most common anticancer mechanism activated by Mediterranean diet spices (Talib et al.). The chemoprevention effect of the Mediterranean diet on colorectal cancer was also investigated by Mahmod et al.. Researchers concluded that components in the Mediterranean diet can reduce the risk of colorectal cancer by reducing inflammation and inhibiting the attachment of pathogenic microbes (Mahmod et al.).

The use of alternative and herbal medicine is increasing among cancer patients. Patients depend on consuming selected herbal infusions or foods containing plant extracts to fight cancer (1). Al-Ataby and Talib showed that daily consumption of lemon and ginger herbal infusion inhibited breast cancer in mice. Phytochemicals in this herbal infusion exhibited high capacity to induce apoptosis, inhibit angiogenesis, and stimulate the immune system (Al-Ataby and Talib). Barley bran grown in Jordan was evaluated by Abuarab and Talib. Results showed anticancer and immunomodulatory effects of barley bran and supported its use as prophylactic agent against cancer (Abuarab and Talib). Aqueous extract of *Elaeagnus* angustifolia flowers inhibited triple-negative breast cancer cells by apoptosis induction as indicted by Fouzat et al.. The anticancer activity of this plant involved activation of P53 and signal transducer and activator of transcription 3 signaling pathways (Fouzat et al.).

Gamal-Eldeen et al. showed that the polysaccharide extract of *Sargassum dentifolium* (an edible brown alga) reduce drug resistance in tongue squamous cell carcinoma by reducing hypoxia.

Gaz-alafi is a local sweet produced mainly in the north of Iraq and west of Iran. Its composition includes secretions from insects and plant products produced from the infected *Quercus brantii* leaves (2). Al Safi et al. showed that aqueous and ethanol extracts of Gaz-alafi are rich in phytochemicals that have anticancer and immunomodulatory effect. Extracts caused regression in tumor growth and stimulation of innate and acquired immunity (Al Safi et al.).

The special issue also discussed the role of intermittent fasting combined with plant extracts to overcome drug resistance. Intermittent fasting is a type of a calorie restriction and involves fasting for 16–48 h. Such fasting stimulates multiple anticancer mechanisms and cause cancer regression (3). Jawarneh and Talib concluded that a combination of Ashwagandha (*Withania somnifera*) root extract and intermittent fasting acts synergistically to overcome cisplatin drug resistance in breast cancer.

Overall, articles included in this issue present a comprehensive scientific contribution to support the use of components in the Mediterranean diet as anticancer nutritional interventions. The special issue also presents the successful use of Mediterranean diet in different combinations to inhibit cancer and reduce drug resistance.

Author contributions

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

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Elaeagnus angustifolia Plant Extract Induces Apoptosis *via* P53 and Signal Transducer and Activator of Transcription 3 Signaling Pathways in Triple-Negative Breast Cancer Cells

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Elaeagnus angustifolia (*EA*) is used as an alternative medicine in the Middle East to manage numerous human diseases. We recently reported that *EA* flower extract inhibits cell proliferation and invasion of human oral and HER2-positive breast cancer cells. Nevertheless, the outcome of *EA* extract on triple-negative breast cancer (TNBC) cells has not been explored yet. We herein investigate the effect of the aqueous *EA* extract (100 and 200 μl/ml) on two TNBC cell lines (MDA-MB-231 and MDA-MB-436) for 48 h and explore its underlying molecular pathways. Our data revealed that *EA* extract suppresses cell proliferation by approximately 50% and alters cell-cycle progression of these two cancer cell lines. Additionally, *EA* extract induces cell apoptosis by 40–50%, accompanied by the upregulation of pro-apoptotic markers (Bax and cleaved caspase-8) and downregulation of the anti-apoptotic marker, Bcl-2. Moreover, *EA* extract inhibits colony formation compared to their matched control. More significantly, the molecular pathway analysis of *EA*-treated cells revealed that *EA* extract enhances p53 expression, while inhibiting the expression of total and phosphorylated Signal Transducer and Activator Of Transcription 3 (STAT3) in both cell lines, suggesting p53 and STAT3 are the main key players behind the biological events provoked by the extract in TNBC cells. Our findings implicate that *EA* flower extract may possess an important potential as an anticancer drug against TNBC.

Keywords: ***Elaeagnus angustifolia*, triple-negative breast cancer, apoptosis, stat3, p53**

INTRODUCTION

Breast cancer (BC) is the most prevalent cancer worldwide and is a commonly diagnosed malignancy in females comprising approximately one-third of all malignancies in women (1). Several genetic alterations, morphological characteristics, clinical outcome, and therapeutic interventions make BC a highly heterogeneous disease. BC is classified into four subtypes (Luminal A, Luminal B, HER-2 positive and triple-negative) to provide clinical utility along with sufficient prognostic and predictive power (2). Of the four subtypes, the triple-negative breast cancer (TNBC) lacks expression of estrogen and progesterone receptors (ER, PR) along with the absence or faint expression of the human epidermal growth factor receptor-2 (HER-2). TNBC accounts for around

12–20% of all breast cancer cases (3, 4); the incidence increases among pre-menopausal females and is more frequent in young women (4, 5). TN tumors are characterized by aggressive behavior with early metastasis to the central nervous system, bone, lung, and liver, along with a short response period to available therapies, poor prognosis and survival as compared to the other BC subtypes (6–8). Unlike ER and HER-2 positive BC, TNBCs are highly resistant to current therapies as they are insensitive to endocrine and molecular-targeted therapies; currently, systemic chemotherapy and surgery are the backbone of TNBC management (6, 9). In general, it is difficult to develop and evaluate novel agents against TNBC due to its extreme biologic heterogeneity. Nevertheless, triple-negative tumors become resistant to therapeutic modalities and can reoccur and develop metastatic abilities. Therefore, there is an urgent need to identify and develop novel potential therapeutic agents against TNBC which are effective with less undesired side effects.

Several complementary and alternative medicines (CAMs) in addition to chemotherapy drugs are largely inspired by nature, mainly plant based phytochemicals are used as a natural source for medical treatments (10, 11). Dietary phytochemicals are naturally occurring bioactive compounds that have the potential to be a competitive alternative for cancer treatment due to their efficacious and protective properties (10, 12). *Elaeagnus angustifolia* (*EA*), is one such a medicinal plant that has been used for centuries in folklore medicine in different parts of the world, especially in the Middle East region (13–15). *EA* is a rich source of vitamins, proteins, calcium, magnesium, potassium, and iron; hence, different parts of *EA*, either fresh or dried are consumed (16, 17). In fact, *EA* is used to treat multiple diseases like asthma, osteoporosis, and rheumatoid arthritis due to their antioxidant, anti-inflammatory, antimicrobial and anticancer properties (16, 18). In this context, it is important to highlight that the bioactive compounds in *EA* like flavonoid, lignanoid and bezenoid can have anti-tumor properties (19–21). However, data exploring the anti-cancer role of *EA* are limited to a few studies. Our group previously reported that *EA* extract suppresses cell invasion of human oral cancer cells *via* the Erk1/Erk2 signaling pathways (22). Another study revealed that hydroalcoholic *EA* flower extract significantly represses angiogenesis, indicating its potential as an anti-cancer drug (23). Our recent investigation on HER2-positive human breast cancer cells pointed out that *EA* aqueous extract significantly inhibits cell proliferation and provokes apoptosis by suppressing both, HER2 and JNK activation (24). However, there are no studies describing the outcome of *EA* extract against TNBC and its underlying molecular mechanisms. Therefore, we herein aimed to explore the potential therapeutic and anti-tumor characteristics of *EA* flower extract on TNBC cells and its mechanism.

In this investigation, we examined the effect of *EA* extract on cell proliferation, cell-cycle progression, apoptosis, and colony formation in two human TNBC cell lines. Our study revealed that EA induced dramatic cell apoptosis in TNBC cells, which occurs *via* p53 and Stat signaling pathways.

MATERIALS AND METHODS

Plant Material Collection and Extraction

Elaeagnus angustifolia flowers were collected from Montreal, Quebec, Canada in June, and the aqueous extract was prepared as described previously by our group (24). Briefly, the flowers were dried and stored in the dark at room temperature. For extract preparation, 3 grams of dried *EA* flowers were boiled in 100 ml of autoclaved distilled water at 100°C for 15 min and stirred continuously. Following boiling, the *EA* flower extract solution was filtered using a sterile filter unit (0.45 μm pore size) and stored at 4°C for future experimental use. Dilutions of *EA* extracts were prepared in cell culture media and for each set of experiments the extract was freshly prepared.

Cell Culture

Two different human TNBC cell lines (MDA-MB-231 and MDA-MB-436) and the non-tumorigenic epithelial cell line (MCF 10A) were commercially obtained from the American Type Culture Collection (ATCC) (Rockville, MD, United States). The cell lines were cultured in Dulbecco's Modified Eagle's Media-high glucose (DMEM, Sigma-Aldrich, St. Louis, MO, United States) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, Massachusetts, MA, United States), 10 mM non-essential amino acids (Gibco, Life Technologies, Massachusetts, MA, United States), 0.5 mM sodium pyruvate (Gibco, Life Technologies, Massachusetts, MA, United States), 2.5 mM L-glutamine (Gibco, Life Technologies, Massachusetts, MA, United States), 1% antibiotic (penicillin-streptomycin, Gibco, Life Technologies, Massachusetts, MA, United States) at 37^{o}C with 5% CO_2 and 85% humidity. All experiments were carried out when the cells had attained 70–80% confluence.

Cell Viability

The TNBC cell lines (MDA-MB-231 and MDA-MB-436) and the non-malignant breast epithelial cell line (MCF 10A) were seeded in 96-well plates with a density of 1×10^4 cells per well. The cells were grown in DMEM medium as described above. MDA-MB-231, MDA-MB-436 and MCF 10A cells were treated with different concentrations (25, 50, 75, 100, 150, and 200 μl/ml) of *EA* extract solution for 48 h. Untreated cells were used as the control and were cultured in 100 μl of media. AlamarBlue^{TM} cell viability assay (Invitrogen, Thermo Fisher Scientific, Waltham, MA, United States) was used to determine the effect of *EA* extract on cell proliferation according to the manufacturer's protocol. After incubating the cells for 4 h with the dye, the shift in fluorescence was measured at two wavelengths, excitation wavelength at 570 nm and emission wavelength at 590 nm using a fluorescent plate reader (Infinite M200, Tecan, Grödig, Austria). The percentage of cell viability was calculated based on the fluorescence of treated cells to untreated cells.

Cell Morphology Analysis

The two TNBC cell lines (MDA-MB-231 and MDA-MB-436) and MCF 10A cells were seeded in 6-well plates (2.5×10^5 cells/well) for 24 h. The old medium was then discarded, and cells were

treated with the half-maximal inhibitory concentration (IC_{50}) of *EA* extract (100 and 200 μl/ml) for 48 h. Cell morphology was examined using a light microscope with 10X magnification after 24 and 48 h. The changes of cell morphology under the effect of *EA* treatment were analyzed by capturing images of the cells with the Leica DFC550 digital camera (Leica Microsystems, Wetzlar, Germany) at 12.5 Megapixel resolution.

Cell Cycle Analysis

For cell cycle analysis, a total of 1×10^6 cells of the TNBC cells were seeded in 100 mm Petri dishes for 24 h. To synchronize the cells into the G0 phase, seeded cells were starved overnight with serum-free DMEM medium. Synchronized cells were treated with *EA* aqueous extract (100 and 200 μl/ml) for 48 h. Post treatment, the cells were fixed with ice-cold 70% ethanol. The DNA was then stained using the FXCycle PI/RNase staining solution (Invitrogen, Thermo Fisher Scientific) at 50 μg/ml according to the manufacturer's recommendations. Cells in the G0/G1, S, and G2/M phases were quantified using the Flow Jo software.

Annexin V Apoptosis Assay

Cellular apoptosis was assessed by the ApoScreen® Annexin V-fluorescein isothiocyanate (FITC)/7-amino-actinomycin D (7-AAD) Apoptosis Kit (SouthernBiotech, United States) as per the manufacturer's protocol. Briefly, TNBC cells were seeded in 100 mm Petri dishes at a density of 1×10^6 cells/dish and incubated overnight. The following day, cells were treated with *EA* extract at a concentration of 100 and 200 μl/ml for 48 h. Adherent and floating cells were harvested by trypsinization, washed twice with cold PBS, and resuspended in Annexin Binding Buffer, then stained with conjugated Annexin V-FITC, PI or both stains for 15 min. After staining, samples were analyzed by BD FACSAria™ II Flow Cytometer and Flow Jo software. The cell population, excluding debris, was gated with forward scatter (FSC-A) and side scatter (SSC-A). Doublets were excluded using FSC-height and FSC-Width plot and singlet cells were then presented as dot plots of FITC-A (annexinV) against PerCP-Cy5.5-A (7-AAD). Quad Gates were used to calculate the percentage of viable cells (annexin V low, 7-AAD low), early pro-apoptotic cells (annexin V high, 7-AAD low) and late apoptosis/necrotic cells (annexin V high, 7-AAD high). Data were presented as density plots of Annexin V-FITC and 7-AAD staining.

Soft Agar Colony Formation Assay

To investigate the ability of TNBC cells to grow in an anchorage-independent manner, soft agar colony formation assay was performed as previously described by our group (25). Briefly, TNBC cells were seeded in 6-well plates (1×10^4 cells/well) with/without 100 and 200 μl/ml of *EA* extract (treated/control, respectively) placed in DMEM medium containing 0.3% agar and plated over a layer of DMEM medium with 10% FBS and containing 0.4% agar. The growing colonies were examined every 2 days for a period of 3 weeks. Colonies in each well were counted under a light microscope, Leica SP8 UV/Visible Laser

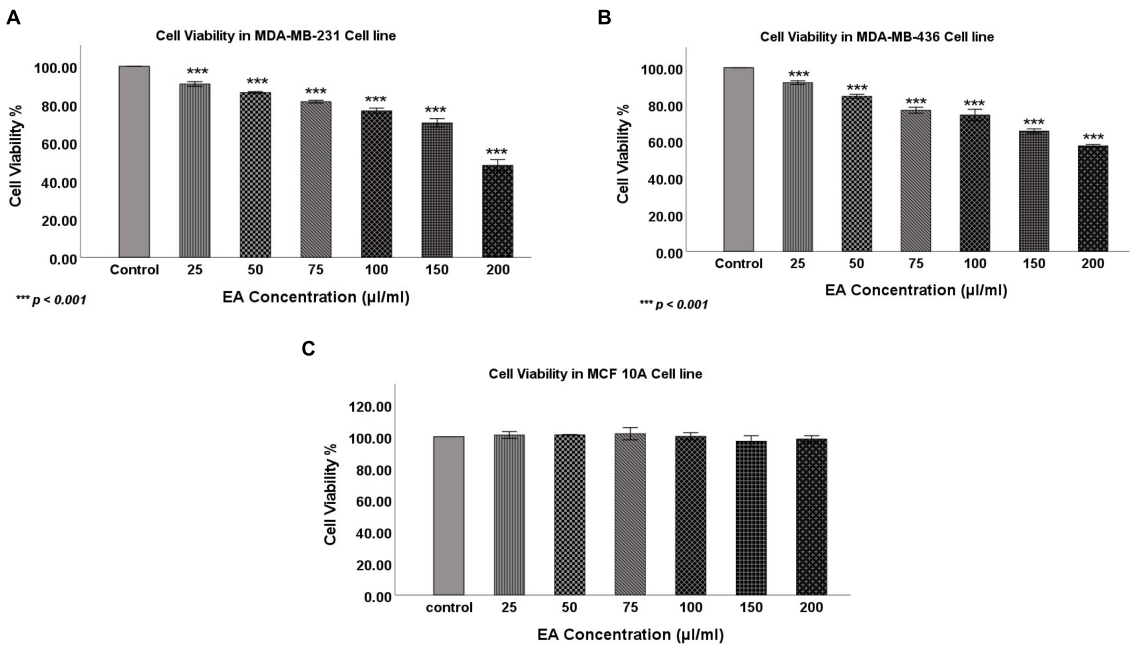

FIGURE 1 | The effect of EA flowers extract at different concentrations on cell viability of the TNBC cell lines **(A)** MDA-MB-231, **(B)** MDA-MB-436 and the **(C)** non-tumorigenic cell line, MCF 10A after 48 h of treatment. Data shows an inverse relation between treatment concentrations and cell viability in both TNBC cell lines, whereas MCF 10A cells' viability was not affected. Data are expressed as a percent of growth relative to control (mean ± SEM; $n = 3$). One-way ANOVA followed by Tukey's *post hoc* test was used to compare the treatment groups and results were considered statistically significant when $p < 0.05$ in comparison with the control. ***$p < 0.001$.

confocal microscope (Leica Microsystems, Wetzlar, Germany), in five predetermined fields.

Western Blot Analysis

The immunoblotting analysis was performed as previously described by our group (20, 25). Briefly, a total of 1×10^6 cells of TNBC cells (MDA-MB-231 and MDA-MB-436) were seeded in Petri dishes and treated with the aqueous extract of *EA* flowers (100 and 200 µl/ml) for 48 h. Protein lysates were extracted from the control and treated cells. Then, equal amounts of protein were run on 10% polyacrylamide gels and transferred onto PVDF membranes. The membranes were incubated with the following primary antibodies overnight; mouse anti-Bax (ThermoFisher Scientific: MA5-14003), mouse anti-Bcl-2 (Abcam: abID# ab692), mouse anti-cleaved caspase-8 (Cell Signaling Technology, CST: mAb #9748), rabbit anti-p53 (Cell Signaling Technology, CST: mAb #2527), mouse anti-Signal Transducer and Activator Of Transcription 3 (STAT3) (Cell Signaling Technology, CST: mAb #9139) and rabbit anti-phosphorylated-STAT3 (Cell Signaling Technology, CST: mAb #9131). To confirm the equal loading of proteins, PVDF membranes were re-probed with rabbit anti-GAPDH (Abcam: abID# ab9485). Post primary antibody staining, membranes were probed with an anti-rabbit IgG-HRP (Cell Signaling Technology, CST:7074S) or anti-mouse IgG-HRP (Cell Signaling Technology, CST: 7076S) secondary antibody.

Chemiluminescence ECL-Western blotting substrate kit (Pierce Biotechnology, Waltham, MA, United States) was used to detect the immunoreactivity as described by the manufacturer. Quantification was carried out by the ImageJ software and the bands' intensities was normalized to GAPDH to determine the relative protein expression in each cell line.

Statistical Analysis

All experiments were analyzed using the IBM Statistical Package for the Social Sciences (SPSS) version 27 software. One-way ANOVA test was performed to analyze the difference between *EA* treated and untreated cells, followed by Tukey's multiple comparisons test. The data was presented as mean ± S.E.M. from three independent experiments ($n = 3$). *P-values* <0.05 were considered significant.

RESULTS

To determine the anticancer activity of the aqueous extract of EA flowers on the TNBC, two cell lines, MDA-MB-231 and MDA-MB-436, were treated with EA extract at different concentrations

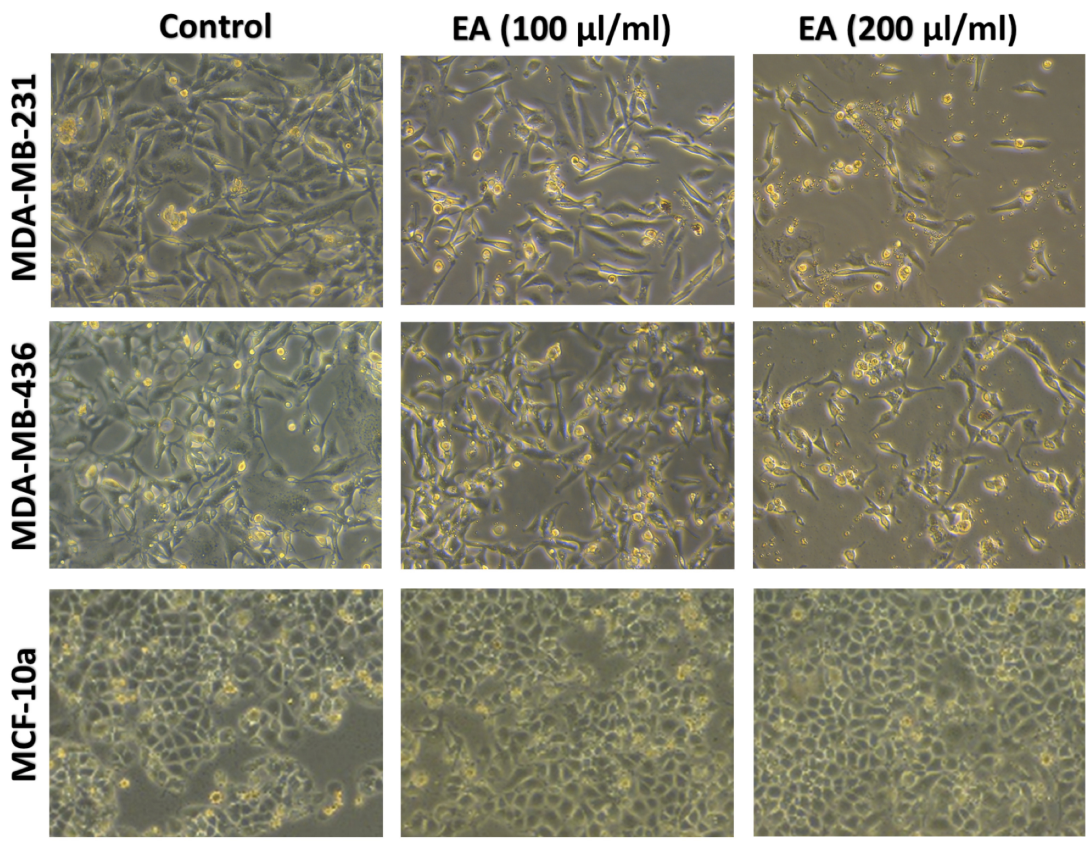

FIGURE 2 | Effect of EA extract on cell morphology of MDA-MB-231, MDA-MB-436 and MCF 10A. We note that treatment for 48 h with 100 and 200 µl/ml of *EA* extract inhibits cell proliferation and induces death in both cell lines, in comparison with untreated (control) which show no cytotoxic effect and display a healthy fibroblast-like phenotype cell. Images were taken at 10x magnification.

(0, 25, 50, 75, 100, 150, and 200 μl/ml) for 48 h. Our data showed that EA extract significantly inhibits the proliferation of both cell lines in a dose-dependent manner (**Figures 1A,B**). More specifically, treatment with 100 and 200 μl/ml inhibited the proliferation of MDA-MB-231 cells by 30 and 55%, respectively, while for MDA-MB-436 cells, proliferation was reduced by 40 and 50%, respectively. Notably, we found that 100 and 200 μl/ml concentrations of the EA extract has no significant effect on cell viability of the non-tumorigenic epithelial cell line, MCF 10A (**Figure 1C**) and hence concentrations of 100 and 200 μl/ml were selected for further investigations in both TNBC cell lines.

Next, we examined the cell morphology of MDA-MB-231, MDA-MB-436 and MCF 10A under the effect of 100 and 200 μl/ml of *EA* extract, using phase-contrast microscopy. As shown in **Figure 2**, untreated TNBC cells (control), MDA-MB-231 and MDA-MB-436, were highly confluent and displayed fibroblast-like phenotype. However, following treatment with 100 μl/ml of *EA* extract, TNBC cells started losing their shape and underwent several morphological changes including deformation, loss of membrane integrity, contact inhibition, cell shrinkage, and formation of apoptotic bodies. At higher concentration of *EA* (200 μl/ml), the morphological changes become more evident with a profound increase in detaching cells and reduction in the number of viable cells, indicating cell death in TNBC. Contrary, this effect was not observed in the non-tumorigenic cell line MCF 10A, as shown in **Figure 2**.

Subsequently, the effect of *EA* on cell cycle progression of TNBC cells was investigated using flow cytometric tool. As shown in **Figure 3**, our data revealed that *EA* extract at 100 and 200 μl/ml concentrations induce a significant cell cycle arrest at the S phase, with a notable decrease in G0/G1 and G2/M phases of both TNBC cell lines, indicating inhibition of cell-cycle progression under the effect of *EA* extract.

To further assess the effect of *EA* extract on cell apoptosis, we performed Annexin V-FITC/7-AAD assay. Our data reveals that *EA* extract significantly induces early and late apoptosis in a dose-dependent manner in both TNBC cell lines (**Figure 4**).

Next, we explored the effect of *EA* extract (100 and 200 μl/ml concentrations) on the colony formation of TNBC cell lines in soft agar over a period of 4 weeks. Our data showed a significant decrease in the number of colonies for both TNBC cell lines under the effect of *EA* extract compared to the control, as shown in **Figure 5**. *EA* extract inhibited colony formation of MDA-MB-231 by 62 and 94% at 100 and 200 μl/ml of *EA* extract, respectively. On the other hand, the number of colonies formed in MDA-MB-436 cells was reduced by 96 and 99% at 100 and 200 μl/ml of *EA* extract, correspondingly, in comparison to the control. These results prove loss of colony-forming ability in both TNBC cell lines upon treatment with *EA* extract, which may reflect the ability to inhibit tumorigenesis *in vivo*.

To further confirm the role of *EA* extract on apoptosis, we examined the expression patterns of key markers of apoptosis

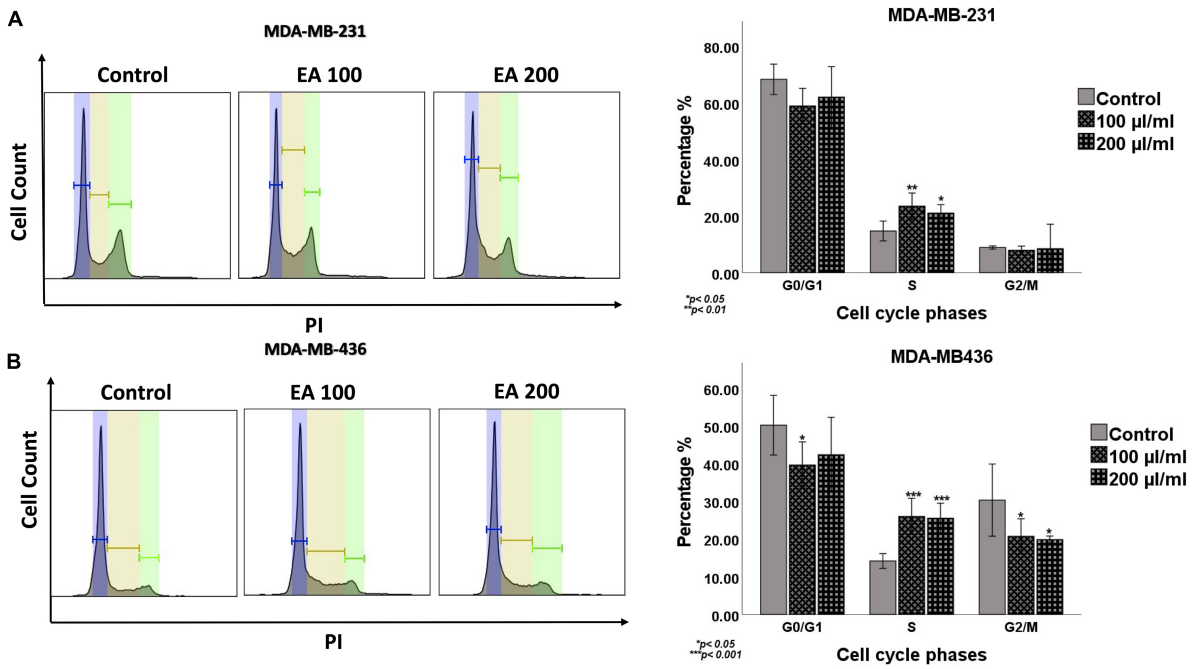

FIGURE 3 | Cell cycle flow cytometry analysis of the TNBC cell lines, **(A)** MDA-MB-231 and **(B)** MDA-MB-436. Cells were treated with 100 and 200 μl/ml of *EA* extract for 48 h followed by staining with propidium iodide (PI). Representative DNA content histogram showing the phases of sub G0, G0\G1, S, and G2/M on tested cell lines upon treatment with *EA* extract compared to their matched control. The cell cycle histogram results reveal that EA extract at these concentrations induce a significant cell cycle arrest at the S phase, with a notable decrease in G0/G1 and G2/M phases. Quantification is represented as the mean ± SEM ($n = 3$). One-way ANOVA followed by Tukey's *post hoc* test was used to compare the treatment groups and statistical significance was indicated when $p < 0.05$. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

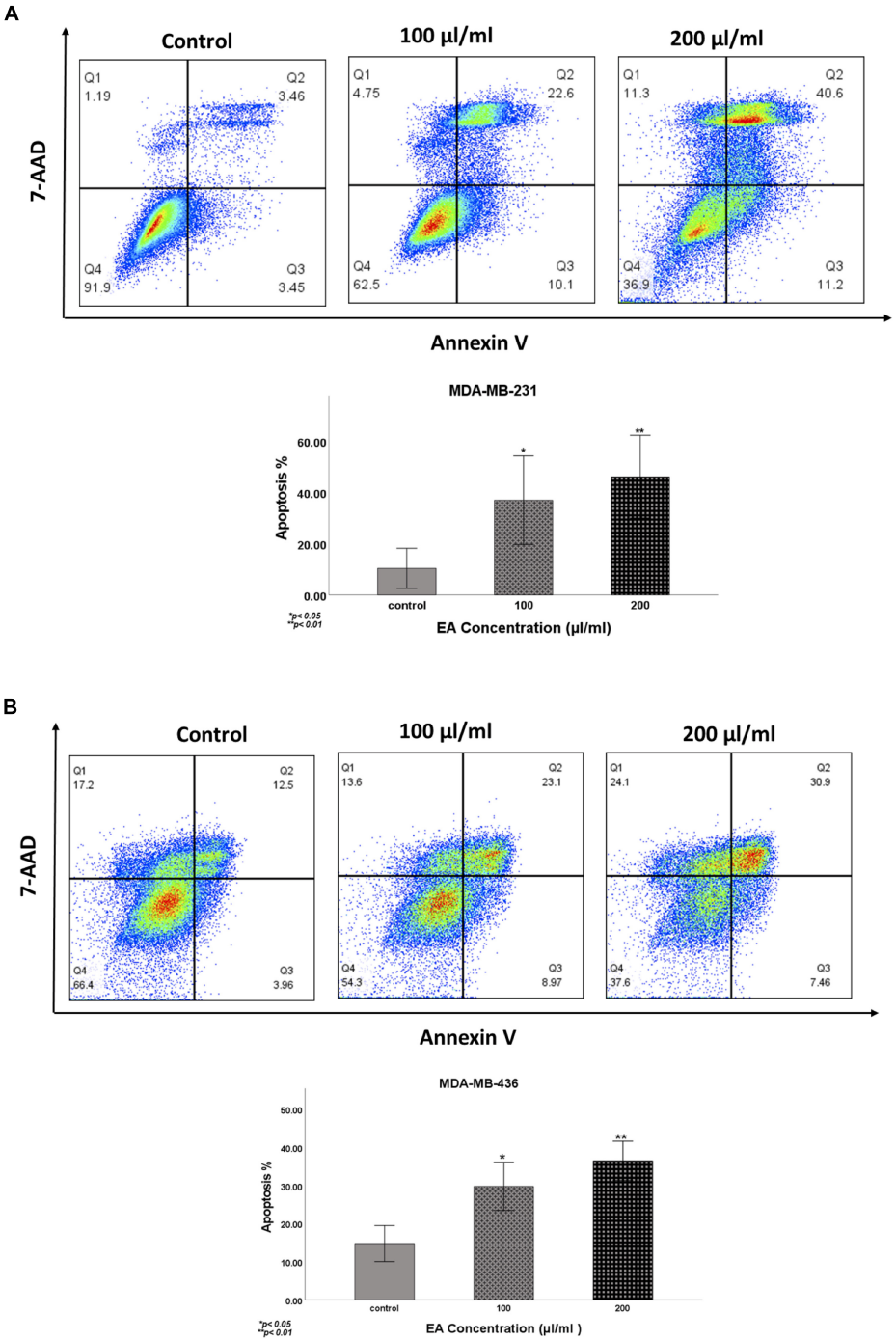

FIGURE 4 | Induction of apoptosis by EA extract in **(A)** MDA-MB-231 and **(B)** MDA-MB-436 cells, as determined by Annexin V-FITC and 7-AAD apoptosis assay. Cells were treated with 100 and 200 µl/ml of *EA* extract for 48 h. Data are presented as Mean ± SEM ($N = 3$). Results were analyzed using One-way ANOVA followed by Dunnett's *post hoc* test. $p < 0.05$ was considered for statistical significance. $^{*}p < 0.05$ and $^{**}p < 0.01$.

in TNBC cells (MDA-MB-231 and MDA-MB-436) following 48 h of treatment with 100 and 200 µl/ml of *EA* extract using western blot analysis. Our data revealed a significant increase in the expression of the pro-apoptotic markers (Bax and cleaved caspase-8) in *EA*-treated cells compared to control, as shown in **Figure 6**. On the other hand, the expression of the anti-apoptotic protein (Bcl-2) was reduced in both cell lines. In parallel, Bax/Bcl-2 ratio was profoundly increased in cells treated with *EA* extract, as seen in **Figure 6**. Taken together, these findings provide evidence that *EA* extract can induce apoptosis in TNBC cells,

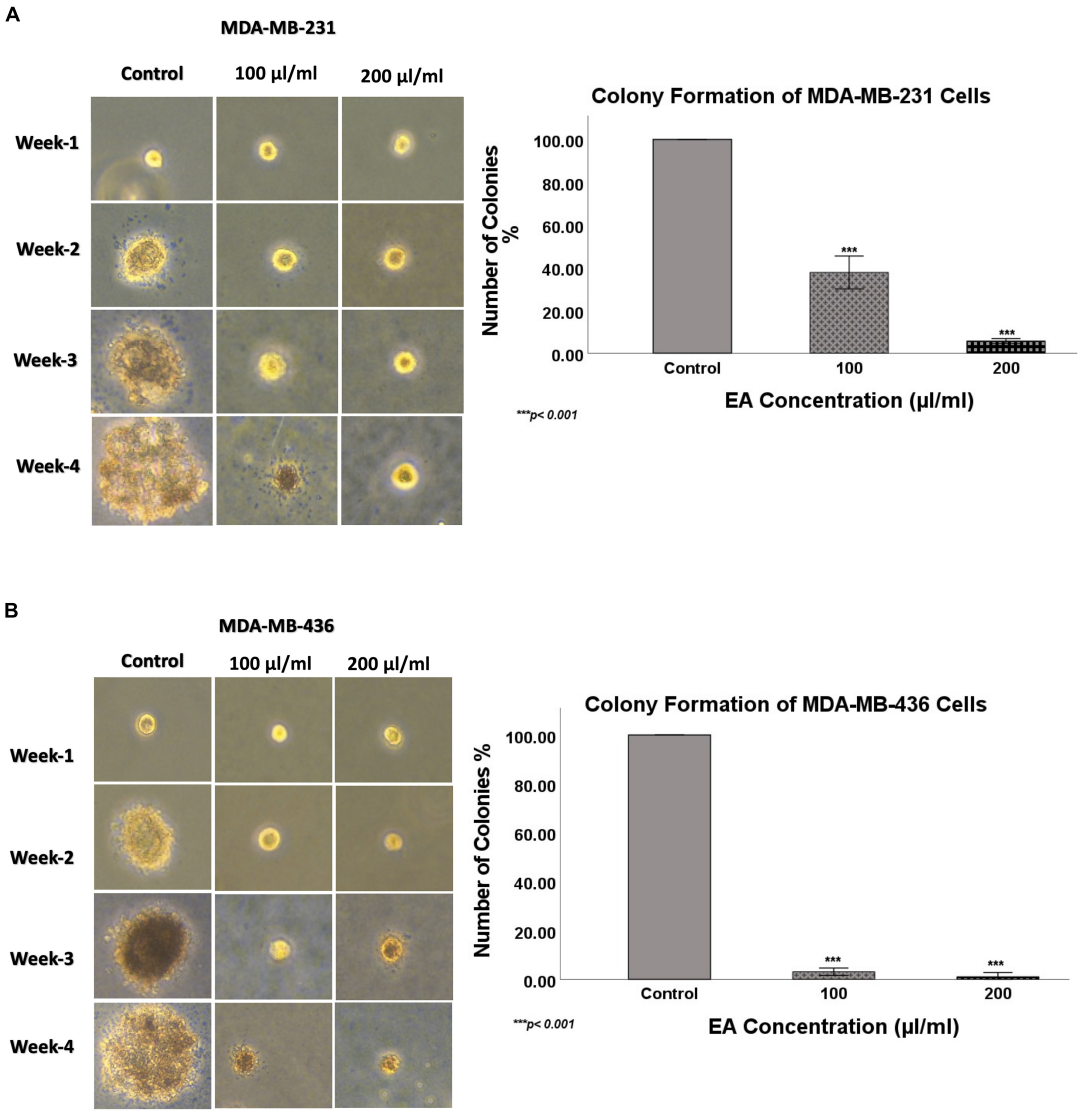

FIGURE 5 | The effect of EA extract on colony formation, in soft agar, in human TNBC cell lines **(A)** MDA-MB-231 and **(B)** MDA-MB-436. *EA* extract inhibits colony formation of MDA-MB-231 and MDA-MB-436, in comparison with their matched control cells. Images were taken at 10x magnification. The colonies were counted manually and expressed as percentage of treatment relative to the control (mean ± SEM; $n = 3$). ***$p < 0.001$.

which is associated with the deregulation of Bcl-2/Bax/caspase signaling pathway.

Regarding the underlying molecular pathways of *EA* extract on cell viability, apoptosis and inhibition of colony formation of TNBC cells, we postulate that p53 and STAT3, which are commonly altered in TNBCs (26, 27), could play a vital role in the regulation of these biological events under the effect of *EA* extract. Thus, we evaluated the expression pattern of p53, STAT3 and p-STAT-3 in MDA-MB-231 and MDA-MB-436 cell lines exposed to *EA* extract in comparison with their matched (untreated cells) controls. We found that treatment with *EA* extract stimulates the expression pattern of p53 whereas, *EA* extract inhibits the expression and phosphorylation of STAT3 in both cell lines in comparison to their control cells.

DISCUSSION

Natural products have continuously proven to be an important and rich source of therapies for a variety of human disorders, including cancer (10, 11). In the present study, we investigated for the first time the effect of *EA* flower extract in TNBC cell lines (MDA-MB-231 and MDA-MB-436) with regards to cell proliferation, morphological changes, cell cycle and apoptosis as well as the underlying molecular pathways. We herein report that *EA* extract can inhibit cell proliferation, alter the normal morphology and deregulate cell cycle progression in addition to the induction of cell apoptosis of TNBC cell lines while having a minimal effect on the growth of non-tumorigenic epithelial cell line, MCF 10A. Moreover, we noted that the aqueous extract of

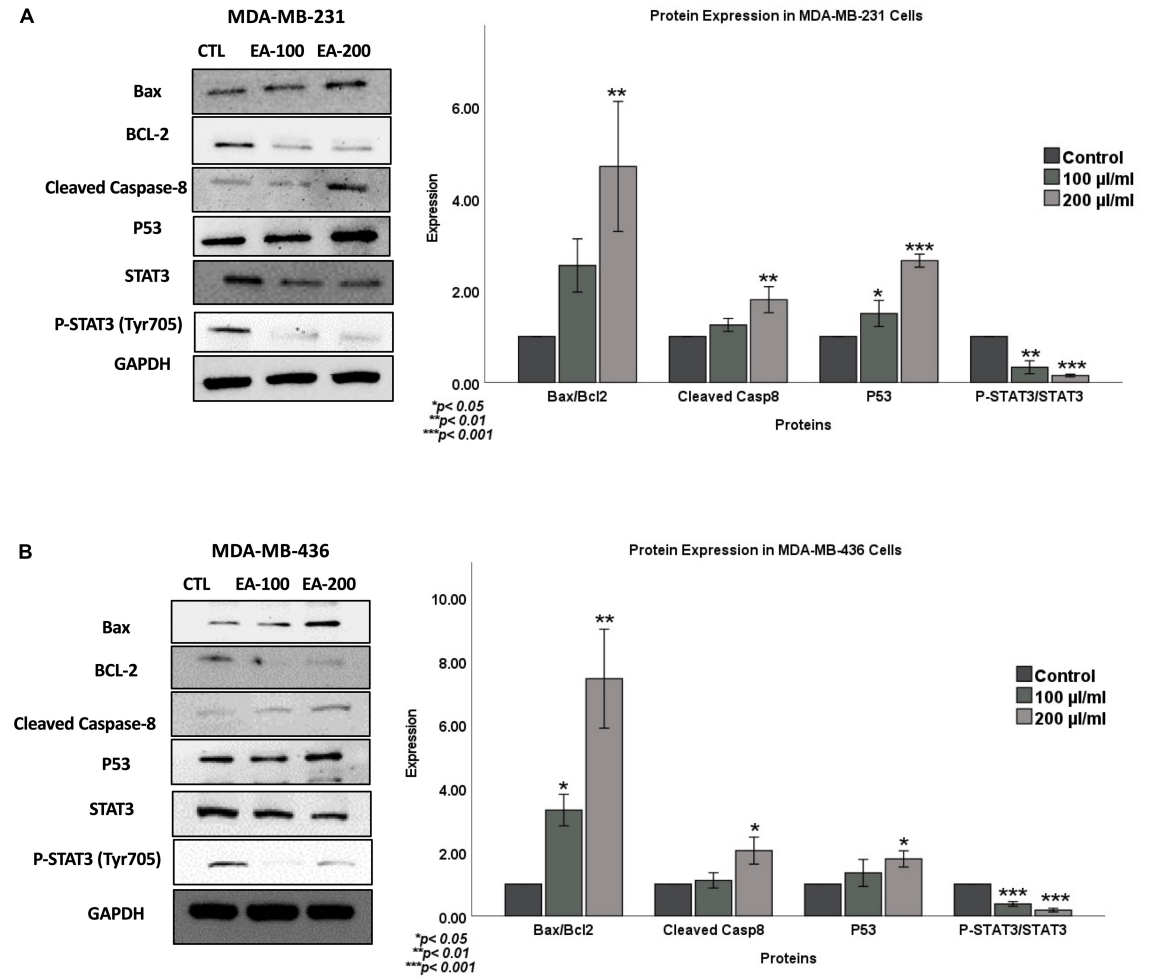

FIGURE 6 | Outcome of EA extract on the expression patterns of Bax, BCL-2, Caspase-8, P53 and STAT3 in **(A)** MDA-MB-231 and **(B)** MDA-MB-436 cell lines. We note that *EA* extract induces an overexpression of the pro-apoptotic markers (Bax and Caspase-8) in comparison with their control, while anti-apoptotic marker Bcl-2 is inhibited. Furthermore, *EA* plant extract enhances the expression of p53 while inhibits phosphorylation of STAT3. GAPDH served as a control in this assay. Cells were treated with 100 and 200 µl/ml of *EA* extract for 48 h as explained in the materials and methods section. Values were corrected for the expression of the housekeeping protein GAPDH and presented as fold change of control. Data were analyzed using One-way ANOVA followed by Dunnett's *post hoc* test. **P-value<0.05* was considered for statistical significance. **p* < 0.05, ***p* < 0.01, *and* ****p* < 0.001. Data are presented as a percentage of treatment relative to the control (Mean ± SEM; *n* = 3).

EA flower can inhibit colony formation of TNBC cells, which correlates with *in vivo* tumor inhibition. In our laboratory, we also found that *EA* extract significantly enhanced the survival rate of both, wild-type and K-RAS mutant *Drosophila melanogaster* flies, which are prone to developing colorectal cancer. This *in vivo* data show clearly that *EA* extract can block colorectal cancer growth, indicating anti-cancer role of *EA* extract (in preparation).

Elaeagnus angustifolia plant is traditionally used for centuries as an analgesic, antipyretic, anti-inflammatory, antioxidant, and diuretic herbal medicine (13–15, 28). Moreover, several bioactive compounds are identified in *EA*, such as phenolic acids and flavonoids, which are thought to play a role in preventing cancer development and progression (18, 20). Studies have demonstrated that these compounds can regulate multiple cellular processes such as DNA repair, cell cycle progression, induction of apoptosis and cell signaling cascades (29, 30). Recently, there has been considerable interest in their potential therapeutic utility as chemo-preventive and/or anticancer agents. Nevertheless, to the best of our knowledge, the effect of this medicinal plant on TNBC has not been previously explored. Therefore, in this study we evaluated the cytotoxic potential of *EA* in TNBC. Particularly, *EA* flowers, which is commonly consumed as tea beverage in several cultures and especially the middle east, were extracted by decoction and tested on TNBC cell lines to examine the outcome of *EA* flowers dietary intake on TNBC (31).

On the other hand, uncontrolled cell proliferation and evasion of apoptosis are well-recognized hallmarks of cancer and vital components of treatment resistance; hence, targeting associated deregulated pathways is considered as the main cancer therapeutics tool (32, 33). Our data reveals that *EA* plant

extract inhibits cell proliferation of TNBC cells, meanwhile it provokes cell apoptosis in our TNBC cell models, which is accompanied by upregulation of proapoptotic proteins (Bax, cleaved caspase-8) and downregulation of anti-apoptotic protein (Bcl-2). Interestingly, our data of non-tumorigenic cells, show that *EA* extract has a very minimal or no toxic effect in the control cell line, MCF10, suggesting a selective cytotoxic activity in breast cancer cells, which is a favorable property in the development of anticancer agents. These findings are in concordance with our recent reports on the anticancer potential of *EA* against human oral and HER2 + breast cancer cells (22, 24). Interestingly, the underlying molecular mechanisms preventing tumor progression in oral and HER2 + cells targeted different pathways. In oral cancer cells, EA inhibited angiogenesis and cell invasion *via* Erk1/Erk2 signaling pathways (22). On the other hand, in HER2 + cells, we found *EA* to inhibit epithelial-mesenchymal transition and provoke apoptosis *via* HER2 inactivation and JNK pathway (24).

Analysis of the underlying molecular pathway reveals that *EA* extract provokes apoptosis, which might be at least partially mediated through the upregulation of the tumor suppressor gene, p53 and the accompanied suppression of STAT3 signaling. P53 controls the transcription of proapoptotic members of the Bcl-2 family, such as Bax, Bid, Noxa, and Puma, resulting in apoptosis induction (34, 35). We thus analyzed the expression of the tumor suppressor gene p53. Notably, we observed that *EA* extract significantly upregulates the expression of p53 and its downstream signaling (Bax). On the other hand, STAT3 possesses oncogenic potential and contributes to cancer cell proliferation, anti-apoptosis, migration, invasion, immune suppression, stemness and resistance to chemotherapy (26, 36, 37). Recent evidence has demonstrated that STAT3 is overexpressed and constitutively activated in TNBC, which is associated with the initiation, progression, and metastasis of TNBC (26, 38). The oncogenic potential of STAT3 is triggered by its phosphorylation on Tyr705, which results in homo- or hetero-dimerization of STAT3 followed by nuclear translocation, binding STAT3 to specific DNA response elements and activating target genes (39–41). STAT3 regulates the expression of several proteins, including proliferation regulatory proteins (cyclin D1 and survivin) and anti-apoptotic proteins (Bcl-2 and Bcl-xl) (41–43). Interestingly, our data demonstrate that EA extract inhibits the expression of STAT3, phospho-STAT3, as well as their target genes, including the anti-apoptotic protein Bcl-2. These findings were correlated with increased apoptosis as indicated by the accumulation of caspase-3 and caspase-8, leading to increased numbers of apoptotic cells as evidenced by the Annexin-V staining. Taken together, these results suggest that EA extract induced apoptosis could be mediated through STAT3 dependent mechanism.

Interestingly, given that STAT3 and p53 have opposing roles, where p53 promotes the apoptotic pathway and activation of STAT3 upregulates survival signals; it has been shown that p53 and STAT3 engage in an interplay where they negatively regulate each other (44, 45). Several studies have shown that blocking STAT3 activity in cancer cells promotes the expression of p53, resulting in p53-mediated cell apoptosis (45, 46). More specifically, Lin et al. reported that expression of wild-type p53 but not mutant p53 significantly reduces tyrosine phosphorylation of STAT3 and inhibits STAT3 DNA binding in DU145 prostate cancer cell line (47). Moreover, several studies have shown an association between STAT3 activation and p53 mutations with therapy resistance in cancer (45, 48, 49). Therefore, dual targeting of p53 and STAT3 could be a promising approach to overcome therapy resistance. Collectively, our data reveal that *EA* could simultaneously upregulate p53 while downregulating STAT3 signaling, suggesting that it may serve as a potential effective treatment in TNBC. Future work includes extracting and analyzing the phytochemical components of the *EA* extract and testing the anti-cancer efficacy of these phytochemicals.

CONCLUSION

Our present study establishes for the first time the anticancer potential of *EA* flower extract against TNBC and its molecular signaling pathway. Moreover, we herein report EA extract provokes cell apoptosis in TNBC cells; this is accompanied by the deregulation of proapoptotic and anti-apoptotic genes. Additionally, our data highlight the role of p53 and STAT3 pathways as a potential target for TNBC therapy *via* natural products. Collectively, this study demonstrates the role of *EA* as a promising therapeutic candidate for breast cancer management, particularly the TNBC subtype.

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

A-EA: conceptualization. AF and OH: methodology, validation, and data curation. A-EA, HA-F, and AK: resources, writing—review and editing, and supervision. AF, OH, and IG: writing—original draft preparation. AK and HA-F: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Anti-hypoxic Effect of Polysaccharide Extract of Brown Seaweed *Sargassum dentifolium* in Tongue Squamous Cell Carcinoma

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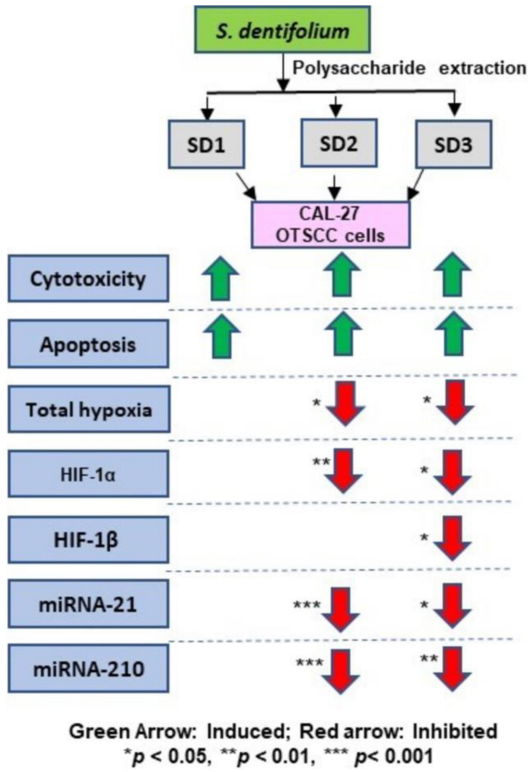
Sargassum dentifolium, (Turner) C. Agarth, 1820, is an edible brown alga collected from red seashores, Egypt. Oral tongue squamous cell carcinoma (OTSCC) is an aggressive malignancy. Hypoxia leads to chemotherapeutic resistance. This work aimed to explore the anti-hypoxia effect of water-soluble polysaccharide fractions of *S. dentifolium* (SD1–SD3) in CAL-27 OTSCC cells. Cell cytotoxicity assay (MTT); cell death mode (DNA staining); total hypoxia (pimonidazole), HIF-1α (ELISA and immunocytochemistry), HIF-1β (ELISA), and hsa-miRNA-21-5p and hsa-miRNA-210-3p (qRT-PCR) were investigated. SD1 and SD2 showed a cytotoxic effect due to apoptosis. SD2 and SD3 decreased total cell hypoxia, inhibited miR-210 ($p < 0.001$ and $p < 0.01$), miR-21 ($p < 0.01$ and $p < 0.05$), and HIF-1α ($p < 0.01$ and $p < 0.05$), respectively. However, only SD3 suppressed HIF-1β ($p < 0.05$). In conclusion, SD2 showed a potential anti-hypoxia effect through amelioration of HIF-1α regulators, which may help in decreasing hypoxia-induced therapeutic resistance.

Keywords: *Sargassum dentifolium*, miR-21, miR-210, HIF-1α, HIF-1β, CAL-27 OTSCC cells

INTRODUCTION

Among brown algae, *Sargassum* is a common tropical/subtropical family that included 150 species (1). A battery of edible *Sargassum* algae that provide a rich source of biologically active polysaccharides, such as *Sargassum stenophyllum*, *Sargassum latifolium*, *Sargassum fulvellum*, and others [reviewed in (2)]. For example, polysaccharide extracts from *Sargassum asperifolium* polysaccharide extracts have been reported to act as tumor anti-initiation activity as well as anti-promotion property *via* their anti-inflammatory activity and a specific anti-progression activity against HepG2 (3). Similarly, the Gamal-Eldeen group (4) reported that *S. latifolium* polysaccharide extracts exhibit promising cancer chemopreventive activity as tumor anti-initiating and anti-promoting agents with specific anti-progression activity against leukemia. Additionally, *Sargassum wightii* polysaccharide extract has been proved to have anti-inflammatory activity (1), antimetastatic activity (5) and a preventive property against oxidative liver injury (6).



GRAPHICAL ABSTRACT | Anti-hypoxic activity of *S. dentifolium* through multiple mediators.

Moreover, *Sargassum duplicatum* and *S. ilicifolium* in a mixture with other plants have been reported to enhance wound healing in skin (7).

Oral tongue squamous cell carcinoma (OTSCC), as one of the widespread oral cavity malignancies, is a strongly aggressive neoplasm that shows a fast local invasion/spread (8) as a result of a high growth rate and metastasis (9). Nearly 50% of patients have been lately diagnosed in Stages III and IV in the initial diagnostic examination (10). OTSCC has a significant elevated incidence in young to middle-age populations (8), with a poor prognosis, characteristic aggressiveness, and high mortality (19% of patients) (11). OTSCC often leads to functional problems in deglutition, mastication, and speech (12). Although chemotherapy may diminish tumor size and metastasis in OTSCC (12), still, the 5-year survival rate is ∼50% (13).

Hypoxia plays a serious role in the pathophysiology of several human disorders, such as cancer chronic lung disease and ischemic cardiovascular disease (14). In tumor microenvironment (TME), uncontrolled rapid proliferation restricts the oxygen availability (hypoxia) in all solid tumors, where a drop in the normal oxygen (2–9%) down to the hypoxic level <2% occurs (15). The adaption of tumor cells to hypoxia resulted in far aggressive and therapeutically resistant phenotypes. Hypoxia stimulates gene expression alterations followed by consequent proteomic changes that affect cellular and physiological functions (15). Among these changes, cells in TME hypoxic regions divide slowly and escape cytotoxic drugs, which are regularly targeting the rapidly dividing cells (16). In TME, hypoxia produces oxygen gradients that participate in the heterogeneity and plasticity of tumors and evoke aggressive and metastatic phenotypes. During hypoxia, the principal event is the stimulated expression of hypoxia-inducible factor-1α (HIF-1α) that implements a functional role in the cellular consequences prompted in response to hypoxia (17, 18).

Herbal nutraceuticals are functional phytochemicals derived from plants and algae (19). They are nontoxic edible supplements that exhibit broad-spectrum medicinal properties and provide efficient protection against many diseases including cancer (20). Growing studies demonstrate that herbal nutraceuticals could act as safe and effective agents against hypoxic cancer cells *via* successfully attenuating their growth, survival, and progression through the inhibition of HIF-1-signaling pathways (20). Recently, many hypoxia-inhibition strategies have been used as a therapeutic approach to treat cancer through regulating/targeting of HIF-1α to overcome the cell resistance due to hypoxia in solid tumors and through suppressing the hypoxia-stimulated resistance to chemotherapies (21). Among these strategies, using algal and herbal nutraceuticals for targeting downstream HIF-signaling pathways has been done through different approaches, including direct inhibition of HIFs using anticancer agents, blocking the dimerization of HIF-1α and β subunits, and silencing by HIFs-siRNA. Many of herbal and marine agents inhibit the hypoxia-associated resistance to chemotherapies by provoking the activation of an HIF-1α degradation cascade and diminishing the overexpressed HIF-1α in hypoxic tumors [reviewed in (20, 22)]. Extracts of marine algae and sponges from the NCI Open Repository have shown substantial activities as inhibitors for the HIF-1 activation in cell-based assays (23), for example, the red alga *Laurencia intricata* product inhibited HIF-1 activation (24).

In our recent work, we have reported the mechanistic anti-hypoxic role of the *S. latifolium* extract in HCT-116 cells, where the extract has been found to inhibit the hypoxia regulators miRNA-21 and miRNA-210, and consequently suppress HIF-1α and HIF-1β (25). We previously investigated the anti-cancer activity of the polysaccharide extract of the edible alga *S. dentifolium* that indicated its apoptotic activity *via* inducing histone acetylation. Additionally, the extract fractions showed potential anti-genotoxic, anti-mutagenic, and antioxidant activities (26). In the current study, we investigated the influence of *S. dentifolium* polysaccharide extracts on the hypoxia pathway in OTSCC as a continuation of our previous study and due to the lack of studies on *S. dentifolium*, which may help in decreasing hypoxia-induced therapeutic resistance in OTSCC.

MATERIALS AND METHODS

Extraction of *Sargassum dentifolium* Polysaccharides

Sargassum dentifolium (Turner) C. Agardh was gathered from Hurghada, Red Sea governorate, Egypt, in December 2018. After

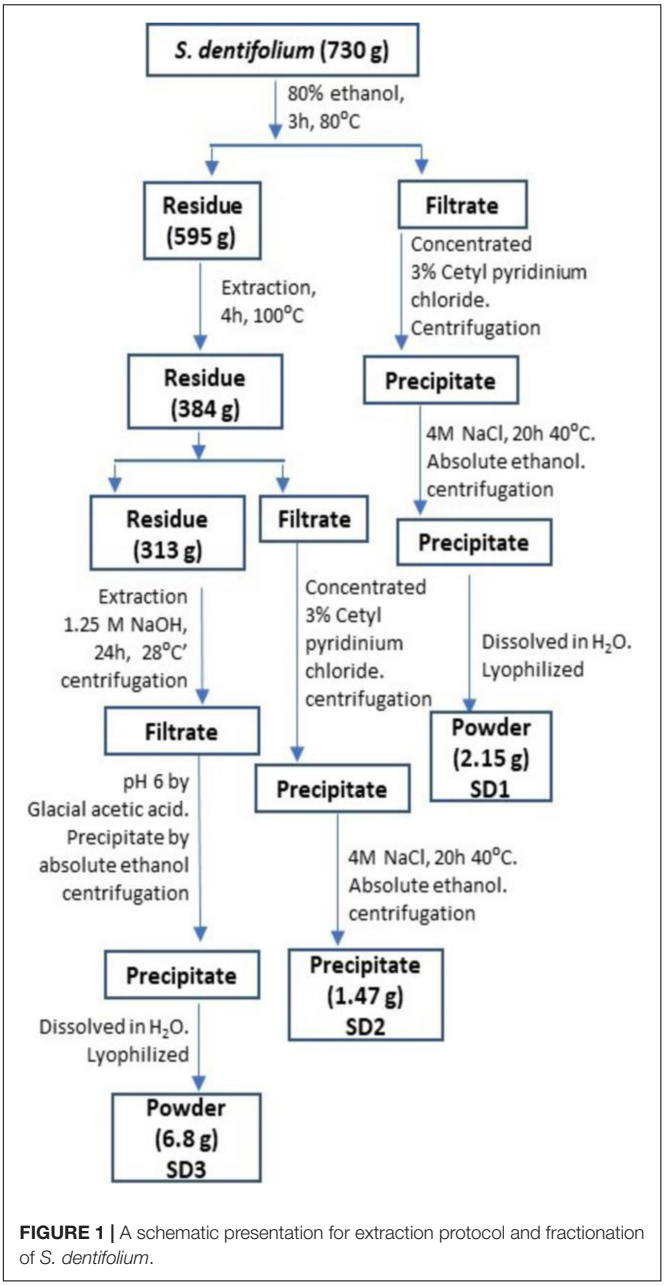


FIGURE 1 | A schematic presentation for extraction protocol and fractionation of *S. dentifolium*.

several washes and drying, the algal mass was grounded by electric mill and sieved through 2-mm mesh. The extraction of water-soluble polysaccharides has been implemented according to the Zhuang group (27), with some modifications (26), as described in a schematic presentation in **Figure 1**. The powdered extract fractions (SD1, SD2, and SD3) were investigated for their anti-hypoxic effect.

Cytotoxicity and Cell Death Mode

Human tongue squamous carcinoma CAL-27 cells (ATCC, United States) were regularly cultured in supplemented DMEM. Chemicals, including celastrol and cell culture materials, were purchased from Sigma–Aldrich (VA, United States), unless mentioned. The cell vitality was tested using a metabolic cytotoxicity MTT assay (28) after 48 h of cell co-culturing with the extract fractions. The data are expressed as % of control cells (mean ± standard deviation). The half-maximal inhibitory concentration (IC_{50}) was calculated for the extract fractions. Cells (5×10^3 cells/well) were co-cultured with IC_{50} of fractions, or celastrol, before being stained with ethidium bromide/acridine orange (100 μg/ml; V/V; EB/AO in PBS) (29). The stained cells were analyzed by a fluorescence microscope ($n = 8$; 200×; Axio Imager Z2, Carl Zeiss, Germany).

Estimation of Total Cellular Hypoxia, HIF-1α, and HIF-1β

Monitoring of the changes in the cellular hypoxia before/after the exposure to extracts was assessed by pimonidazole, a hypoxia-detection reagent, by microplate fluorometer for a qualitative assessment of hypoxia. CAL-27 cells were treated with 30% of IC_{50} of fractions for 6, 12, 24, and 48 h. In another experiment, cells (5×10^4 cells/well) were seeded with 30% of IC_{50} of fractions for 48 h. The cells were lysed by Cell Lysis Solution (#LSKCLS500; Merck, United States) that was supplemented with Protease Inhibitor Cocktail (#P8340; Merck, United States). The cell lysates were then submitted to the analysis with either Human HIF-1α ELISA Fluorescent Kit (#ab229433; Abcam, Germany) or Human ARNT/HIF-1 beta Colorimetric ELISA Kit (#LS-F9594; LifeSpan Biosciences, United States). For the immunocytochemical analysis, CAL-27 cells were cultured onto 8-chamber slides and then treated with 30% of IC_{50} of SD2 for 48 h. The cells were fixed with absolute methanol and then stained using a rabbit monoclonal anti-HIF-1α antibody (Abcam, ab179483), Goat Anti-Rabbit IgG-Phycoerythrin (Abcam, ab72465), and Hoechst 33342 (DNA counterstaining). The cells were analyzed under a fluorescence microscope (Zeiss, Goettingen, Germany), attached to a digital camera (AxioCam MRc3 S/N 4299, Zeiss, Germany) and equipped with an image analyzer (ZEN-11 edition software).

miR-210 and miR-21 Expression

CAL-27 cells (1×10^6 cells) were treated for 48 h, 30% of IC_{50} of fractions, and then, after, the total RNA was extracted by a miRNeasy RNA extraction kit (#217004, Qiagen, Germany). One microgram of RNA was submitted to reverse transcription by a miScript II RT kit (#218161, Qiagen, Germany). PCR amplification (3-ng cDNA) implemented by a miScript Sybr green PCR kit (#218073, Qiagen, Germany). Primers for U6 (#600750, Agilent technologies), as well as hsa-miR-21 and hsa-miR-210 (#MS00009079 and #MS00003801 Qiagen, Germany), were used. Calculations of the relative miRNA expression were carried out by ΔΔCt protocol (30) after normalization with U6 expression in control cells.

Statistical Analysis

Experimental analyses were repeated independently ($n = 6$), and the results were presented as mean ± SD. Graphpad Prism software V6 was used. Data were analyzed by Dunnett's

multiple comparisons test after one-way ANOVA. The data were considered significant when $p < 0.05$.

RESULTS

Cell Viability and Cell Death Mode

The possible cytotoxicity effect of the extract fractions, compared with the anticancer drug celastrol, was investigated by MTT after 48 h of co-culturing with CAL-27 cells. The readings showed that the fractions exhibited a variable cytotoxic effect, where they reduced the cell viability with IC_{50} of 63.36, 50.27, and 99.49 μg/ml for SD1, SD2, and SD3, respectively (**Figure 2A**). Celastrol showed a concentration-dependent cytotoxicity with IC_{50} 8.73 μg/ml. Except in the analysis of a cell death mode, 30% of IC_{50} of each fraction was used in all of the next investigations. The treatment with this IC_{50} percentage leads to a high viability percentage ranged from 84 to 90%, which represents a safe dose to evaluate the protein and genetic changes, as concluded from the concentration/viability equation. AO/EB was used as dual DNA-staining to monitor the cell death mode in CAL-27 cells. After 48 h of incubation with IC_{50} of each agent, the overall analysis of the harvested cells indicated that the treatment with celastrol resulted in a high remarkable increase in the percentages of early apoptotic cells (30.71%; $p < 0.001$) and late apoptotic/necrotic cells (19.12%; $p < 0.001$), compared to their corresponding control, 6.67 and 3.33%, respectively (**Figure 2B**). In the other hand, the treatment of cells with IC_{50} of fractions led to a noticeable elevation in the early apoptotic population in SD1-treated cells (40.01%; $p < 0.001$) and in SD2-treated cells (27.23%; $p < 0.001$) and a parallel increase in the percentage of late apoptotic/necrotic cells in SD1- and SD2-treated cells ($p < 0.01$ and $p < 0.05$, respectively), as shown in **Figure 2B**.

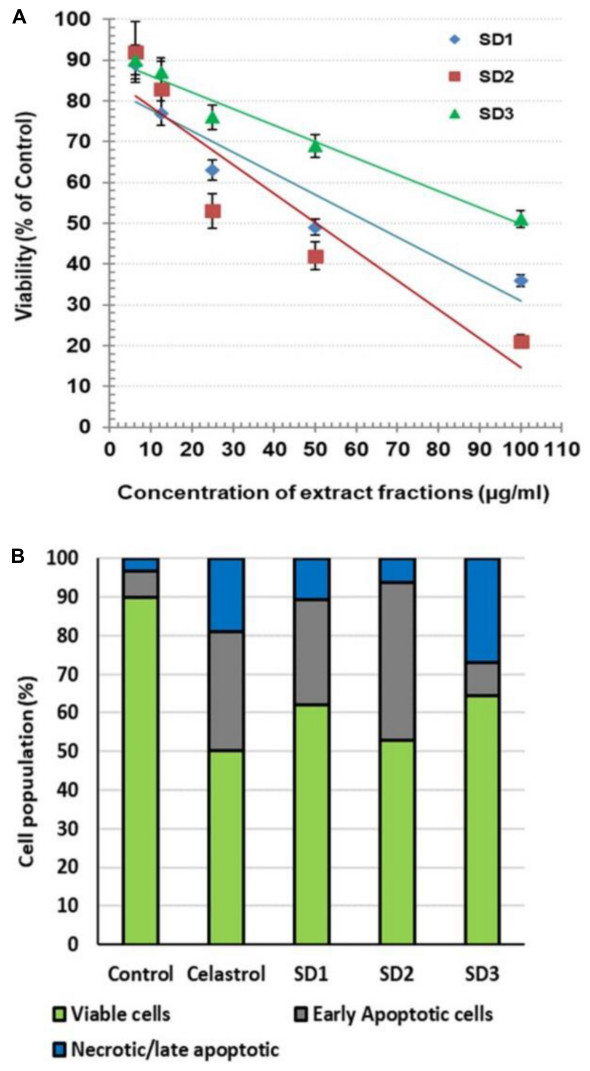


FIGURE 2 | (A) Cytotoxic effect of *S dentifolium* extract fractions (SD1, SD2, and SD3) on CAL-27 cells after 48 h of incubation, as assayed by MTT. The viability is expressed as percentage of control (mean ± SD; $n = 8$). **(B)** A segmented histogram indicates the percentage of cell population distribution of vital, early apoptotic, and late apoptotic/necrotic cells after being treated with IC_{50} of celastrol or *S dentifolium* fractions (SD1, SD2, and SD3) for 48 h compared to control.

Monitoring of Cellular Hypoxia

Follow-up of time-dependent alterations in the cellular hypoxia was carried out by pimonidazole, an exogenous nontoxic 2-nitroimidazole. It interacts with the proteins and forms fluorescence adducts, especially with thiol groups, in the hypoxic cells. The fluorescence intensity of these adducts was qualitatively estimated by a microplate fluorescence reader to assess hypoxia occurrence. The cells were co-cultured with 30% of IC_{50} of each fraction for 6, 12, 24, and 48 h. The experiment indicated that SD1 led to a nonsignificant decrease in cell hypoxia ($p > 0.05$), as shown in **Figure 3A**. In the other hand, SD2 and SD3 resulted in a dramatic time-dependent inhibition in the cell hypoxia, especially after 24 and 48 h ($p < 0.01$; $p < 0.001$, respectively), **Figure 3A**, where the high maximal inhibitory time for hypoxia (t_{50}) for SD2 and SD2 was 25.38 and 31.71 h, respectively.

miR-210 and miR-21 Expression

To explore the influence of the fractions on the epigenic regulators of cellular hypoxia, the expression of miR-210 and miR-21 was traced by qRT-PCR. As a master hypoxamiR, the oncomiR miR-210 is upregulated, generally, in tumor cells. Perusing of the effect of extract fractions (30% of IC_{50}) after 48 h, the results revealed that miR-210 expression was significantly diminished in SD2- and SD3-treated cells, reaching.21 ± 0.03 ($p < 0.001$) and.42 ± 0.06 ($p < 0.01$) fold of the control cells, respectively (**Figure 3B**). As a potent oncomiR, miR-21 shows a high expression in various tumor cell types. Furthermore, it acts as a pivotal hypoxamiR during a hypoxia cascade. The treatment with 30% of IC_{50} of fractions for 48 h resulted in a dramatic downregulation of miR-21 expression in the cells treated with SD2 and SD3 down to 0.32 ± 0.04 ($p < 0.01$) and 0.57 ± 0.08 ($p < 0.05$) fold of the control cells, respectively (**Figure 3B**). These findings suggest that SD2 is a potent inhibitor of hypoxia and its regulators.

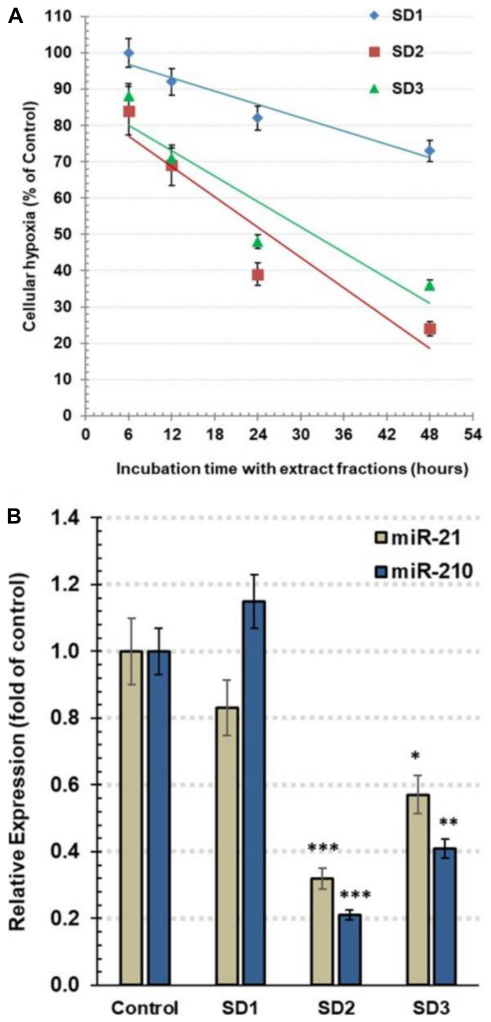


FIGURE 3 | (A) Estimation of the total hypoxia degree by pimonidazole in CAL-27 cells. The cells were co-cultured with 30% of IC_{50} of each fraction for 6, 12, 24, and 48 h. The results are expressed as percentage of control (mean ± SD; $n = 6$), where control was 1,374 IFU. **(B)** The relative expression of hsa-miRNA-21-5p and hsa-miRNA-210-3p in CAL-27 cells after being treated with 30% of IC_{50} of fractions for 48 h. Data are expressed as mean ± SD, $n = 8$. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the corresponding control.

Detection of HIF-1α and HIF-1β

Hypoxia-inducible factor-1α and HIF-1β are hypoxia indicators that are controlled, generally, by miR-210 and by miR-21 among other miRNAs. Using ELISA, the influence of the fractions on the protein concentrations of HIF-1α and HIF-1β was investigated in CAL-27 cells after 48 h from treatment. Commonly, the elevated HIF-1α is an indication of cellular hypoxia. SD2 was the most potent HIF-1α inhibitor among the tested fractions ($p < 0.01$) after 48 h of treatment (**Figure 4A**). The immunochemical analysis of the cells confirmed the diminished HIF-1α concentration in SD2-treated cells (**Figure 4B**), compared to untreated cells.

DISCUSSION

Herbal nutraceuticals suppress the pathways that induce HIF-1α synthesis and enhance its degradation and reduce its downstream-signaling events (20). Above exerting these anti-cancer activities, herbal nutraceuticals have been reported to (a) reverse hypoxia-stimulated drug resistance, (b) ameliorate the therapeutic index of cancer therapies against hypoxic tumors, and (3) diminish the harmful adverse effects of those therapies. The mechanisms of action of these nutraceuticals and their potent chemo- and radio-sensitization on hypoxic tumor cells are variable and depend on the nutraceuticals type and properties [reviewed in [20], [22]]. Therefore, the current study investigated the effect of *S. dentifolium* polysaccharide extracts on the hypoxia pathway in OTSCC, which may provide a new adjacent agent that lowers OTSCC hypoxia-associated resistance to chemotherapies.

Under normoxic conditions, in the ubiquitin-dependent mechanism, O_2-dependent prolyl hydroxylation targets α subunit of the ubiquitin–proteasome pathway and, hence, degrades HIF-1α (31). Subsequently, HIF-1α remains in a steady state and in a low concentration that prevents the formation of the transcriptional functional complex: HIF-1α/HIF-1β. In the other hand, in severe hypoxic conditions, HIF-1α subunit is mainly the responding part of the HIF-1 complex, and the degradation pathway is inhibited; therefore, HIF-1α concentration is rapidly elevated (31). HIF-1α aggregates in the cytoplasm and then transfers to the nucleus to heterodimerize with the β subunit; binds to hypoxia response elements of target genes, which results in overexpression of hypoxia-regulated genes (14). HIF-1α implicates in the regulation of the cell proliferation, motility, survival, apoptosis, cellular metabolism, and angiogenesis, while the HIF-1β subunit occurs constitutively within cytoplasm. In the current study, exploring the effect of *S. dentifolium* fractions on the cell viability indicated that the fractions exhibited a variable cytotoxic effect on CAL-27 cells in the following order: SD2 > SD1 > SD3. The most potent fraction of the lowest IC_{50} was SD2 due to high percentages of early and late apoptosis.

Hypoxia markers, such as misonidazole, pimonidazole, nitroimidazole, were used to trace cellular the O_2 profile (32). They have the capability to detect hypoxia due to their production of detectable adducts at low O_2 tension. For example, pimonidazole is covalently bound to thiol-containing cellular macromolecules in hypoxia cells when the O_2 tension is below 10-mm Hg, <1.3% (32). In the current study, screening of hypoxia status in CAL-27 cells by pimonidazole after co-culture with *S. dentifolium* fractions for different intervals indicated that SD2 was more potent than SD3 in the time-dependent inhibition of hypoxia, especially after 24 and 48 h. It is known that the regulation of HIF-1α is disturbed as a response to the cellular hypoxia status and/or as a subsequent reflection of genetic alterations that occur in tumor pathophysiology, including tumor invasion, angiogenesis, and cell survival (32). Therefore, exploring the status of HIF-1α protein may be the target mechanism of the extract fraction to inhibit cellular hypoxia. In this study, HIF-1α was found to be basically higher in CAL-27 cells compared to the treated cells. HIF-1α protein was

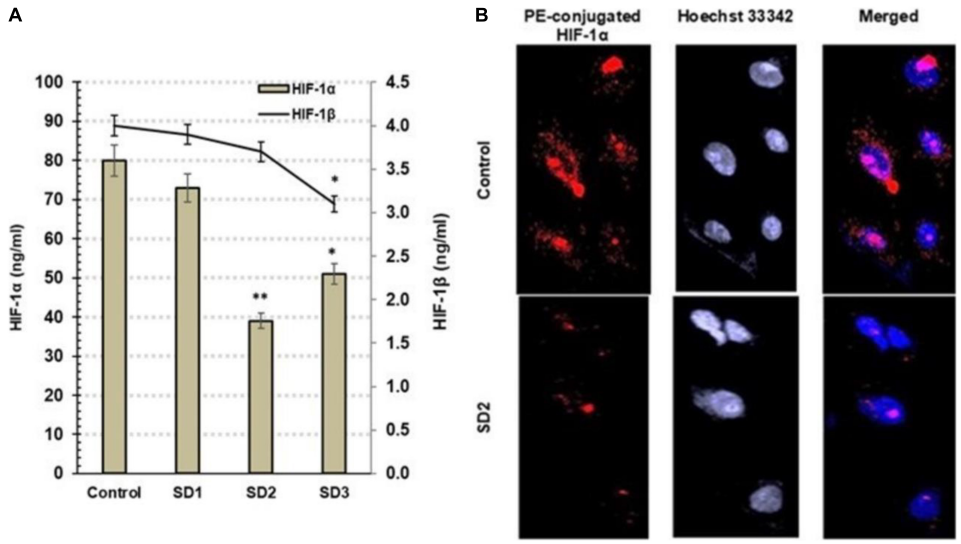


FIGURE 4 | (A) Estimation of HIF-1α and HIF-1β by ELISA in CAL-27 cells after being treated with 30% of IC_{50} of each extract fraction for 48 h, compared to control cells, after 48 h. Data are expressed as (mean ± SD in ng/ml, $n = 6$). *$p < 0.05$, and **$p < 0.01$ compared with the corresponding control. **(B)** SD2 treatment led to an inhibited HIF-1α; the immunocytochemical staining was implemented by a PE-conjugated HIF-1α antibody (red) and the nuclei counterstaining with DAPI (blue). SD2-treated cells showed a lower HIF-1α concentration than control. The cells were analyzed under fluorescence microscope (magnification ×200).

significantly diminished when the cells were treated with SD2 or SD3. The study findings confirmed that SD2 strongly inhibited hypoxia through targeting HIF-1α.

HIF-1β is identical to vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT) (33). It is known that HIF-1β is over-expressed in experimental hypoxic conditions and metastatic tumor cells (34). Contrary to early reports, HIF-1β has a critical function in hypoxic response, where recent reports advocate that HIF-1β concentrations are not consistent during hypoxia. Instead, HIF-1β is fluctuated in response to hypoxic conditions, parallel to HIF-1α, as its heterodimeric partner (34, 35). In the present study, HIF-1β concentration showed a nonsignificant alteration in SD1- and SD2-treated cells, while SD3 treatment led to a noticed inhibition in HIF-1β concentration. Recently, HIF-1β has been reported to be required for full activation of NF-κB in cells in response to canonical and non-canonical stimuli (36), where HIF-1β binds TRAF6 gene and regulates its expression independently from HIF-1α. Moreover, exogenous TRAF6 expression is capable to recover all of the cellular hypoxic phenotypes in absence of HIF-1β. These findings elaborated that HIF-1β is an essential regulator of NF-κB (37). Therefore, the inhibition of HIF-1β by SD3 may provide not only a role in the inhibition of the hypoxia pathway but also a suppression of the NF-κB activation process, a suggestion that needs further investigations.

The majority of miRNAs are alleged to function through RNA-target silencing complexes as a consequence of the impaired base pairing of 30-UTR of certain mRNAs to their target genes; hence, it closes the mRNA translation of these genes or stimulates a direct damaging cleavage (8). A fundamental role is implemented by miRNAs in the upregulation/downregulation of genes in the balance of cell growth/apoptosis (8). Imperfect expression of miRNA often accelerates cancer progression (38). miRNAs are mainly tending to be either oncogenes or tumor suppressors in cancers, including oral cancer (38). OncomiRs, oncogenic miRNAs, are dramatically overexpressed in a way that evoked the neoplastic initiation successively into the tumor progression stage. In the other hand, the tumor suppressive miRNAs play an important role in halting or blocking the tumor development (39). miRNAs expression in OTSCC dominantly controls the activation of gene transcription regulators: protein kinase Ca and HIF-1α that are mainly stimulating the transcription of hypoxamiRs: miR-210 and miR-21 (38). Disturbance of miRNAs in OTSCC resulted in a concomitant induction in cell division, growth rate, anti-apoptosis, metastasis, and drug resistance (38).

miR-210 is an essential responding factor to the hypoxia occurrence in the microenvironment of the endothelial cells, which controls the cells when they are dividing, differentiating, and migrating (40). Previously, fundamental microRNAs functions were recognized in critical cellular pathways in TME. Overexpressed miR-210 was observed to participate in the stabilization of HIF- 1α in TME, in hypoxia (41), and that this overexpression was noticed in head and neck cancerous patients (42). The effect of the extract fractions on the expression of hypoxia-controlling miRNAs (miR-21 and miR-210) was determined. The treatment of OTSCC cells with the fractions SD2 and SD3 dramatically diminished the miR-210 expression. Similarly, the treatment with SD2 and SD3 led to a dramatic down-expression of miR-21 in the cells. miR-21 is overexpressed in most of cancers and possesses several oncogenic functions in carcinomas including OTSCC (43), where miR-21 is correlated with depleted apoptosis (44). Other reports have been reported that using anti-miR-21 to block miR-21 triggers apoptosis and diminishes anchorage-based division of OTSCC cells and likewise suppresses mass xenografts formation of OTSCC in

immunocompromised animals (38, 45). A previous report claimed that, in OTSCC, miR-21 decreased the apoptosis through silencing of tropomyosin 1 gene (45). Patients with oral cancer show a successful initial response to chemotherapy; however, a later relapse regularly occurs due to hypoxia-stimulated drug resistance, which limits the chemotherapy effectiveness and its application (46). Therefore, approaches to minimizing the cellular hypoxia through edible complementary agents may facilitate a lower drug resistance and potentiate the efficiency of radio/chemotherapies in oral cancer. Accordingly, the findings of this study deduce *S. dentifolium* extracts as a promising adjacent candidate to chemotherapies for inhibiting hypoxia-induced drug resistance in TME.

CONCLUSION

The extract fractions of the edible alga *S. dentifolium* were used in this investigation. Through separate mechanistic amelioration of HIF-1α and HIF-1β and their regulators—miRNA-21 and miRNA-210 - the fractions SD2 and SD3 revealed a possible anti-hypoxia impact in Tongue Squamous Cell Carcinoma (CAL27) cells. The findings imply that *S. dentifolium* could be used as a complementary dietary agent in cancer therapy to reduce hypoxia-related therapeutic resistance in solid tumors.

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

AG-E: study concept/design and writing manuscript. BR: statistical analysis. SE-D, NH, AA, HJB, and MA: practical work. ER: alga extraction. All authors contributed to the article and approved the submitted version.

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Daily Consumption of Lemon and Ginger Herbal Infusion Caused Tumor Regression and Activation of the Immune System in a Mouse Model of Breast Cancer

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The Mediterranean diet includes the consumption of various fruits and vegetables. Lemon and ginger are highly popular in Mediterranean cuisine. The current study aims to evaluate both anticancer and immunomodulatory activities of lemon and ginger combination. The antiproliferative activities of the combination were tested against different cancer cell lines using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. The degree of apoptosis induction and vascular endothelial growth factor expression were detected using ELISA. Balb/C mice were inoculated with the EMT6/P breast cancer cells and received combination water extract orally for 14 days. The effect of the water extract on splenocytes proliferation was measured using the mitogen proliferation assay. Macrophage function was evaluated using the nitro blue tetrazolium assay and pinocytosis was assessed using the neutral red method. Gas chromatography coupled to the tandem mass spectrometry was used to determine the composition of the combination. The lemon and ginger combination showed significant apoptosis induction and angiogenesis suppression effects. Fifty percent of the mice taking this combination did not develop tumors with a percentage of tumor reduction of 32.8%. This combination showed a potent effect in stimulating pinocytosis. Alpha-pinene and α-terpineol were detected in high percentages in the combination water extract. The lemon and ginger combination represents promising options to develop anticancer infusions for augmenting conventional anticancer therapies. Further testing is required to understand the exact molecular mechanisms of this combination.

Keywords: ginger, lemon, pinocytosis, apoptosis induction, angiogenesis, immunomodulatory activities

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INTRODUCTION

Cancer is considered one of the widespread causes of death in the 21st century. Statistics reported 18.1 M new cancer cases and 9.6 M cancer deaths in 2018 (1). These numbers reflect the limited curative effect of conventional anticancer treatments (2). Many studies demonstrate that increased adherence to the Mediterranean dietary pattern is associated with health benefits (3). Herbal and alternative medicine is highly popular among cancer patients and it depends mainly on taking

drinks and foods containing active ingredients (4). Food plays a vital role in cancer initiation and progression and some studies revealed a clear relationship between reduced cancer risk and dietary polyphenols (5). Many studies proved the effects of food in cancer treatment and prevention. A combination consisting of melatonin with a ketogenic diet showed promising results against drug-resistant breast cancer (6). While the combination of thymoquinone and piperine works synergistically against breast cancer by activating many mechanisms, namely, inhibition of blood vessels formation, stimulation of apoptosis, and modulating the immune response (7). Drinking of beverages, like tea and coffee or other products rich in antioxidants showed protective effects against neurodegenerative, cardiovascular, and cancer (8). Also, many natural products like curcumin had various biological influences, including anticancer activity. It acts as a regulator of p53 in breast cancer and is involved in regulating different molecular mechanisms (9).

Moreover, various dietary compounds exhibit anticancer effects by different mechanisms, namely, inhibition of tissue invasion and metastasis, modulation of the immune response, stimulating programmed cell death, and producing synergistic response with other anticancer agents (10–12). People with the greatest adherence to Mediterranean foods and drinks have more potential to avoid chronic diseases (13). Such a diet includes a high proportion of fruits and vegetables that provide a rich source of antioxidants. Lemon and ginger are important sources of active phytochemicals and both are popular ingredients in the Mediterranean diet (14, 15).

Lemon (*Citrus limonum*) relates to the Rutaceae family. The essential oil of lemon fruit peels is limonene (43.07%), a major bioactive component with a vital antifungal function (16). Citrus fruits are abundant in biologically effective phytochemicals that may protect against many cancer types. Previous studies confirmed the anticancer potential of citrus peels with potent activity reported for lemon peel (17). Furthermore, daily consumption of citrus fruits is connected with a diminished risk for gastric cancer (18). The ethyl acetate and petroleum ether extracts of citrus lemon have anticancer activity against various human cancer cell lines (19).

Ginger (*Zingiber officinale Roscoe*) relates to the Zingiberaceae family. In India and Nepal, ginger, lemon, and the black salt mixture were used widely to treat nausea (20). Gingerols and shogaols are essential components in ginger, both of them have biological activity, like anticancer, oxidative stress reduction, antimicrobial, anti-inflammatory, and antiallergic to multiple central nervous system activities (21).

Gas chromatography (GC) and GC−mass spectrometry (MS) of the essential oils from fresh and dried ginger rhizome revealed the presence of Zingiberene, which is considered a significant compound in both ginger oils (22). The methanol extract of ginger has an antiproliferation effect against human colorectal cancer cell lines (HCT116, SW480, and LoVo cells) (23). As well, an ethanol-water extract of dried ginger root presents antitumor activity against ovarian cancer cells (24).

Although lemon and ginger herbal infusion is highly popular globally, studies about the health benefits of this infusion are missing. In this study, a combination consisting of lemon and ginger water extract was tested for its anticancer and immunomodulatory activities *in vitro* and *in vivo* using a mouse model of breast cancer.

MATERIALS AND METHODS

Plant Material and Extract Preparation

Dry herbs of the lemon and ginger combination used to prepare herbal infusions were purchased from the local market. These herbs are available in small bags ready for soaking in hot water. A water extract was prepared by soaking the herbal bag in hot water for 5 min and then drying the extract completely, using a rotary evaporator. The dried extract was weighed and stored at −20°C.

Cell Line, Culture Conditions, and Mice

Six cell lines were used to examine the impact of the lemon and ginger combination water extract on their survival. Human epithelial breast cancer cell line (MCF-7), human breast adenocarcinoma cell line (MDA-MB-231), human colon carcinoma cell line (HCT-116), human adenocarcinoma alveolar basal epithelial cell line (A549), and EMT-6/P (mouse epithelial breast cancer) cell line were used in this study. MCF-7 cell line was cultured in the complete Roswell Park Memorial Institute (RPMI)-1640 medium. The MDA-MB231, HCT-116, and A549 cell lines were cultured in the complete Dulbecco's modified eagle medium (DMEM) medium-high glucose. Mouse epithelial breast cancer cell line (EMT-6/P) was cultured in a minimal essential medium. Kidney epithelial cells from the African green monkey (Vero) were used as normal control and cultured in the complete DMEM medium. All the six cell lines were incubated at 37°C in a 5% CO_2 and 95% humidity incubator. All media mentioned above were supplemented with 10% fetal calf serum, 1% L-glutamine, 0.1% gentamycin, and 1% penicillin-streptomycin solution.

Untreated cells (wells only contain cells plus tissue culture media) were used as the negative control. While cells treated with vincristine sulfate are considered as positive controls. Vincristine is a standard anticancer drug derived from the plant called *Catharanthus roseus*, it was classified as a plant alkaloid. Both of these controls were used to compare the activity of our extract (25).

Balb/C healthy female mice aged between 4 and 6 weeks and weighing between 23 and 25 g were utilized in this research. The mice were kept in separated cages with wooden shavings used as bedding. The animal room's environmental parameters were 50–60% humidity, 25°C temperature, and continuous air ventilation. Animal care and use were conducted according to standard ethical guidelines and the Research and Ethical Committee approved all of the experimental protocols at the Faculty of Pharmacy in Applied Science Private University.

Antiproliferative Assay

Each of the six-cell lines (actively growing) was collected by the trypsinization method and seeded into 96-well flat-bottom plates at a density of 13,000 cells/well for 24 h. At the end of the

incubation period, cells were treated (in triplicate) with gradually increasing concentrations of lemon and ginger water extract (25–0.19 mg/ml). The extract was sterilized using 0.2-um syringe filters. Cells were kept with the extracts for 48 h; later, the cell survival was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. A microplate reader (Biotek, Winooski, VT, United States) was used to detect the resulting change in color at 550 nm. The percentage survival was measured for treated cells and compared with untreated cells. Untreated cells were used as negative controls and cells treated with vincristine sulfate (0.05–0.00039 mg/ml) were used as positive controls (26).

Determination of Vascular Endothelial Growth Factor Expression in MDA-MB231 Cells

The MDA-MB231 cells were seeded into three separated tissue culture flasks at the concentration of 150,000 cells per ml. After overnight incubation, the old media were discarded and cells were treated with one of the following treatments: lemon and ginger combination water extract (3.5 mg/ml), vincristine sulfate (0.025 mg/ml), as a positive control, and the third flask considered as a negative control [tissue culture media + 0.1% dimethyl sulfoxide (DMSO)]. The three flasks were placed in the incubator for 48 h. After that, the media of each flask were transferred into sterile tubes and vascular endothelial growth factor (VEGF) levels were measured using ab222510 Human VEGF Simple Step Elisa® Kit version 1 catalog (27). A standard curve was obtained using the Human VEGF Simple Step ELISA Kit at various concentrations.

Apoptosis Detection in MDA-MB231 Cells

MDA-MB231 cells were dispensed into three separated tissue culture flasks at a concentration of 150,000 cells/ml. After 24 h, cells were treated with one of the subsequent treatments.

Lemon and ginger combination water extract (3.5 mg/ml), vincristine sulfate (0.025 mg/ml) and the negative control (tissue culture media + 0.1% DMSO). Cells were incubated for 48 h with different treatments (27). Then the media of each flask (three flasks) were removed and the attached cells were harvested. Caspase-3 activity was measured using ab39401 Caspase-3 Assay Kit (Colorimetric). Fold-increase in caspase 3 activity was measured by comparing extract results with the negative control.

Antitumor Activity on Experimental Animals

The duration of this study was 4 weeks, conducted on 20 healthy females Balb/C mice. Throughout the first 2 weeks, controlling the diet of 10 mice by giving them: a lemon and ginger combination of 14.3 mg/kg/day orally (gavage feeding). In the last 2 weeks, the same dose was administrated daily, but after inoculating (subcutaneously) each mouse with (100,000 cells/0.1 ml) EMT-6/P cell line (at day 14) (7).

The *in vivo* chosen dose was to mimic the way of preparation and concentration given to humans daily. The remaining untreated 10 mice were considered as a negative control group [inoculated with cancer at day 14 and treated with 0.1 ml phosphate-buffered saline (PBS) orally]. Tumors growth was monitored using a digital caliper, tumor sizes were measured and the volume of each tumor was calculated based on the following formula: $(A \times B^2 \times 0.5)$ (28). At the end of the 4th week, the mice were humanely killed and their tumors have been removed, weighed, and kept in 10% formalin.

Preparation of Murine Splenocytes

A healthy Balb/C mouse was sacrificed and its spleen was removed aseptically. A tissue grinder was used to prepare a splenocytes suspension; mainly by passing the spleen through it. Cells were washed three times then re-suspended in 0.15 M NH_4Cl solution to break down RBCs. After 10 min, the cells were repeatedly centrifuged and re-suspended in RPMI-1640 media for further use in other assays (29).

Lymphocytes Proliferation Assay

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay kit (Bioworld, Philadelphia, PA, United States) was used as the main function in this assay. Splenocytes suspension was prepared (5×10^6 cell/ml) in RPMI-1640 and followed by seeding in a 96-well culture plate in the presence of 2 μg/ml Con A or 4 μg/ml lipopolysaccharide (LPS). To this, 100 μl of decreasing concentrations (25–3.125 mg/ml in RPMI-1640) of lemon and ginger combination water extract were added separately (in triplicates) followed by incubation for 48 h. Following the incubation, 10 μl MTT (5 mg/ml) solution were added to each well. The plate was coated with aluminum foil and then incubated for 4 h. One hundred μl DMSO was then added to each well to end the reaction and the absorbance was measured at 550 nm (29). Results were summarized as a percentage of survival (%) compared to the untreated cells (negative control). The same procedure was repeated but without Con A and LPS.

Macrophage Isolation From Peritoneal Fluid

Peritoneal macrophages (PEM) were obtained from 5 Balb/C mice, which were previously injected with 3 ml of thioglycollate (intraperitoneal) 72 h before the experiment day. The mice were euthanized by cervical dislocation. The outer layer of the peritoneum is shear by forceps and scissors then pull it out quietly to discover the inner layer that lines the peritoneal cavity. Moreover, 5 ml ice-cold PBS was injected into the abdominal cavity. After gentle massaging to the peritoneum area (to expel and migrate cells into the PBS solution), the fluid was collected by inserting a 5 ml syringe into the peritoneum and start collecting the fluid, whereas, moving the tip of the needle gently to dodge hampering by the fat tissue or others. Later, it was placed in a centrifuge tube held on ice. If any visible blood contamination was detected in the fluid sample, then the sample should be removed (30).

The method was repeated five times and the fluids were pooled. After the centrifugation of the pooled fluid (3,000 RPM, 10 min, 4°C), each cell pellet was suspended in RPMI 1640

medium. The cells were counted and seeded at a concentration of 5×10^6 cells/well in a 96-well microplate, then incubated to adhere for 3 h at 37°C in a 5% CO_2 humidified incubator. After that, the non-adherent cells were washed away with a medium and the adherent cells were used in the different assays outlined below (29).

In vitro Phagocytic Assay

The NBT (nitro blue tetrazolium) reduction assay was performed according to Rainard's method (31). The Peritoneal macrophages (PEM) (5×10^6 cells/well of a 96-well plate) were cultured with different concentrations of the lemon and ginger combination water extract (12.5–1.56 mg/ml) for 48 h at 37°C. After that, 20 μl yeast suspension (5×10^7 cells/ml in PBS) and 20 μl NBT (1.5 mg/ml in PBS) was added to every single well. The Wells that received 20 μl PBS and 20 μl DMSO were used as controls. The cells were later incubated for one hour at 37°C, the supernatant was then discarded and the adherent macrophages were washed with RPMI 1640 three times. The cells were air-dried before the 120 μl of 2M KOH and 140 μl DMSO were added to each well. The absorbance of the blue solution was measured at 570 nm (OD 570) in the plate reader. The percentage of NBT reduction (which reflects phagocytic activity) was calculated according to the following equation (32).

$$\text{Phagocytic index} = (\text{ODsample} - \text{ODcontrol})/\text{ODcontrol} \times 100$$

In vitro Pinocytosis Assay

This experiment was conducted to assess the impact of the Lemon and Ginger combination water extract on the innate immunity expressed by the pinocytic activity of macrophages. The effect of the extract on macrophage function was measured using the neutral red uptake method. The peritoneal mice macrophages were collected and cultured for 48 h with decreasing concentrations of herbal water extract (12.5–1.56 mg/ml) in a 96-well plate. One hundred μl of neutral red solution (7.5 mg/ml in PBS) was added to each well; then, the wells were incubated for 2 h. The supernatant was discharged and the cells in the 96-well plate were washed with PBS two times to remove the neutral red that was not brought into the macrophage. Then, 100 μl of cell lysis solution (ethanol and 0.01% acetic acid at the ratio of 1:1) were added to each well for the cells to lyse. After the incubation of the cells at room temperature overnight, the optical density was measured at 540 nm. The pinocytic activity was shown in terms of absolute OD values (reflecting dye uptake) (32).

Gas Chromatography Coupled to Tandem Mass Spectrometry Analysis

To identify the compounds in the extract, a chromatograph 2010 was used (ultra; Shimadzu, Tokyo, Japan). This is equipped with an 8030 mass detector, and the process is easily implemented with the aid of MS Lab Solution software. The cleaning process of the glass apparatus is thorough and involves applying soap with warm water three times, before being heated at 105°C for 1 h, and then cooled to 25°C. Sample bottles and equipment were cleaned with acetone and DMSO before the start of the experiment. The extract was shaken and mixed using the ultrasound path for 6 min, then it was filtered using glass wool. After drawing the extract into small vials, 1 μl was injected into the GC-MS. The chromatographic separation was achieved using a capillary Rtx-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, Restek, Bellefonte, PA, United States). The stationary phase of the column is composed of 5:95 diphenyl:dimethylpolysiloxane blend. The operating GC conditions were kept at 60°C for 5 min and set to reach 240°C at the rate of 3°C per min. The sample was injected at the injection temperature of 250°C and the injection volume was 1.0 μl in the 1:30 split ratio. Furthermore, helium (at a flow rate of 1.0 ml/min) was used as a carrier gas. The MS was obtained with electron impact ionization (70 eV) at full scan mode (40–500 m/z). The ion source and the transfer-line temperature were maintained at 200°C. The MS was taken through a centroid scan of the mass ranging from 40 to 800 amu. The components were identified based on retention index, library mass search database (NIST and WILEY), and comparing with the mass spectral data (33).

Statistical Analysis

Data analysis was performed by employing mean ± SEM. The statistical significance among the groups was measured using SPSS (Statistical Package for Social Sciences, Chicago, IL, United States) one-way ANOVA. A P value of <0.05 was considered significant. Furthermore, the IC_{50} values were calculated for the lemon and ginger combination water extract in the different cell lines using non-linear regression in SPSS (version 25).

RESULTS

Gas Chromatography Coupled to Tandem Mass Spectrometry Analysis

Alpha-pinene, alpha-terpineol, and terpinen-4-ol compose the essential components in the lemon and ginger combination water extract. The analysis of the lemon and ginger combination water extract, using GC-MS/MS, showed the presence of different compounds.

The lemon and ginger combination extract; contain a high concentration of alpha-pinene, alpha terpineol, and terpinen-4-ol with percentages of 11.5, 7.5, and 5.4%, respectively (**Table 1**). While geraniol, geranial, neral, δ-elemene, camphene, and borneol have a percentage value of 3.75, 3.02, 4.4, 4.06, 3.35, and 4.1%, respectively. Minor detected compounds are citronellol (1.6%), sabinene (2.26%), myrtonol (2.85%), and beta-pinene (2.9%).

Compounds Found in Lemon and Ginger Behave in a Synergistic Manner to Inhibit Different Cancer Cell Lines *in vitro*

Inhibition of cell proliferation, in correlation to dosage, was observed after the treatment of six cell lines with serial dilutions of water extract (25–0.195 mg/ml).

TABLE 1 | Major compounds identified in the lemon and ginger combination extract using GC-MS/MS method.

No	Compound	Formula	M.W	R.T	%
1	2-Heptanol	$C_7H_{16}O$	116.2	13.02	1.88
2	Tricyclene	$C_{10}H_{16}$	136	15.53	1.66
3	α-Pinene	$C_{10}H_{16}$	136	17.22	11.5
4	Camphene	$C_{10}H_{16}$	136	18.19	3.35
5	Sabinene	$C_{10}H_{16}$	136	18.54	2.26
6	B-Pinene	$C_{10}H_{16}$	136	19.25	2.9
7	Methylheptenone	$C_8H_{14}O$	126	20.54	1.6
8	o-Cymol	$C_{10}H_{14}$	134	21.8	1.1
9	Terpinolene	$C_{10}H_{16}$	136	22.0	0.75
10	Linalool	$C_{10}H_{18}O$	154	22.4	0.31
11	*Trans*-2-Pinanol	$C_{10}H_{18}O$	154	26.21	0.58
12	Camphore	$C_{10}H_{16}O$	152	26.4	0.80
13	Borneol	$C_{10}H_{18}O$	154	28.9	4.1
14	Terpinen-4-ol	$C_{10}H_{18}O$	154	29.9	5.4
15	Isogeranial	$C_{10}H_{16}O$	152	30.05	8.02
16	Cryptone	$C_9H_{14}O$	138	30.8	5.21
17	α-Terpineol	$C_{10}H_{18}O$	154	31.6	7.5
18	Myrtenol	$C_{10}H_{16}O$	152	32.0	2.85
19	Citronellol	$C_{10}H_{20}O$	156	32.4	1.6
20	Neral	$C_{10}H_{16}O$	152	32.8	4.4
21	Geraniol	$C_{10}H_{18}O$	154	33.2	3.75
22	Geranial	$C_{10}H_{16}O$	152	33.82	3.02
23	Bornyl acetate	$C_{12}H_{20}O_2$	196	33.97	1.51
24	δ-elemene	$C_{15}H_{24}$	204	34.40	4.06
25	Citronellyl acetate	$C_{12}H_{22}O_2$	198	35.01	3.95
26	α-Cubene	C_8H_6	102	35.45	2.11
27	Aryl-curcumene	$C_{15}H_{22}$	202	35.48	1.02
28	α-Funebrene	$C_{15}H_{24}$	204	36.02	5.8
29	Cubenol	$C_{15}H_{26}O$	222	36.48	2.22
30	Epiglobulol	$C_{15}H_{26}O$	222	37.44	1.5
31	Viridifloral	$C_{15}H_{24}O$	220	37.89	0.73
32	Limonene	$C_{12}H_{10}$	154.21	37.99	0.41

GC-MS/MS, gas chromatography coupled to the tandem mass spectrometry; MW, molecular weight, RT, retention time.

The lemon and ginger combination, at the highest concentration 25mg/ml, significantly (P value = 0.002) lowered the proliferation of MCF-7, MDA-MB-231, HCT-116, A549, EMT-6/P cell lines when compared with VERO normal cell line (**Figure 1**).

The IC_{50} values of lemon and ginger combination for MCF-7, MDA-MB-231, HCT-116, A549, EMT-6/P cell lines, and VERO normal cell line were 4, 3.5, 14, 6.5, 11, and >25 mg/ml, respectively (**Figure 1**). IC_{50} values were 3.5–11 mg/ml (**Table 2**).

Lemon and Ginger Water Extract Decreased Vascular Endothelial Growth Factor Expression (*in vitro*) in Cancer Cells

Vascular endothelial growth factor (VEGF) was measured in the MDA-MB231 cell line to investigate whether the inhibition of angiogenesis may improve the antiproliferative effect. In the negative control group, VEGF returned a high outcome of (336 pg/ml).

The lemon and ginger combination, concentrated at 3.5 mg/ml, significantly (P value = 0.02) lowered the level of VEGF expression (197 pg/ml). Vincristine lowered the value of VEGF to 193 pg/ml (P value = 0.01) (**Figure 2**).

Lemon and Ginger Water Extract Induced Apoptosis by Enhancing Caspase-3 Activity

The colorimetric assay of caspase-3 activity was conducted using the caspase-3 assay kit to estimate the impact of lemon and ginger combination water extract on the caspase-3 levels of the MDA-MB231 cell line. At the concentration of 3.5 mg/ml, the combination extract showed a value that was 4.4 times significantly (P value = 0.003) greater than that of the negative control. In contrast, vincristine showed a value equivalent to 5.6-folds (P value = 0.001) of the negative control (**Figure 3**).

Lemon and Ginger Water Extract Enhances Lymphocyte-Proliferation in the Presence and Absence of Mitogens

The combination of lemon and ginger induced lymphocytes cell proliferation in the presence of Con A and LPS (**Figure 4**). At the high concentration (25 mg/ml), the lemon and ginger combination water extract did not stimulate the proliferation of lymphocytes, with a cell viability index of 0.9 and 1 compared to the negative control on Con A and LPS stimulated cells, respectively. The viability cell index of the lemon and ginger combination water extract was 0.7 in the condition of no mitogenic effect (**Figure 4**).

The Effect of Lemon and Ginger Combination Water Extract on Stimulating of Peritoneal Macrophages

The lemon and ginger combination water extract showed no phagocytic activity with a percentage value of 27.6% at different doses ranging from 12.5 to 1.56 mg/ml (**Figure 5**). While the results of the pinocytic assay revealed that the lemon and ginger combination water extract caused an increase in pinocytic activity ($P = 0.004$) with an absorbance value of 0.57 nm compared to the control absorbance value of 0.24, at different doses ranging from 12.5 to 1.56 mg/ml (**Figure 6**).

The Prevention and Curative Effects of Lemon and Ginger Combination Water Extract Against EMT-6/P Cells Implanted in Mice

A significant decrease in tumor size was noticed in tumor-bearing mice treated orally with the lemon and ginger combination compared with the negative control that showed tumor growth increased by (107.0197%) (**Table 3**).

The lemon and ginger combination recorded a percentage in tumor reduction of (32.8%) with no recorded deaths and a percentage of mice with no detectable tumor of (50%) (**Figures 7**, **8**). On the other hand, treated mice showed normal activity with no side effects.

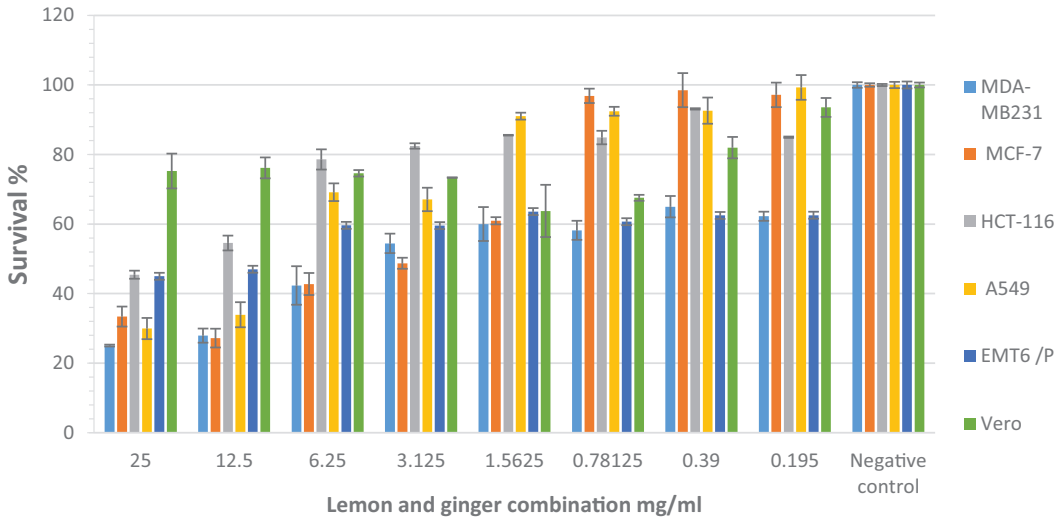

FIGURE 1 | The antiproliferative activity of different concentrations (25–0.195 mg/ml) of the lemon and ginger water extract seen after the treatment of six cells with serial dilutions of water extract. *Significant values when compared with Vero cells proliferation (*P*-value = 0.05).

TABLE 2 | The IC_{50} values (mg/ml) for lemon and ginger extract and vincristine against various cell lines.

Analyzed plants	MCF-7 cell line	MDA-MB231 cell line	HCT-116 cell line	A549 cell line	EMT-6/P cell line	Vero-normal cells
Lemon and ginger IC_{50} (mg/ml)	4 ± 0.600	3.5 ± 0.500	14 ± 1.300	6.5 ± 2.000	11 ± 1.700	>25
Vincristine (positive control)	0.013 ± 0.008	0.025 ± 0.002	0.021 ± 0.009	0.008 ± 0.001	0.041 ± 0.006	>0.05

Ten mice were used in each group.
mm^3, cubic millimeter.

DISCUSSION

Herbs are viewed to be of high value to the health of individuals and communities in general. The medicinal benefit of plants lies in some chemical substances which have specific physiological effects on the human body (33). The water extract of lemon and ginger combination showed potent antiproliferative activity against the MCF-7, MDA-MB231, HCT-116, A549, EMT-6/P, and Vero (normal cells) cell lines (**Table 1**).

No previous studies have examined the antiproliferation activity of the lemon and ginger combination water extract on the aforementioned cell lines. However, many studies have

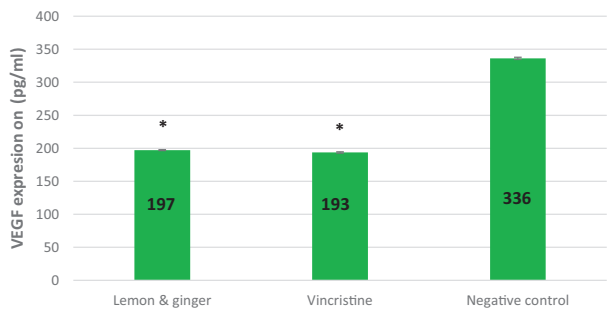

FIGURE 2 | The effect of lemon and ginger treatment on the expression of vascular endothelial growth factor (VEGF). The concentration of VEGF (pg/ml) in the MDA-MB231 cell line treated with lemon and ginger combination (3.5 mg/ml) and vincristine (0.025 mg/ml), and in untreated control cells (negative control). Each treatment was performed in triplicated. Results are shown as means (bars) SEM (lines). *Significant values.

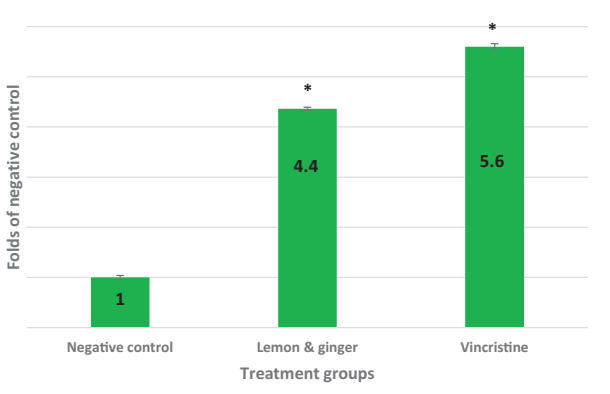

FIGURE 3 | Folds of caspase-3 activity and apoptosis induction in IC_{50} concentrations of vincristine (0.025 mg/ml), lemon, and ginger combination (3.5 mg/ml) in MDA-MB231 cell line. *Significant values (*P*-value = 0.05).

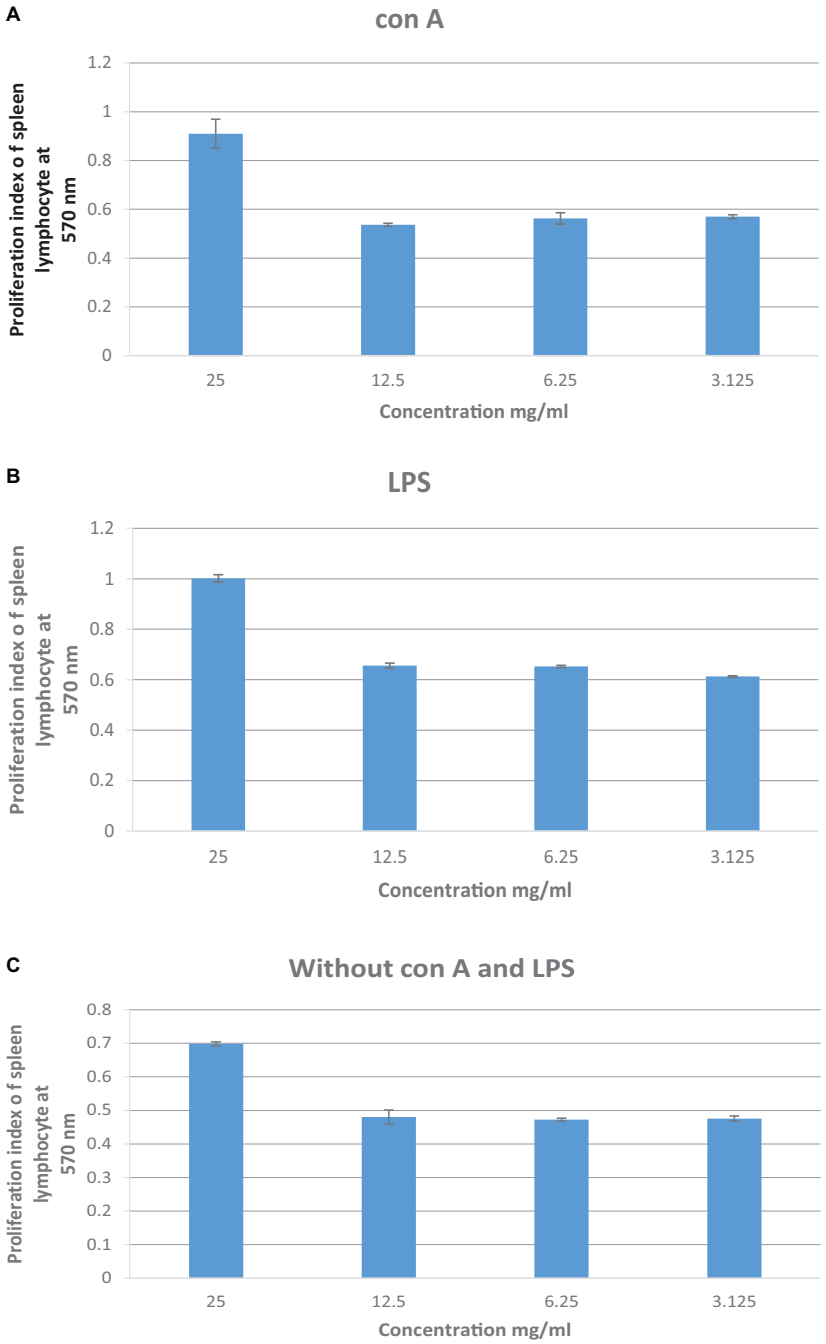

FIGURE 4 | (A) The effect of lemon and ginger combination water extract at various concentrations (25–3.125 mg/ml) on the proliferation index of splenic lymphocytes in the presence of Con A (2 μg/ml). **(B)** The effect of lemon and ginger combination water extract at various concentrations (25–3.125 mg/ml) on the proliferation index of splenic lymphocytes in the presence of LPS (4 μg/ml). **(C)** The effect of lemon and ginger combination water extract at various concentrations (25–3.125 mg/ml) on the proliferation index of splenic lymphocytes in the absence of mitogens. Results are expressed as means of three independent experiments (bars) ± SEM (lines). Proliferation index = treated cell absorbance/negative control absorbance.

investigated the antiproliferation activity of each extract against selected cell lines.

Lemon has shown the presence of different indolo-furoquinoxaline derivatives, which exhibited antiproliferative activity against leukemia and human breast cancer cells. On the other hand, there was no significant activity against the normal cell line (34).

Meanwhile, ginger has been reported to have a cytotoxic effect against human breast cancer cell lines (MDA-MB-231). The ability of ginger to inhibit cell growth was due to the

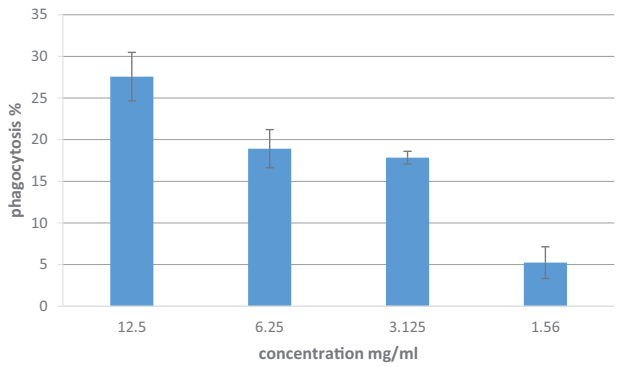

FIGURE 5 | *In vitro* phagocytic assay utilizing nitro blue tetrazolium (NBT) reduction test of peritoneal macrophage treated with different concentrations of lemon and ginger combination water extract for 48 h. Results are expressed as means of three independent experiments (bars) ± SEM (lines).

presence of [6]-gingerol as it hindered the activity of matrix metalloproteinases (MMP)-2 and MMP-9 in the human breast cancer cells (35).

The lemon and ginger combination water extract revealed synergic effect in reducing cancer cells proliferation *in vitro*. This finding is compatible with a previous study, which reported the ability of lemon and garlic water extract to induce apoptosis and prevent carcinoma development (36). However, the potent anticancer response of the lemon and ginger combination water extract was observed to be mediated by diverse anticancer mechanisms, which may be explained by the existence of various phytochemicals compounds. The observed proliferation inhibition in the cells treated with the lemon and ginger combination water extract is basically because of the impact of different antiproliferation factors. Alpha-pinene was detected in lemon and ginger combination utilizing GC/MS with a percentage value of 11.5%.

The cytotoxic potential of alpha-pinene was examined in two different human cell lines (human ovarian cancer cell line SK-OV-3 and human hepatocellular carcinoma cells Bel-7402) (37). Also, it was studied against the human histiocytic lymphoma U937 cell line (38). Furthermore, a previous study confirmed that alpha-pinene has an antiproliferation effect through provoking oxidative stress and associated signaling cascade against human lung cancer (A549) and human liver cancer (HepG2) (39). These previous findings are consistent with this study. On the other hand, α-pinene was found to have no antiproliferation activity against melanoma cells *in vitro* (40). This may be justified by the fact that solid tumors have different responses to phytochemicals as α-pinene.

Other highly concentrated compounds in the combination extract are alpha-terpineol (7.5%) and terpinen-4-ol (5.4%).

Alpha-terpineol has demonstrated antioxidant ability against peroxyl radicals and has been shown to have a cytostatic impact against six human cancerous cell lines from five diverse histologic and embryonic origins (breast, lung, prostate, ovarian, and leukemia) (41). Furthermore, terpinen-4-ol was found in the combination extract to a significant degree. It caused necrotic cell death coupled with less activity of apoptosis process in both murine malignant mesothelioma cell line AE17 and murine B16 melanoma cells. The inhibitory impact of terpinen-4-ol is achieved through the elicitation of the G1 cell cycle arrest (42).

Further analysis of the lemon and ginger combination water extract revealed the presence of geraniol, citral, δ-elemene, camphene, and *borneol* in notable percentages and the previously reported activity of these components confirms the results of this study.

Based on a previous study, the growth of prostate cancer cells was inhibited by the effect of geraniol compound on inducing the apoptosis and autophagy process (43). Citral prevents the proliferation of breast cancer cells (MDA MB-231 cells) *in vitro*, mainly through the initiation of apoptosis, antimetastasis, and antiangiogenesis potentials (44). In colorectal

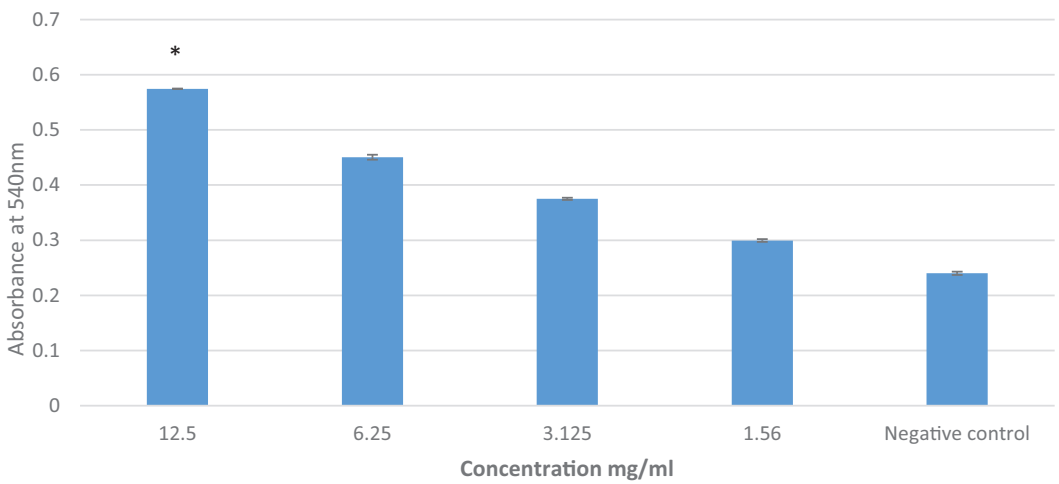

FIGURE 6 | The effect of lemon and ginger combination water extract on macrophage pinocytosis. Results are expressed as means of three independent experiments (bars) ± SEM (lines). *Significant effect of the herbal drink on the pinocytosis activity at the highest concentration when compared with negative control.

TABLE 3 | Effect of lemon and ginger combination water extract on tumor size and cure percentage.

Treatment	Initial tumor size mm^3	Final tumor size mm^3	% change in tumor size	% of mice with no detectable tumor	Average tumor weight (g)
Control	432.6216 ± 22.3	895.6118 ± 43.1	107.0197	20%	0.715
Lemon and ginger combination	242.5815 ± 19.7	162.9365 ± 20.5	-32.8323	50%	0.288

adenocarcinoma (DLD-1), the antiproliferative activity of δ-elemene was implemented by the activation of mitochondrial caspase-dependent and caspase-independent pathways (45). On the other hand, camphene exhibited anticancer activity in melanoma cells both *in vitro* and *in vivo*. It can disrupt the mitochondrial membrane potential and enhances the caspase-3 effect (46). In a previous study, borneol (NB) improved the absorption of selenocysteine; as a result, it increased his apoptotic activity against human hepatocellular carcinoma cells. Also, they reduced tumor growth by provoking ROS-mediated DNA damage (47).

In the current study, the potential inhibition of cancer cell growth by lemon and ginger combination may due to the effects of the nine major compounds previously mentioned and to the other active minor components.

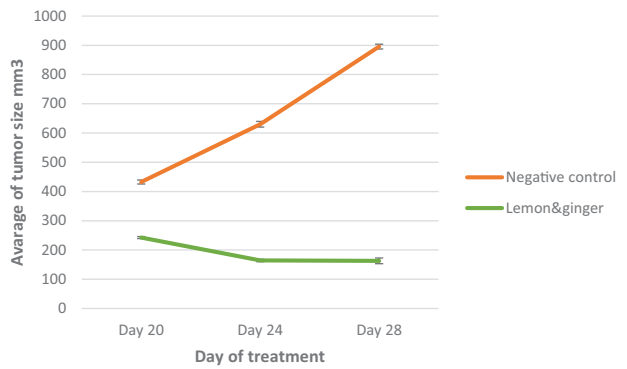

FIGURE 7 | A plot of change in average tumor size (mm^3) vs. time in (days of treatment in EMT-6/P cell line). *Significant values (*P*-value = 0.05).

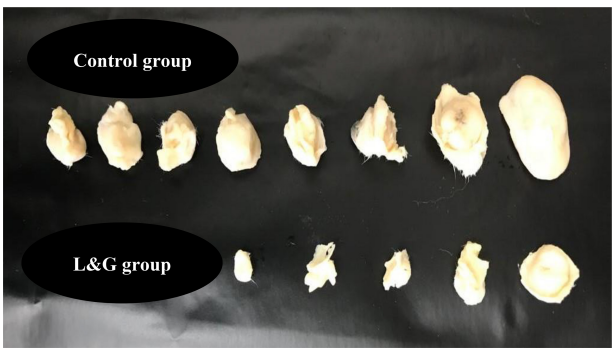

FIGURE 8 | The effect of lemon and ginger combination water extract (L & G) on tumor size and cure percentage. Combination therapy resulted in a high cure percentage and small tumors size. There were 10 mice in each group.

To gain further in-depth knowledge of the mechanisms of action of the combination extract under study, the lemon and ginger combination was further tested for its ability to inhibit angiogenesis. The VEGF is a potent signal protein that stimulates angiogenesis. VEGF upregulation is a well-known mechanism in many types of tumors and the inhibition of this pathway is an interesting target in cancer prevention and therapy (48). In our study, the lemon and ginger combination showed a strong ability to suppress the MDA-MB-231 human breast cancer angiogenesis.

Further examination showed that the lemon and ginger combination water extract had a potent anticancer effect *in vitro*. The combination has potent apoptosis induction activity and potent VEGF inhibition in the MDA-MB231 cell line.

In MDA-MB-231 cells, nuclear factor kappa B (NF-κB) is involved in the upregulation of VEGF mRNA then increases the angiogenesis (49). The activations of NF-κB were attenuated utilizing α-pinene treatment (50). In HeLa cells, alpha-terpineol repressed TNF-α and NF-kB translocation into the cell nucleus. Repression of NF-kB activation can result in the decrease of cyclin D expression, an essential protein in the induction of the G phase of the cell cycle (51). Meanwhile, geraniol is a strong agent with antiangiogenic properties (52). These results are inconsistent with the findings of this study.

Another mechanism responsible for the observed anticancer activity is apoptosis (programmed cell death) induction. In cancer, this process is inoperative, due to the upregulation of antiapoptotic genes and the downregulation of apoptotic genes; therefore, cells continue dividing and proliferating (53). One of the targeted pathways in cancer treatment is the activation of apoptosis.

In our study, the lemon and ginger combination herbal drink was found to have the ability to induce apoptosis in the MDA-MB231 cell line (4.4 times greater than that of the negative control).

Both hesperidin (flavonoid) (54), and eriocitrin (flavonoid) (55) in lemon cause apoptosis induction and inhibit the human hepatocellular carcinoma. The high antiproliferative activity of lemon has been explained in previous studies, which have shown that the methanol extract of lemon has anticancer activity toward MCF-7 breast cancer cells *via* Bax-related caspase-3 activation. Meanwhile, the ethanol extract of lemon peel exhibited a weak cytotoxic effect (IC_{50} > 500 μg/ml) against human leukemia HL-60 cells (56). Alpha-pinene, which is found in high percentages in the lemon and ginger combination, was also capable of inducing apoptosis, as evidenced by the rise in caspase-3 activity (57). Moreover, geraniol has the most dominant apoptosis-inducing activity among terpenoids against shoot primordia of *Matricaria chamomilla*; it causes DNA fragmentation in the cells. The activity of geraniol is concentration- and time-dependent (58). These results correspond with this study's conclusion.

The immune system is a sophisticated defense system in vertebrates, having the role of protecting them from numerous types of foreign infectious agents that they encounter during their lifetimes. It uses a variety of cells, tissues, and organs and is capable of recognizing and eliminating invasions (59). There has been a growing interest in identifying and characterizing natural compounds with immunomodulatory activities. Three immune assays have been conducted, namely, lymphocytes proliferation, phagocytosis, and pinocytosis assays.

Lymphocytes proliferation assay was conducted three times: splenic lymphocytes with extract alone, splenic lymphocytes with extract and Con A, and splenic lymphocytes with extract and LPS, for lemon and ginger combination water extract.

Lemon and Ginger Combination Water Extract Had a Limited Effect on Lymphocyte Proliferation and Phagocytosis Activity

The volatile oil of ginger exhibited significant suppression of the mitogen-stimulated T-lymphocyte proliferation in mice (60), which agrees with our result. While citrus peels had a mitogenic response to con A (56). In addition to that, both lemon citrus and ginger stimulate innate immunity (61, 62). These previous studies conflicted with our results may be due to the antagonistic effect of the combination on the activity of both lemon and ginger.

For pinocytic activity, the potent pinocytic activity was the lemon and ginger combination. Various groups of phytochemicals are found in the lemon and ginger combination water extract. This could explain the activation of a specific anticancer response, such as immune enhancement It is worth mentioning that *a*-pinene exhibited anti-Leishmania activity *via* macrophage stimulation with minimum cytotoxicity (63). Citral influences cytokines production by activation of murine macrophages through the prevention of the transcription factor NF-κB (64). In the cell-based assay, terpinen-4-ol was able to modulate apoptosis and immune system activity in a way that reduced the cell growth of melanoma cells (65). In agreement with these previous studies, the results of this study showed that the lemon and ginger extract possesses strong antiproliferation and pinocytosis activity.

An *in vivo* study was conducted to evaluate the activity of lemon and ginger combination water extract on the EMT-6/P cell line implanted in Balb/C mice. This study results showed a significant reduction in tumor sizes in all treated mice. On the other hand, no previous study has examined the *in vivo* tumor-prevention activity of lemon and ginger combination water extract.

Besides cancer chemotherapy strategies, using dietary agents such as fruits and vegetables help the prevention of tumor growth. Extracts derived from fruits and vegetables are demonstrated to have antiproliferative effects (18). It is extensively acknowledged that chemopreventive agents, as lifestyle and dietary habits, have more superior potential in the long term than chemotherapeutic agents, especially in prostate cancer growth and progression (65).

In vivo, the lemon and ginger combination water extract induced a significant decrease in percentage change in tumor size, a high percentage of mice with no detectable tumor, and low average tumor weight (g) (**Table 2**).

A cohort study confirms that the daily consumption of citrus fruits is associated with a decreased risk of gastric cancer (18). Notably, daily oral feeding of 100 mg/kg of whole ginger water extract prevented both the growth and progression of the human prostate cancer cell line PC-3 xenografts by approximately 56% in nude mice (66). The lemon and ginger combination is mostly composed of alpha-pinene, according to GC-MS/MS analysis. *In vivo*, alpha-pinene pretreatment reduced the production of the pancreatic tumor necrosis factor, interleukin (IL)-1beta, and IL-6 during the induction of acute pancreatitis by cerulean (67). Another previous study found that the tumor volumes from mice treated with α-pinene were around 40% less than those from the mice in the control group (68). There is an interaction between the work of lemon and ginger combination resulted in antitumor activity *in vitro* and *in vivo*. Furthermore, the anticancer activity could be explained by the stimulation of apoptosis and pinocytosis and reduction of VEGF expression.

CONCLUSION

Lemon and ginger herbal combination is a healthy drink with anticancer and immunomodulatory health benefits. Its anticancer effect is mediated by apoptosis induction and angiogenesis inhibition. The immunomodulatory effect is mediated through the activation of the innate immune system. The health benefits of this herbal drink are mainly due to the presence of biologically active phytochemicals in ginger and lemon. Further studies are needed to have a better understanding of the mechanisms of action of the herbal drink.

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 Institutional Research Board at Applied Science Private University.

AUTHOR CONTRIBUTIONS

The project idea was developed by WT. IA-A performed the sample collections and ran the laboratory experiments. Both authors developed the experimental design, analyzed the data, and wrote and revised the manuscript.

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Consumption of Food Components of the Mediterranean Diet Decreases the Risk of Breast Cancer in the Makkah Region, Saudi Arabia: A Case-Control Study

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Background: Breast cancer is one of the leading causes of death worldwide, it affects both men and women. In Saudi Arabia, breast cancer has been the most prevalent type of cancer in women, for the past few years. Dietary habits and cultural beliefs vary according to region, and further studies are required to demonstrate the relationship between these dietary habits and cultural beliefs and the risk of developing breast cancer. This study is aimed to discover the relationship between preventive dietary factors of the Mediterranean diet and rates of breast cancer among postmenopausal women in the Makkah region of Saudi Arabia.

Methods: A case-control study was conducted in King Abdulla Medical City Hospital, Makkah, Saudi Arabia and included 432 Saudi female participants: 218 in the control group and 214 breast cancer patients. All participants were postmenopausal, around the same age, and all were ethnically Arab Saudis. Data were obtained using a self-administered validated questionnaire.

Results: Study results showed that a diet that includes 1–2 servings of legumes weekly, 1–5 servings of fish weekly, 1–5 servings of dairy products daily, 3–5 servings of fruits and vegetables daily, and more than one cup of black tea and coffee per day significantly ($p < 0.05$) reduces the risk of breast cancer.

Conclusion: This study demonstrates that consuming a Mediterranean diet, which includes legumes, fish, fruits and vegetables, black tea, coffee, and low intake of dairy products, works as a preventive factor against breast cancer in postmenopausal females from the Makkah region.

Keywords: breast cancer, postmenopausal, Mediterranean diet, dietary habits, nutrition

INTRODUCTION

Breast cancer is one of the leading causes of death worldwide, with 2.3 million newly diagnosed women in 2020 (1). In Saudi Arabia, breast cancer is the second most common type of cancer, after rectal cancer, and is responsible for 3,629 (14.8%) new cases diagnosed in both genders. Breast cancer affects women more than men and represents 29.7% of newly diagnosed cancer cases in women (2). In 2014, the tumor registry in King Faisal Specialist Hospital and Research Center reported that breast cancer represented the highest percentage of cancer cases at 11.7% (3). The rapid increase of cases among different age groups, during the past few years, makes breast cancer one of the most critical topics to study in the medical field. About 5–10% of cancer cases are caused by genetic factors, while 90–95% cases are related to environmental factors and unhealthy lifestyle elements, such as diet, obesity, and alcohol consumption (4).

In addition to a high intake of olive oil, the Mediterranean diet focuses on a plant-based consuming pattern with high fiber intake from fruits and vegetables, legumes, and cereals and high omega-3 from fish and kinds of seafood. Furthermore, moderate intake of dairy products and low intake of red meat and poultry are also recommended in the Mediterranean diet (5). A recent case-control study showed that following the Mediterranean diet rich in olive oil, fish, fruits, and vegetables reduces the risk of breast cancer in pre- and postmenopausal women (5).

Several risk factors contribute to increased risk of breast cancer, which include alcohol, obesity, a sedentary lifestyle, exogenous estrogen and progesterone, menarche at an early age (<12 years old), previous surgeries or biopsies, previous mammography screenings, and never giving birth or breastfeeding are all risk factors for breast cancer (6, 7). However, a healthy diet and lifestyle have been shown to reduce the incidence of breast cancer. Therefore, verifying the risk factors for breast cancer is of vital importance. Several studies have assessed the influence of lifestyle and nutrition on breast cancer (8, 9). Fruits, vegetables, dairy products, and olive oil were found to be preventive against breast cancer (10). A case-control study in Iran showed that a high level of vitamin D in the fourth quartile of serum 25(OH)D decreased the risk of breast cancer (11). However, that study showed that high consumption of meat and fat increased the risk of breast cancer (12).

Regional differences in diet and lifestyle habits play a role in breast cancer development; women in Saudi Arabia have different dietary habits depending on the region they live in and their cultural beliefs. Further studies are required to investigate the effect of dietary habits on breast cancer in different cultures. Additionally, women after menopause could be at higher risk of breast cancer than premenopausal women (6). Therefore, this research is aimed to explore the causation and preventive factors of breast cancer in postmenopausal women from the Makkah region, particularly the impact of consuming a Mediterranean-based diet on the prevalence of breast cancer.

MATERIALS AND METHODS

Study Design and Setting

This case-control study took place in King Abdullah Medical City Hospital in the Makkah region of Saudi Arabia from June 2014 to November 2016. This hospital is the biggest in the region and the only center that provides screening and treatment of cancer locally.

Participants

A total of 432 female participants were recruited (214 cancer cases and 218 controls). Only Saudi postmenopausal women who were newly diagnosed with breast cancer and aged above 45 years old were included. A postmenopausal woman is defined as any woman who no longer has her periods for at least a year. The mean ages of the case and control groups were 57 ± 7.3 years old and 56.9 ± 8.6 years old, respectively, which was not significantly ($p > 0.05$) different. Additionally, no significant differences ($p > 0.05$) were found in caloric intake (1,916 Kcal $\pm$ 437.7 and 1,837 Kcal $\pm$ 392.4, respectively) between the case and control groups. Diagnoses of breast cancer patients were confirmed by biopsy by the oncologist in the same hospital. To control the racial factors, only ethnically Arab women were invited to this study. The control group was healthy women without any acute or chronic disease and they were chosen from the hospital workers and patients' families or friends. They were selected from the same region and age range as the cases with a single year of age in the match and without any acute or chronic disease. **Figure 1** is a flowchart that shows inclusion and exclusion criteria for the recruitment of participants. The response rates for the case and control groups were 93.4 and 93.2%, respectively.

The study data were collected after the investigation was approved by the Institutional Review Board of Umm Al-Qura University (approval number AMSEC-2-20-5-2014), following the Declaration of Helsinki rules. Eligible women read and signed the consent form before starting the data collection.

Data Collection

Data for this study were collected using convenience sampling and face-to-face interviews. Participants were asked to fill out a questionnaire, supervised by a registered dietitian. The questionnaire recorded personal information and questions

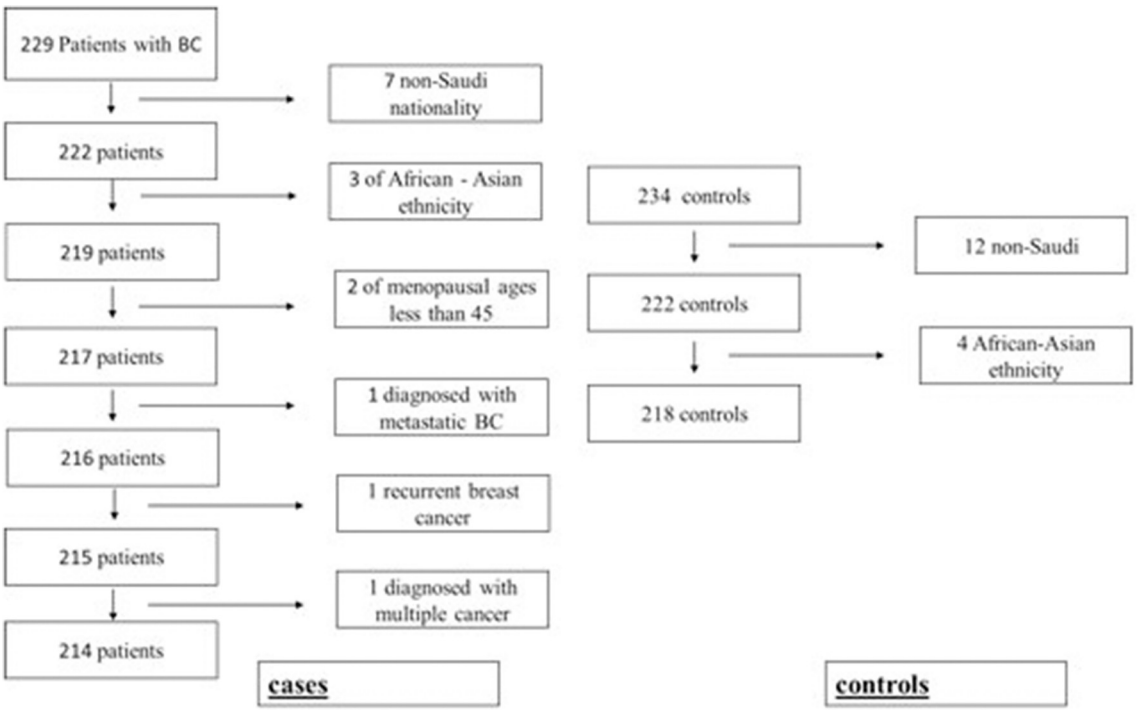

FIGURE 1 | Flowchart for recruiting cancer patients and control group participants.

about dietary habits; a validated dietary questionnaire from the 2017 work of Azzeh et al. (13) was used. The questionnaire included questions about daily intake of foods, such as fruits and vegetables, meat and processed meat, poultry, dairy products, and beverages, such as coffee and black tea (*Camellia sinensis*). In the Saudi community, black tea is generally offered in an 80-ml cup, while coffee is offered in a 50-ml standard cup. Other food categories that include fish and seafood, olive oil, green leafy vegetables, and legumes were recorded weekly. Daily bread consumption and preferences for either white or whole wheat bread were also assessed. All participants were taught about the serving size for each food item prior to starting the questionnaire. The registered dietitian measured the height and weight of the participants using the Detecto physician scale, available at the hospital (Detecto, Webb City, Missouri, USA). Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared.

Statistical Analysis

Statistical tests in this study were completed using IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY, USA); a value of $p < 0.05$ was set for the significant differences. A suitable test was used to decide the value of p for each parameter. The data were arranged using a case-control status to verify the differences between the cases and the controls. To assess the risk factors related to breast cancer, the odds ratio (OR), 95% confidence intervals (95% CI), and β-coefficient were determined. All variables were adjusted according to the confounders described in Alsolami et al.'s article (14), which were as follows: BMI (continuous), total caloric intake (continuous), employment, income, education level, family size, smoking, physical activity, cancer background, menstruation start, and contraceptives usage.

RESULTS

Results of this study showed that the employment rate in the control group (41.2%) was higher ($p < 0.001$) than the case group (13%; **Table 1**). The percentage of participants in the control group who had a low monthly income of <5,000 SR (4.8%) was lower ($p < 0.001$) than that of the case group (21.8%). The literacy percentage in the control group (0.5%) was lower ($p < 0.001$) than that of the case group (7.4%). The case group had a noticeably larger ($p < 0.001$) family size as compared to the control group. Menarche at an early age (<10 years old) was remarkably higher ($p < 0.001$) in the case group as compared to the control group. The consumption of hormonal contraceptives was higher ($p < 0.001$) in the case group as compared to the control group (21.8 vs. 12.7%, respectively). No differences ($p > 0.05$) were shown regarding marital status, family history of breast cancer, age of menopause, and breastfeeding duration in either control group or case group. The complete details for the baseline characteristics of the participants were published previously in Alsolami et al.'s research (14).

Table 2 records the dietary habits of all participants regarding the main food groups. Fruits and vegetables (measured in servings per day), dairy products (servings per day), red and processed meat consumption (servings per day), and legumes

TABLE 1 | Baseline characteristics of the participants ($n = 432$ total).

Parameter	Control	Case	*P*-value
Body Mass Index (BMI) (kg/m^2)	27.7 ± 6.3	35.4 ± 10	<0.001
Employment			
Yes	178 (41.2%)	56 (13%)	<0.001
No	40 (9.3%)	158 (36.5%)	
Family income			
<5,000 SR*	21 (4.8%)	94 (21.8%)	<0.001
5,000–10,000 SR	90 (20.8%)	88 (20.4%)	
10,000–2,0000 SR	80 (18.5%)	24 (5.6%)	
>20,000 SR	27 (6.2%)	8 (1.9%)	
Education			
Illiterate	2 (0.5%)	32 (7.4%)	<0.001
Primary	3 (0.7%)	96 (22.2%)	
Intermediate/secondary	23 (5.3%)	38 (8.8%)	
Postsecondary	190 (44%)	48 (11.1%)	
Family size			
5 or less	115 (26.6%)	40 (9.3%)	<0.001
6 or more	103 (23.8%)	174 (40.3%)	
Smoking			
Yes	3 (0.7%)	38 (8.8%)	<0.001
No	215 (49.8%)	176 (40.7%)	
Regularly exercise			
Yes	81 (18.8%)	56 (13%)	0.009
No	137 (31.7%)	158 (36.6%)	
Cancer awareness			
Yes	214 (49.5%)	174 (40.3%)	<0.001
No	4 (0.9%)	40 (9.3%)	
Contraceptive use			
Hormonal	55 (12.7%)	94 (21.8%)	<0.001
Not-hormonal	31 (7.2%)	32 (7.4%)	
Don't use	132 (30.6%)	88 (20.4%)	
Age of started menstruation			
≤10 years old	55 (12.7%)	94 (21.7%)	<0.001
11–14 years old	31 (7.2%)	32 (7.4%)	
≤15 years old	132 (30.6%)	88 (20.4%)	

Values are expressed as frequency (%, from the total) or mean ± SD.
Values of p are obtained by the Mann-Whitney test for the parametric variables or X^2 for non-parametric variables.
**SR = Saudi Riyal (1 SR equals 0.27 American dollar).*

TABLE 2 | Dietary habits for the main food groups between the case and control participants.

Parameter	Control ($n = 218$)	Case ($n = 214$)	*p*-value
Meals/day			
<2	57 (26.1%)	70 (32.7%)	0.134
>3–5	161 (73.9%)	144 (67.3 %)	
Fruits and vegetables (servings/day)			
<1	24 (11%)	38 (17.8%)	<0.001
1–2	150 (68.8%)	158 (73.8%)	
3–5	42 (19.3%)	14 (6.5%)	
>5	2 (0.9%)	4 (1.9%)	
Dairy products (servings/day)			
<1	4 (1.8%)	58 (27.1 %)	<0.001
1–2	193 (88.5%)	134 (62.6%)	
3–5	19 (8.7%)	16 (7.5%)	
>5	2 (0.9%)	6 (2.8%)	
Red and processed meat (servings/day)			
<1	14 (6.4%)	16 (7.5%)	<0.001
1–2	152 (69.7%)	94 (43.9%)	
3–5	35 (16.1%)	48 (22.4%)	
>5	17 (7.8%)	56 (26.2%)	
Poultry (servings/day)			
<1	24 (11%)	28 (13.1%)	0.093
1–2	147 (67.4%)	158 (73.8%)	
3–5	40 (18.3%)	26 (12.1%)	
>5	7 (3.2%)	2 (0.9%)	
Legumes (servings/week)			
<1	10 (4.6%)	40 (18.7%)	<0.001
1–2	171 (78.4%)	118 (55.1%)	
3–5	33 (15.1%)	48 (22.4%)	
>5	4 (1.8%)	8 (3.7%)	
Bread type			
White	103 (47.2%)	110 (51.4%)	0.388
Brown	115 (52.8%)	104 (48.6%)	

Values are expressed as frequency (%).
Values of p are obtained by X^2 test.

(servings per week) showed significant results ($p < 0.001$) between the two groups. However, no differences ($p > 0.05$) between the two groups were found regarding the number of meals consumed per day, poultry intake, and whole wheat vs. white bread.

Table 3 represents dietary habits by using the second group of common food items and drinks in the study groups. Results showed that there was a difference ($p < 0.05$) between the groups based on consumption of fish and seafood (measured in servings per week), leafy vegetables (servings per week), and olive oil (servings per week). Data measuring the consumption of black tea (cups per day) and coffee (cups per day) also showed a difference ($p < 0.05$) between the case and control groups. Only 8.3% of the control group ate <1 serving of fish and seafood every week when compared to 29.9% of the case group. Around 3.7% of the case group consumed <1 serving per week of green leafy vegetables, while only 1.4% from the control group ate <1 serving per week. Participants in the control group consumed olive oil ($p < 0.001$) more than the case group; 9.6% of the control group consumed <1 serving of olive oil per week, while 26.2% of the cancer cases consumed <1 serving of olive oil per week. About 56 and 36.4% of the control group and the case group, respectively, consumed 1–2 servings of olive oil per week. Participants with breast cancer tended to drink less black tea (in cups per day) as compared to healthy participants ($p < 0.009$): 18.7% of the participants with breast cancer consumed <1 cup of tea per day, while 10.1% of the healthy subjects consumed the same amount of tea daily. Subjects from the control group also consumed more coffee: only 9 control subjects (4.1%) drank <1 cup per day as compared to 48 subjects (22.4%) with breast cancer.

TABLE 3 | Dietary habits of the study groups related to common foods and drinks.

Parameter	Control ($n = 218$)	Case ($n = 214$)	*p*-value
Fish and seafood (servings/week)			
<1	18 (8.3%)	64 (29.9%)	<0.001
1–2	153 (70.2%)	133 (62.1%)	
3–5	44 (20.2%)	13 (6.1%)	
>5	3 (1.4%)	4 (1.9%)	
Leafy vegetables (servings/week)			
<1	3 (1.4%)	8 (3.7%)	<0.001
1–2	140 (64.2%)	126 (58.9%)	
3–5	53 (24.3%)	32 (15%)	
>5	22 (10.1%)	48 (22.4%)	
Olive oil (servings/week)			
<1	21 (9.6%)	56 (26.2%)	<0.001
1–2	122 (56%)	78 (36.4%)	
3–5	40 (18.3%)	40 (18.7%)	
>5	35 (16.1%)	40 (18.7%)	
Black tea (cup/day)			
<1	22 (10.1%)	40 (18.7%)	0.009
1–2	45 (20.6%)	48 (22.4%)	
3–5	19 (8.7%)	7 (3.3%)	
>5	132 (60.6%)	119 (55.6%)	
Coffee (cup/day)			
<1	9 (4.1%)	48 (22.4%)	<0.001
1–2	63 (28.9%)	86 (40.2%)	
3–5	29 (13.3%)	11 (5.1%)	
>5	117 (53.7%)	69 (32.3%)	

Values are expressed as frequency (%).
Values of p are obtained by X^2 test.

TABLE 4 | Potential dietary habits as predictors for breast cancer.

p-value	95% CI	OR	β	Independent variable
Dairy products (servings/day)				
<1	0	1		
1–2	−1.727	0.178	0.037–0.859	0.032
3–5	−3.269	0.038	0.004–0.372	0.005
>5	−0.301	0.740	0.02–27.748	0.871
Legumes (servings/week)				
<1	0	1		
1–2	−3.135	0.043	0.01–0.191	<0.001
3–5	−1.038	0.354	0.074–1.694	0.194
>5	−1.146	0.318	0.031–3.314	0.338
Fruits and vegetables (servings/day)				
<1	0	1		
1–2	−0.519	0.595	0.228–1.55	0.288
3–5	−1.837	0.161	0.043–0.605	0.007
>5	−0.460	0.631	0.032–12.401	0.762
Leafy vegetables (servings/week)				
<1	0	1		
1–2	−0.899	0.407	0.006–27.9	0.677
3–5	−1.450	0.234	0.003–16.867	0.506
>5	1.112	3.040	0.041–223.42	0.612
Fish and seafood (servings/week)				
<1	0	1		
1–2	−1.557	0.211	0.82–0.545	0.001
3–5	−2.631	0.072	0.202–0.265	<0.001
>5	−1.267	0.282	0.015–5.194	0.394
Red and processed meat (servings/day)				
<1	0	1		
1–2	−0.216	0.806	0.137–4.746	0.811
3–5	0.383	1.466	0.222–9.695	0.691
>5	1.001	2.72	0.405–18.280	0.303
Olive oil (servings/week)				
<1	0	1		
1–2	−0.405	0.667	0.218–2.042	0.478
3–5	0.763	2.145	0.574–8.014	0.256
>5	0.422	1.524	0.426–5.454	0.517
Black tea (cups/day)				
<1	0	1		
1–2	−2.811	0.06	0.01–0.371	0.002
3–5	−2.820	0.06	0.009–0.395	0.003
>5	−1.062	0.346	0.106–1.131	0.079
Coffee (cups/day)				
<1	0	1		
1–2	−1.842	0.159	0.031–0.812	0.027
3–5	−2.487	0.083	0.013–0.544	0.009
>5	−1.940	0.144	0.028–0.736	0.02

The reference is the control group.
All variables were adjusted for all other confounders.
β, beta coefficient; CI, confidence interval; OR, odds ratio.

Potential dietary habits related to breast cancer incidence are shown in **Table 4**. The consumption of 1–2 servings of dairy products per day was shown to be preventive against breast cancer (OR = 0.178, 95% CI = 0.037–0.859, $p = 0.032$), as was the consumption of 3–5 servings of dairy products daily (OR = 0.038, 95% CI = 0.004–0.372, $p = 0.005$). Results also showed a preventive effect of consuming 1–2 servings of legumes per week (OR = 0.043, 95% CI = 0.01–0.191, $p < 0.001$), 3–5 servings of fruits and vegetables per day (OR = 0.161, 95% CI = 0.043–0.605, $p = 0.007$), 1–2 servings of fish and sea food per week (OR = 0.211, 95% CI = 0.82–0.545, $p = 0.001$), and 3–5 servings of fish and sea food per week (OR = 0.072, 95% CI = 0.202–0.265, $p < 0.001$). Drinking 1–2 cups of tea (OR = 0.06, 95% CI = 0.01–0.371, $p = 0.002$) or 3–5 cups of tea daily was shown to reduce the incidence of breast cancer (OR = 0.083, 95% CI = 0.009–0.395, $p = 0.003$). The daily intake of 1–2 cups of coffee (OR = 0.159, 95% CI = 0.031–0.812, $p = 0.027$), 3–5 cups of coffee (OR = 0.083, 95% CI = 0.013–0.544, $p = 0.009$), or even more than 5 cups of coffee per day (OR = 0.144, 95% CI = 0.028–0.736, $p = 0.02$) had shown to reduce the incidence of breast cancer.

DISCUSSION

In Saudi Arabia, the rate of breast cancer ranges from three to eight confirmed cases for every 1,000 patients. Breast cancer accounts for 14.8% of all cancers reported among Saudi nationals and about 29% of cancers among women of all ages (2). It is critically important to assess the dietary factors associated with breast cancer in the Makkah region. This study showed that consuming healthy foods that include black tea, coffee, fruits and vegetables, fish and seafood, legumes, and dairy products can be preventive factors against breast cancer.

Our study results showed that consuming one to five servings of dairy products daily, a major source of vitamin D for Saudi women, had up to a 96% preventive effect against breast cancer. Dairy products in Saudi Arabia are fortified with vitamin D, which has been shown to reduce the risk of breast cancer by multiple mechanisms: either by promoting cell differentiation, decreasing cancer cell growth, stimulating cell death (apoptosis), or by reducing the formation of blood vessels in the tumor (angiogenesis) (15, 16). A recent review study supported our study findings that vitamin D had an inverse relationship with breast cancer (17). A descriptive study conducted in Saudi Arabia by AlFaris et al. (18), in contrast, demonstrated the results of the intake of vitamin D on breast cancer incidence that did not support our findings. This can be explained by vitamin D deficiency among both the control group and the cancer group in the previous study. The study also showed a relationship between symptoms of vitamin D deficiency and breast density, where women with mild to moderate breast density appeared to develop more deficiency symptoms.

Dairy products are also a good source of calcium, which plays a role in reducing the risk of breast cancer. Although the exact mechanism is still unclear, a meta-analysis of eleven studies by Hidayat et al. (19) showed a connection between calcium intake and breast cancer. The ability of calcium to regulate the cells' apoptosis, proliferation, and differentiation makes it a significant preventive factor. Both calcium and vitamin D were found to have anticarcinogenic effects in a review study by Cui (20). However, a recent meta-analysis published in 2019 by Chen et al. on the relationship between breast cancer and milk/yogurt intake did not support our results (21). This discrepancy may be due to the number of servings consumed by the subjects of Chen et al.'s study (21) and the amount of vitamin D added to the dairy products. Some studies have indicated that environmental pollutants, growth factors, and the amount and type of fat in milk can raise the risk of breast cancer. Another relevant question is whether cows that produce the milk were given bovine growth hormone, which results in an increase in the insulin-like factor-1 in milk and therefore might cause malignant cells to proliferate (21).

Conjugated linoleic acid (CLA) in dairy products is considered a chemoprotective agent. CLA also has antioxidant and anti-inflammatory effects that can decrease the risk of developing breast cancer (22). Research on the mechanism of CLA in reducing the risk of developing breast cancer is remarkably diverse, as described in a meta-analysis conducted by Zhou et al. (23). Another study by McCann et al. (24) did not support a clear correlation between CLA consumption and the development of breast cancer in pre- and postmenopausal women. They explained their result as being due to the high level of CLA consumption necessary for a preventive effect, as compared to the relatively low consumption by a typical test subject. Aro et al. (25) in their study found that breast cancer risk was higher in postmenopausal women who consumed a low amount of CLA from dairy products, but not in premenopausal women who consumed the same amount of CLA.

The study results also showed that the consumption of one to two servings of legumes per week had a negative association with breast cancer incidence. Various phytochemicals in legumes have an inhibitory effect on cell proliferation. Xu and Chang (26) comprehensively studied the effect of antioxidants and phytochemicals from different common types of legumes against nine different types of cancer, such as breast cancer, and they found that legumes are an outstanding source of natural antioxidants for the reduction of oxidative stress and cancer prevention. The fiber content in legumes was additionally found to inhibit the enterohepatic circulation of estrogen, leading to the reduction of the circulating estrogen level and resulting in a reduction of breast cancer risk (27). Fiber is also associated with decreasing cell mutation by binding with bile acid, which is thought to promote cell proliferation (28).

Fruits and vegetables are rich sources of fibers that are known to protect against breast cancer. Women who have a daily intake of three to five servings of fruits and vegetables are 83.9% less likely to develop breast cancer. An important study by Farvid et al. (29) concluded that high fiber intake from fruits and vegetables reduces the risk of developing breast cancer in women. Antioxidants in fruits and vegetables have been shown to neutralize free radicals and prevent DNA damage that might lead to cancer (29). Additionally, Naja et al. (28) concluded that the consumption of both fruits and vegetables reduces breast cancer risk. Many mechanisms could explain the preventive effect of fruit and vegetable consumption. Fiber content may bind to estrogen, thereby inhibiting the estrogen enterohepatic reabsorption. Antioxidants in fruits and vegetables also reduce oxidative stress and inflammation by protecting the DNA from damage and by inducing detoxifying enzymes. Vitamin C, found especially in citrus fruits, has been shown to benefit the immune system, while vitamin E and carotenoids have been found to have chemopreventive effects (29).

Women in the Makkah region consume seafood and fish regularly, especially on holidays. Our study indicates that consuming up to 5 servings of fish and seafood weekly reduces the risk of breast cancer by 78.9–92.8%. The positive effect of fish consumption appeared to be limited to certain common types of cancer, such as breast cancer (30). Engeset et al. (31), however, found no evidence of an inverse correlation between overall fish intake and the risk of breast cancer. Fish is a known source of omega 3 fatty acids, or polyunsaturated fatty acids (PUFAs), which can reduce the risk of breast cancer (32). Their study showed that PUFAs inhibited the epidermal growth factor receptor, which in turn reduced the proliferation of breast cancer.

Our study found that the consumption of coffee and black tea had a negative relationship to the development of breast cancer. In black tea, several antioxidant compounds and chemoprotective components are well known; catechins, particularly gallate epigallocatechin, exerted important antioxidant properties by decreasing the number of reactive oxygen species (33). Other coffee components, such as cafestol and kahweol, are known to have antioxidant and anticarcinogenic effects. Kahweol restricts the proliferation of the breast cells and causes apoptosis; it also increases the synthesis of reactive oxygen species to produce cytotoxicity (34). Other proposed mechanisms by which coffee may reduce the risk of breast cancer are though to be associated with induction of

apoptosis and reducing inflammatory markers in the circulation (33). Yang et al. (35) explained the anticancerogenic mechanisms of black tea: its antioxidant effect protects the cell and DNA from being damaged by the free radicals. The phenolic compounds in coffee are known to have antioxidant, antimutagenic, and anticarcinogenic effects against several forms of cancer. A follow-up study by Ganmaa et al. (36) found a weak inverse link between caffeine consumption and the risk of postmenopausal breast cancer. Another article from Saudi Arabia supported our findings that caffeine content in coffee and tea provides effective prevention of breast tumor growth and/or recurrence (37).

A systematic review and meta-analysis published in 2015 indicated that the increased consumption of total saturated fat positively affected breast cancer incidence (38). Moreover, high consumption of meat has also been shown to increase the risk of developing breast cancer (39). However, study results did not show a remarkable effect of high fat intake from meat and processed meat and the risk of breast cancer. It is noteworthy to perform further studies regarding this point considering the different types of meat and processed meat consumed locally.

This study is limited by the semiquantitative measurement of food intake, which is a common problem with studies that use food frequency questionnaires and recall bias. This study was also limited by the regional sample collection, relatively small sample size, and recruitment of exclusively postmenopausal women for both case and control groups. Furthermore, it is noteworthy to perform further studies about the association between the adherence to the Mediterranean diet and breast cancer incidence and/or mortality rate.

CONCLUSIONS

Our study in the Makkah region concluded that the consumption of fish and seafood, fruits and vegetables, legumes, coffee, black tea, and dairy products can have preventive effects against breast cancer. Dietary factors of the Mediterranean diet that did not show a significant effect on breast cancer incidence included olive oil, whole wheat, and leafy vegetables. It is recommended to perform additional studies using cohort study design in various regions of Saudi Arabia and with more participants.

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 studies involving human participants were reviewed and approved by Institutional Review Board of Umm Al-Qura University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FA conceived and designed the study. DH, AQ, KG, WA, AAA, AFA, HAA, and MG conducted research, provided research materials, and collected and organized data. HMA, MA, MH, AYA, SM, MQ, and WB analyzed and interpreted data. All authors wrote the initial and final drafts of the article and critically reviewed and approved the final draft of the manuscript.

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Corrigendum: Consumption of Food Components of the Mediterranean Diet Decreases the Risk of Breast Cancer in the Makkah Region, Saudi Arabia: A Case-Control Study

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A Corrigendum on

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In the original article there was an error in affiliation 10 as published. Instead of Makkah the city should be "Jeddah."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Immunomodulatory and Anticancer Activities of Barley Bran Grown in Jordan: An *in vitro* and *in vivo* Study

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The Mediterranean diet is regarded as one of the most healthful dietary patterns in the world, owing to a combination of foods high in antioxidants and anticancer constituents. Barley bran is one of the components of the Mediterranean diet. It has nutritional and beneficial effects in different pathological conditions. Many studies were achieved to assess the nutritious values of barley bran, but there is no research indicating immunomodulatory and anticancer activities of barley bran grown in Jordan. The present study aims to examine and assess the potential immunomodulatory and anti-tumor activities of ethanol, *n*-hexane, aqueous/methanol, and water extracts obtained from barley bran. The Maceration method was utilized to prepare ethanol, *n*-hexane, aqueous/methanol, and water extracts. Various phytochemical groups were determined by using qualitative phytochemical tests. The antiproliferative activity of extracts was determined against MCF-7, HCT-116, A549, and EMT6/p by the MTT assay. The Folin-Ciocalteu reagent was used to detect the total phenolic content in extracts. Furthermore, immunomodulatory activity was assessed by determining the effect of extracts on splenocytes proliferation in the presence and absence of mitogens. The nitro blue tetrazolium assay and the neutral red method were used to assess the effect of each extract on the phagocytic activity of macrophages and pinocytosis, respectively. For the *in vivo* part, three different concentrations (10, 20, and 30% w/v) of barley bran were used to test the prophylactic effect in four Balb/C mice groups inoculated with EMT6/p cell-line subcutaneously. Also, serum samples were collected to assess the effect on cytokines (IFN-gamma, IL-2, IL-4, and IL-10). Barley bran extracts inhibited cancer cell proliferation. According to immunoassays, *n*-hexane and aqueous/methanol extracts could significantly rise lymphocyte proliferation and pinocytosis activity of macrophages. The activity of phagocytosis was increased by *n*-hexane and ethanol extracts. For the *in vivo* part, the average tumor size and weight of mice given the 30% barley bran group was significantly reduced ($p < 0.05$) compared with the control group. During our study, higher levels of TH1 cytokines (IFN- γ, IL-2) and lower levels of TH2 cytokine (IL-4) and T regulatory cytokine (IL-10) were obtained due to consumption of barley bran in food. Barley bran can be used as a prophylactic agent because it has anti-cancer and immunomodulatory activities.

Keywords: anticancer nutrition, immunomodulatory effect, natural products, tumors, animal model

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INTRODUCTION

Cancer is the second cause of death in Jordan after cardiovascular diseases (1). It is a broad term disease that causes cells to divide uncontrollably. The mortality and morbidity of cancer disease are estimated to increase as the young population ages with longer life expectancy (1). Cancer can be caused by external and internal factors. External factors include radiations, smoking tobacco, and pollutants in drinking water, food, air, chemicals, certain metals, and infectious agents. On the other hand, the internal factors are genetic mutations, body immune system, and hormonal disorders (2). There are many types of cancer; colorectal cancer is stated to be the most prevalent type in males followed by breast cancer in females (3). According to the latest published annual report by Jordan's Cancer Registry (JCR) for the period 2006 to 2015, there is a rise in the number of cancer cases. **Table 1** demonstrates the most common cancers in Jordan among both genders with breast cancer being the most widespread, one among females with 1,145 registered cases. Cancer is a big problem; so many pieces of research are made about it.

Cancerous cells can form tumors, damage the immune system, and cause other changes that avoid body function. Cancerous cells may present in one area, and then spread via the lymphatic system to other areas (4). Cancer is not a new disease. Many people suffer from cancer throughout the world. The term cancer came from a Greek word karkinos used by Hippocrates (460–370 B.C.) to identify carcinoma tumors, but he was not the first to discover this disease (5).

Cancer was first identified by the ancient Egyptians in 1,500 BC; the first form identified was breast cancer and, subsequently, other mummies with bone cancer (around 1,600 BC); in that time, treatment was only palliative, but no effective cure was found to treat it (5).

Treatments are continuously developing. Examples of methods include chemotherapy, surgery, radiation therapy, stem cell transplantation, immunotherapy, cancer vaccination, and photodynamic therapy. Treatment methods rely on the cancer stage, type, and location (6). The drugs that are used to treat cancer are unsafe, meaning that they can cause vomiting, nausea, diarrhea, fatigue, hair loss (associated with chemotherapy), oral mucositis, oral infection (candida colonization mostly), xerostomia (loss of saliva production), and osteoradionecrosis, usually related to radiation treatment of head and neck cancers, while some rectal, bladder, and bowel toxicities were detected during radiation therapy of rectal and prostate cancers (6). Also, patients with cancer can suffer from anemia, infertility, immunosuppression [defected white blood cells (WBC) numbers] and lung dysfunction (7). Continuous efforts have been made to reduce the harmful side effects of drugs that are used to treat cancer. These side effects inspired scientists to pursue new potential natural products, either on their own or as adjuvant drugs, for anti-cancer treatment with least side effects. Plant-derived products are used because they are relatively safe, simple, low cost, and less-toxic products (8).

The first serious trials were performed in the 1950s, with the discovery of anticancer agents (vinblastine, vincristine, podophyllotoxins, and vinca alkaloids), which prompted the United States National Cancer Institution (NCI) to pay more attention and establish a programmed plant collection in 1960 (9). Many of these natural species are used in our daily diet and have shown an anti-cancer and immunomodulatory effect, such as green tea (Camellia sinensis)—inhibition of metastasis, milk thistle (Silybum marianum)—caused cell-cycle arrest and 90% reduction of tumor, garlic (Allium sativum)—deceased the rate of tumor growth, and Dandelion (Taraxacum officinale)—stimulated the immune system by rising tumor necrosis factor (TNF)-α and interleukin (IL)-1α (10).

These natural products have immunomodulatory properties, leading to immunotherapy. Immunotherapy helps the immune system to recognize and kill cancer cells. It is defined as a therapy that can activate or suppress the immune system. It aims to enhance the immunological anti-cancer response by combining vaccines with immunostimulatory cytokines or by blocking the pathways used by cancer cells to suppress the response (11). Furthermore, The Mediterranean diet combines foods common in Mediterranean-bordering countries. It includes fruits, vegetables, unrefined grains, and olive oil. It has been associated with a lower incidence of colon, prostate, stomach, and breast cancer (12).

Barley (Hordeum vulgare) is a main cereal grain cultivated in temperate climates worldwide. It is found at different places in the Fertile Crescent. It is utilized as animal feed and as a part of several health foods. Barley is similar to the Latin word farina "flour," while bran means "barley-house" (13). Barley includes a variety of phytochemicals in various amounts, which are usually determined by genotypic or environmental variables, or by the combination of both factors. Phytochemicals in barley are classified into several key classes, including phenolic acids, flavonoids, lignans, tocols, phytosterols, and folates (14). Studies showed that barley has a number of biological activities, including angiotensin-converting enzyme inhibitory activity, tyrosinase inhibitory activity, antioxidant ability, and xanthin oxidase inhibitory activity (13). Barley also has anticancer activities against different cancer cell lines such as A549 (15). A study

TABLE 1 | Ten most common cancers among Jordanians, both genders, 2015*.

Rank	Site	Frequency	Percentage (%)
1	Breast	1,145	20.6
2	Colorectal	668	12.0
3	Lymphoma	390	7.0
4	Lung	378	6.8
5	Urinary Bladder	296	5.3
6	Thyroid	228	4.1
7	Prostate	215	3.9
8	Leukemia	200	3.6
9	Stomach	157	2.8
10	Uterus	156	2.8

Data from the Jordan's Cancer Registry, Ministry of Health.

showed that barley has immunomodulatory activity as it has bioactive components (16).

Bran is usually obtained by millers by separating the bran (the outer layer of the grain) and the embryo from the endosperm so that bran was thrown away in the milling process, leaving defatted grain to be used (17). Studies showed that the barley bran can be used before cancer development as a prophylactic agent and can also be used during cancer formation (17). Unlike chemotherapeutic agents that suppress the immune system, making the patient more susceptible to secondary diseases, barley bran can amplify the immune system (immune system boosters) (18). Phytochemicals in barley bran have not been previously tested as a combination. In this study, we hypothesized that the active phytochemicals of barley bran may have the potential to inhibit cancer cells and activate the immune system.

MATERIALS AND METHODS

Barley Bran Supply and Extracts Preparation

Barley bran was generously donated by Engineer Amer Abu Namous Establishment for marketing natural products in Amman, and, as declared by the workers there, barley is given to them in huge packages to grind and distribute it to the shops. It was cleaned well from Weevils by spreading it under the warm sun for 5 days in a row with alternative flipping over; after that, it was retained in dry jars until use.

Different extracts were prepared by using solvents of different polarities. Ethanol (70%), n-Hexane, aqueous/methanol (70:30), and water were utilized to macerate barley bran (1L per 100 g) for 14 days at room temperature with daily stirring. After that, residue was eliminated; the supernatant was filtered and concentrated using a rotary evaporator, entirely dried using lyophilizer, and saved at −20°C until utilized.

Qualitative Phytochemical Screening of Barley Bran Extracts

Phytochemical screening was achieved for ethanol, n-hexane, aqueous/methanol, and water extracts. Qualitative chemical screening tests were done to identify the existence of the main classes of substances, such as saponins, tannins, terpenoids, alkaloids, flavonoids, phenols, steroids, and carbohydrates. Identification of the extracted components was performed using the standard methods described (19).

Quantitative Analysis of Barley Bran n-Hexane and Aqueous/Methanol Extracts by Liquid Chromatography-Mass Spectrometry (LC-MS)

The samples were prepared by dissolving them in 2 ml of DMSO (dimethyl sulfoxide) and finishing the 50-ml amount with acetonitrile. Each sample was centrifuged at 4,000 rpm for 2 min, and then 1 ml was moved to the autosampler. The amount of the injection was 3 μL. The study was carried out using the impact II ESI-Q-TOF system fitted with the BurkerDalotonik Elute UPLC system from BurkerDaltonik (Berman, Germany). The device worked using the Ion Source Apollo II ion funnel electrospray source (capillary voltage, 2,500 v; nebulizer gas, 2 bars; dry gas flow, 8 L/min; dry temperature, 200°C; mass accuracy, <1 ppm; mass resolution, 50,000 FRS; the TOF repetition rate, 20 kHz). A Burker's solo 2-C-18 UHPLC column (100 mm x 2.1 mm x 2 μm) was used to conduct chromatographic separation at a flow rate of 0.51 ml/min and a column temperature of 40°C. For the detection of ms/z and retention time, all standards were used.

Cell Lines and Cell-Culturing Condition

To investigate the potential anticancer effect of barley bran extracts, five cell lines were utilized (MCF-7, HCT-116, A549, EMT6/P, and VERO). MCF-7 [(ATCC® HTB-22™) with 14 passages] is the human epithelial breast cancer cell line. HCT 116 [(ATCC® CCL-247™) with 4 passages] is the human colon carcinoma cell line. A549 [(ATCC® CCL-185™) with 9 passages] is the adenocarcinomic human alveolar basal epithelial cell line, while EMT6/p [ATCC® CRL-2755™) with 4 passages] is the mouse epithelial breast cancer cell line. VERO is the normal kidney epithelial cells extracted from an African green monkey. Doxorubicin was used as positive control. The cells were cultured in a complete medium and incubated at 37°C in 5% CO_2, a 95% humidity incubator. The MCF-7 cell line was cultured in a complete RPMI 1640 medium, while the EMT6/P cell line was cultured in a complete MEM medium. The rest of the cell lines were cultured in complete DMEM-medium high glucose.

Experimental Animals

This study was carried out according to standard ethical guidelines, and all of the experimental protocols got the approval by the Research and Ethical Committee at the Faculty of Pharmacy-Applied Science Private University. To accomplish this study, Balb/C mice within age range of 4–6 weeks and body weight of 23–25 grams were utilized. Wooden shaving cages were utilized as bedding to save the mice. The environmental parameters in the animal room were 25°C temperature, 50–60% humidity with continuous air ventilation.

Antiproliferative Assay

After culturing, trypsinization, and cell counting for a particular cell line, the cells were seeded into a 96-well tissue culture plate (100 μL/well) at an exact concentration of 15,000 cells/well using a multichannel pipette. After 24 h of incubation, the media in each well was totally removed, and the attached cells were treated in triplicate with decreasing concentrations of different extracts of barley bran (5–0.078 mg/ml), resulting in a total volume of 200 μL/well. Following incubation for 48 h, An MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit (Bioworld, UK) was utilized to measure cell viability. The principle of the assay is to identify the reduction of MTT by mitochondrial dehydrogenase to form blue formazan crystals, reflecting normal function of the mitochondria and cell viability. The MTT assay was done by removing old media from each well, washing it with PBS, then adding 100 μl of culture media and 10 μl of thiazolyl blue tetrazolium solution. After 3 h of plate incubation, 100 μl of DMSO was added to dissolve the formazan particles that were formed in live cells. Then, the plate was incubated for 1 h, and placed on ELISA microplate absorbance reader at 550 nm to measure the optical density (OD).

To calculate the percentage of survival cells and IC50 values, Microsoft Excel software was utilized.

$$\text{Percentage of cell viability (\%)} = (\text{OD of treated cell}/\text{OD of control cell}) * 100$$

Preparation of the Positive Control Doxorubicin

Doxorubicin (Dox) was utilized as a positive control because it has a broad spectrum of antitumor activity. It belongs to anthracyclines, which are cytotoxic agents. To prepare desired concentrations of 200 μM, the stock solutions were diluted before use by DMEM.

Calculation of Inhibitory Concentration (IC_{50})

Half maximal inhibitory concentration (IC_{50}) is the molar concentration of a substance at which there is 50% cell death in comparison to cells of negative control. Regarding the NCA plant screening programs, if the IC_{50} value of a crude extract incubated (48–72 h) is <20 μg/ml in carcinoma cells, usually, a crude extract is considered to have *in vitro* cytotoxic activity (20). The calculation and analysis of IC_{50} values were conducted by non-linear regression test in a statistical package for the social sciences (SPSS) version 22 (Chicago, Illinois).

Total Phenolic Content (TPC) by Folin-Ciocalteu (F-C) Reagent

The amount of TPC in barley bran extracts was detected using the F-C procedure described by Roslan et al. (21). The principle of this method signifies the reaction between F-C reagent that is made of a mixture of sodium tungstate (Na_2WO4) and sodium molybdate (Na_2MoO4) with phenolic compounds to give a blue color. Also, F-C was found to be reactive against other antioxidants (besides phenolics) as aldehydes, ketones, thiols, unsaturated fatty acids, proteins, amino acids, amines, nucleotides, carbohydrates, vitamins, and some nitrogenous compounds (21).

Firstly, 5 mg/ml of each extract was prepared as a stock solution. Five various dilutions were also prepared from each stock as 2.5, 1.25,0.625,0.312,0.156 mg/ml. Briefly, 12.5 μL of each dilution was mixed with 250 μL of 2% sodium carbonate solution in a 96-well microplate in triplicate. They were permitted to react at RT for 5 min. Then, 12.5 μL of 50% F-C reagent was added and permitted again to stand at RT for 30 min. A plate reader was used to read the absorbance of reaction mixture at 630 nm (21). Gallic acid standard solution was used at different concentrations, ranging from.1 to 1. mg/ml in distilled water (**Appendix 3.1**) to produce a standard curve. Data are expressed as equivalent of gallic acid (mg) for each milliliter of each extract (21).

Preparation of Murine Splenocytes

The spleen was removed aseptically after a Balb/C mouse was sacrificed. Spleen cells were passed through the mesh of a tissue grinder suspended in RPMI-1640 media. The cells suspension was centrifuged at 1,500 RPM and 4°C for 10 min. After centrifugation, supernatant was eliminated and cells were re-suspended with RBC cell lysis to remove red blood cells. Pipetting of the suspension was essential for many times. After 10 min of another centrifugation, the resulted pellets were re-suspended in 5-ml RPMI-1640 media, and splenocytes were available to be counted and seeded for various assays.

Lymphocytes Proliferation Assay

Lymphocytes Proliferation Assay in the Presence of Con A or LPS

Based on the manufacturer's instructions, this assay was conducted using the MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide] assay kit (Bioworld, UK). Briefly, splenocytes suspension was prepared in RPMI-1640 (supplemented with 50 U/ml streptomycin, 50-U/ml penicillin, and 10% FBS) and seeded into a 96-well culture plate at a specific concentration of a 2-x-106 cell/ml in the presence of 5-μg/ml Con A or 4-μg/ml LPS. Then, 100 μL of various concentrations (0.625–5 mg/ml) of barley bran extracts were added in an in-triplicate manner. The plate was incubated for 48 h under 5% CO_2 and humidified atmosphere of 95% air at 37°C temperature. After the incubation, each well was treated with 10 μL of MTT (5 mg/ml) solution and incubated again for 4 h. After that, it was treated with 100 μL of DMSO to dissolve the developed formazan particles, and the absorbance was measured using ELISA microplate absorbance reader at 550 nm. Results were expressed as an stimulation index compared with negative control (22).

Lymphocytes Proliferation Assay Without the Presence of Con A and LPS

In this assay, the same procedures were performed as the previous assay but with omission of Con A and LPS addition.

Macrophage Isolation From Peritoneal Fluid

Owing to the high presence of tissue macrophages, the peritoneal cavity is a favored site for the collection of these cells. Forty-eight h before peritoneal macrophages (PEM) collection, mice were injected intra-peritoneally with 5 ml of a 3% (w/v) brewer thioglycollate medium (23). After that, ice-cold sterile phosphate –buffer saline (pH 7.4) was utilized to isolate peritoneal macrophages (PEM). The mice were euthanized by cervical dislocation, and their abdominal cavities were visualized to introduce 5 ml of ice-cold PBS into the cavity. Following gentle massaging, the fluid was removed and put in a centrifuge tube held on ice. The process was repeated many times. After centrifugation at 1,500 RPM, 4°C for 10 min, the cell pellets were re-suspended in a completed RPMI 1640 medium.

In vitro Phagocytic Assay [Nitro Blue Tetrazolium (NBT) Reduction Test]

The NBT reduction assay was assessed, depending on the method defined by Madakkannu and Ravichandra (24). In Brief, peritoneal macrophages were seeded into a 96-well tissue culture plate at a definite concentration of 5-x-10^6 cells/well, and cultured with various concentrations of barley bran extracts (5–0.625 mg/ml) for 48 h at 37°C. After that, each well was

treated with 20-μL yeast suspension (5-×-10^7 cells/ml in PBS) and 20-μL nitro blue tetrazolium (NBT) (1.5 mg/ml in PBS), except the control wells, which were only treated with 20-μL yeast suspension (5-×-10^7 cells/ml in PBS). Following cells were incubated for 60 min at 37°C, the supernatant was eliminated and the adherent macrophages rinsed with RPMI 1640. The cells were air-dried before 140-μL DMSO, and 120 μL of 2M KOH was added to each well. At the end, the plate was put on a microplate reader at 550 nm to measure the optical density (OD). The percentage of NBT reduction, which represented the phagocytic activity, was calculated according to the following equation:

$$\text{Phagocytic index} = (\text{OD sample} - \text{OD control})/\text{OD control} * 100.$$

Pinocytic Activity Assay by the Neutral Red Method

The neutral red method was used to evaluate macrophages pinocytic activity of barley bran extracts, which were defined by Madakkannu and Ravichandran (24). Peritoneal macrophages were seeded into a 96-well tissue culture plate at an exact concentration of 5-x-10^6 cells/well, treated with various concentrations of barley bran extracts (5–0.625 mg/ml), and then incubated at 37°C for 48 h. Thereafter, each well was treated with 100 μL of neutral red solution (7.5 mg/ml in PBS) and incubated for 2 h. Following incubation, the supernatant was eliminated, and each well was rinsed with PBS two times to get rid of neutral red that was not pinocytized by macrophage. Then, 100 μl of cell lysis solution (ethanol and.01% acetic acid at the ratio of 1:1) was added to each well to break the cells. The plate was kept at RT overnight. At the end, the microplate reader was utilized to measure the optical density (OD) at 540 nm. Pinocytic activity was described by the absolute OD values, which reveal the dye uptake (24).

Preparation of Food for the *in vivo* Part (Experimental Diet)

Three percentages were prepared by weight; the 1st group was 900-g mouse fodder and 100-gm barley bran; the 2nd group was 800-g mouse fodder and 200-g barley bran; and the 3rd group was 700-g fodder and 300-g barley bran (**Figure 1**).

Firstly, the fodder and barley bran were separately weighed and grinded and then well-mixed regarding to the upper categories, and then water was added to each category (the water percent was depending on the appropriate texture for kneading). After that, the dough was rolled into rollers, clipped into small similar discs, and exposed to the sun for a range of 3–5 days to dry. At the end, it was kept in a −20°C freezer until the time of usage.

Tumor Prophylaxis Assay

Forty female Balb/C mice were divided into 4 groups: Group 1: the 10% group (were fed fodder mixed with 10% barley bran), Group 2: the 20% group (were fed fodder mixed with 20% barley bran), Group 3: the 30% group (were fed fodder mixed with 30% barley bran), and Group 4: the control group (the healthy mice were fed usual fodder and inoculated with tumor) (**Table 2**). This study was divided into prophylactic and treatment phases. Each phase lasted 15 days.

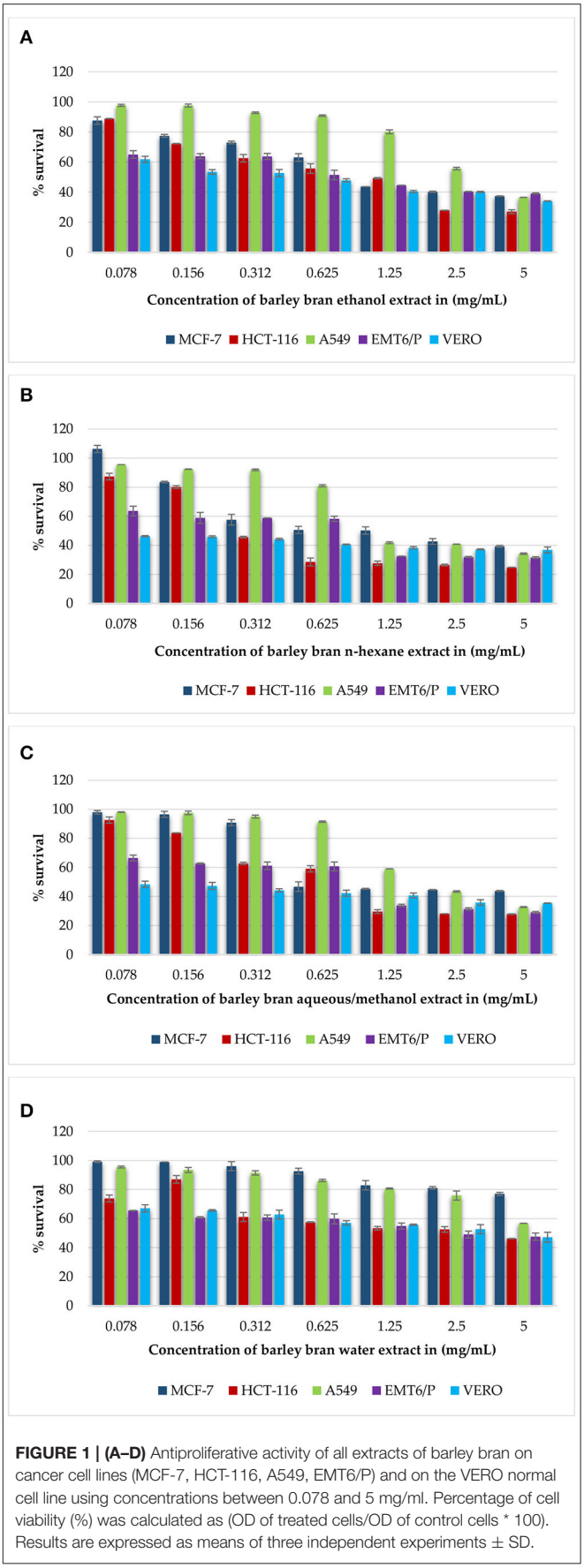


FIGURE 1 | (A–D) Antiproliferative activity of all extracts of barley bran on cancer cell lines (MCF-7, HCT-116, A549, EMT6/P) and on the VERO normal cell line using concentrations between 0.078 and 5 mg/ml. Percentage of cell viability (%) was calculated as (OD of treated cells/OD of control cells * 100). Results are expressed as means of three independent experiments ± SD.

TABLE 2 | Study groups with percentages received, an *in vivo* experiment.

Group	Number of mice	Name	Prophylaxis/treatment
Group 1	10	10% BB	10% BB in fodder
Group 2	10	20% BB	20% BB in fodder
Group 3	10	30% BB	30% BB in fodder
Group 4	10	Control group	0% BB in fodder

TABLE 3 | The percentage yield obtained from the extraction of 1,010 gm of barley bran using the maceration method (% yield = weight after extraction/weight before extraction * 100%).

Extraction solvent	% of dried extracts yields
Ethanol	1.83%
n-hexane	0.6%
Aqueous/methanol	2.4%
Water	3.5%

The feeding process began from September 29, 2020 to November 1 (the day of the sacrifice).

About 1,000,000 EMT6/p cells of tumorigenic dose per 1 ml of MEM media were injected subcutaneously (S.C.) on October 13 (2 weeks of feeding pre-inoculation) in the abdominal area of each 11–12-week-old female Balb/C mouse.

Tumors were permitted to be grown for 14 days; here, we showed the number of tumors, the size, and the weight averages of each barley bran group. After that, we compared them with the control group. Volumes were measured using the following below equation (25); digital caliper was utilized to evaluate tumors size.

Tumor volume = $A^* B^2 {}^*0.5$

where:

A = length of the longest aspect of the tumor

B = length of the tumor aspect perpendicular to A.

Determination of IFN-γ, IL-2, IL-4, and IL-10 Levels in a Serum Sample

Serum levels of IFN-γ, IL-2, IL-4, and IL-10 were evaluated for demonstrative samples of mice from all research groups using a mouse TH1/TH2 ELISA kit (affymetrixebioscience, Canada). The same quantitative sandwich enzyme immunoassay technique was applied for IFN-γ, IL-2, IL-4, and IL-10. A 96-well plate (Corning™ Costar™ 9018) was coated with a monoclonal capture antibody specific for mice IFN-γ, IL-2, IL-4, and IL-10 and incubated overnight at 4°C; after that, aspirated, washed three times, and soaked with a wash buffer for 1 min. ELISA/ELISPOT diluent was utilized to block the wells and incubated at room temperature for 1 h. Standards control and samples were pipetted into the wells and incubated at room temperature for 2 h. After washing was applied, detection antibody specific for mice IFN-γ, IL-2, IL-4, and IL-10 was added and incubated at room temperature for 1 h. After another washing, an avidin-HRP (enzyme) was added and incubated at room temperature for half an hour. After that, another washing was applied; a substrate solution of TMB (Tetramethylbenzidine) was added and incubated at room temperature for 15 min. Finally, the stop solution was added, and ELISA microplate absorbance reader was utilized to measure the absorbance at 450 nm. The intensity of the color measured is in proportion to the quantity of mouse IFN-gamma or mice IL-2, IL-4, and IL-10 bound in the initial stage. The samples absorbance values were then read off the standard curve (**Appendices 3.2–3.5**).

A detailed and step-by-step procedure was done according to Catalog No. 887314 for mice IFN-γ, IL-2, IL-4, and IL-10, respectively.

Statistical Analysis

Data were expressed using the mean ± standard deviation of triplicate independent experiments utilizing the SPSS 22 one-way ANOVA, followed by post hoc test. When the *P*-value was < 0.05 ($p < 0.05$), the differences between groups were significant. IC50 values were evaluated using nonlinear regression in SPSS (Statistical Package for the Social Science, Chicago, Illinois version 22).

RESULTS

Preparation of Different Extracts From Barley Bran

Percentage Yield of Extracts

After the extraction of 1,010 gm of barley bran, high differences were detected in the percentage yields (**Table 3**). We used 500 gm of barley bran for ethanol extract, and 200 gm of barley bran for n-hexane, aqueous/methanol, and water extracts. By utilizing the maceration method, the percentage yields for barley branethanol, n-hexane, aqueous/methanol, and water extracts were (1.83%), (0.6%), (2.4%), and (3.5%), respectively. The highest yield was (3.5%) for the water extract, while the lowest yield was (0.6%) for n-hexane extract.

Qualitative Phytochemical Screening of Barley Bran Extracts

The phytochemical bioactive constituents of ethanol, n-hexane, aqueous/methanol, and water extracts were qualitatively observed using the standard methods (**Table 4**). The screening results revealed that ethanol extract has phenols and flavonoids. N-hexane extract has terpenoids, steroids, and flavonoids. Aqueous/methanol extract has saponins, phenols, flavonoids, alkaloids, and carbohydrates. Finally, water extract has saponins, tannins, and flavonoids.

Quantitative Analysis of Barley Bran n-Hexane and Aqueous/Methanol Extracts by Liquid Chromatography-Mass Spectrometry (LC-MS)

About 5 mg of barley bran n-hexane and aqueous/methanol extracts was utilized for further analysis. This analysis of n-hexane and aqueous/methanol extracts using LC-MS showed

TABLE 4 | Qualitative phytochemical screening results of secondary metabolites obtained from barley bran extracts.

Phytochemical screening tests	Ethanol extract	n-hexane extract	Aqueous/ methanol extract	Water extract
Saponins	−	−	+	+
Tannins	−	−	−	+
Terpenoids	−	+	−	−
Steroids	−	+	−	−
Phenols	+	−	+	−
Flavonoids	+	+	+	+
Alkaloids	−	−	+	−
Carbohydrates	−	−	+	−

Results were rated as: + (positive), − (negative).

TABLE 5 | Major compounds identified in barley bran *n*-hexane extract using the LC-MS method.

No.	Compounds	Formula	RT	%
1	y-Linolenic acid	C18H30O2	29.51	9.316754
2	Stearic acid	C18H36O2	32.82	34.31192
3	Palmitic acid (NMR)	C16H32O2	33.43	56.37133

RT, retention time.

TABLE 6 | Major compounds identified in barley bran aqueous/methanol extract using the LC-MS method.

No.	Compounds	Formula	RT	%
1	Anthranilic acid	C7H7NO2	3.14	3.695253
2	3,5-Dimethoxy-4-hydroxyacetophenone	C17H14O7	4.32	4.555799
3	HexA-Chrysoeriol (or Kaempferide) (PUT)	C22H20O12	7.08	3.048765
4	5,6,4'-Trihydroxy-7,3'-dimethoxyflavone	C17H14O7	10.35	88.70018

RT, retention time.

the existence of high concentrations of stearic acid, palmitic acid, and 5,6,4'-Trihydroxy-7,3'-dimethoxyflavone with the following percentages: 88.7, 56.37, and 34.3%, respectively (**Tables 5**, **6**). Some other compounds were identified in lower concentration like y-Linolenic acid, 9.3%; 3,5-Dimethoxy-4-hydroxyacetophenone, 4.6%; anthranilic acid, 3.7%; and HexA-Chrysoeriol (or Kaempferide), 3.04%.

Antiproliferative Activity of Barley Bran Different Extracts on Different Cell Lines

Gradual decreasing in the concentrations of barley bran extracts (5–0.078 mg/ml) on the MCF-7 cell line caused a rise in the average percentage survival in a dose-dependent manner. At concentration of 5 mg/ml, the inhibition percentages of ethanol, n-hexane, and aqueous/methanol extracts were 63, 61, and 57%, respectively. Ethanol, n-hexane, and aqueous/methanol extracts showed the high activity with IC_{50} values of 0.98 mg/ml, 0.77 mg/ml, and 0.85 mg/ml respectively, whereas aqueous extract was the least effective against the MCF-7 cell line with IC_{50} value more than 5 mg/ml (**Figures 1A–D**).

Similar results were obtained when the HCT-116 cell treated with the same concentrations of barley bran extracts. At concentration of 5 mg/ml, the inhibition percentages of ethanol, n-hexane, aqueous/methanol, and water extracts were 73, 76, 72, and 54%, respectively. Ethanol, n-hexane, and aqueous/methanol extracts showed the high activity with IC_{50} values of.93 mg/ml, 0.38 mg/ml, and 0.67 mg/ml, respectively, whereas aqueous extract was the least effective against the HCT-116 cell line with IC_{50} value of 1.3 mg/ml (**Figures 1A–D**).

The dose-dependent inhibition was also obtained after treating A549 cells with increasing concentrations of barley bran. At a concentration of 5 mg/ml, the inhibition percentages of ethanol, n-hexane, and aqueous/methanol extracts were 64, 66, and 68%, respectively. Ethanol, n-hexane, and aqueous/methanol showed the moderate activity with IC_{50} values of 2.8 mg/ml, 1.23 mg/ml, and 2 mg/ml, respectively, whereas water extract was the least effective against A549 cell line with IC_{50} value more than 5 mg/ml (**Figures 1A–D**).

The mouse breast cancer cell line (EMT6/P) was also treated with barley bran, and results showed similar response comparative with other cell lines. At a concentration of 5 mg/ml, the inhibition percentages of ethanol, n-hexane, aqueous/methanol, and water extracts were 61, 69, 71, and 53%, respectively. Ethanol, n-hexane, and aqueous/methanol showed the high activity with IC_{50} values of.84 mg/ml,0.62 mg/ml, and.71 mg/ml, respectively, whereas water extract was the least effective against the A549 cell line with IC_{50} value of 2.2 mg/ml (**Figures 1A–D**).

The VERO cell line was subjected to various concentrations of extracts, ranging from (5–0.078 mg/ml). The Vero cell line showed more resistance to the tested barley bran signified by the percentage of survived cells. About 66, 64, 60, and 53% survival percentages were reported for ethanol, n-hexane, aqueous/methanol, and water extracts, respectively (**Figures 1A–D**).

The half maximal inhibitory concentration (IC_{50}) is the concentration of a compound causing 50% cell death in comparison to the negative control. IC_{50} values were calculated for all extracts. N-hexane with IC_{50} values of.77 mg/ml, 0.62 mg/ml, 0.38 mg/ml, 1.23 mg/ml was the most effective extract against the MCF-7, EMT6/p, HCT-116, and A549 cell lines, respectively. The toxicity of all extracts was limited with IC_{50} values more than 5 mg/ml. Doxorubicin was utilized as positive control in our antiproliferative trials. Doxorubicin showed good activity against all types of cell lines MCF-7, HCT-116, A549, EMT6/P, and Vero cells lines with respective IC_{50} values of 5.66, 10.84, 101.7, 0.57, and more than 200 μg/ml. **Table 7** demonstrates the IC_{50} for all extracts and doxorubicin utilizing five cell lines.

To identify the safe active concentration for each extract and compare to the VERO normal cell line concentration, selectivity index (SI) also was identified for ethanol, n-hexane, aqueous/methanol, and water extracts of barley bran against

TABLE 7 | The IC_{50} of ethanol, *n*-hexane, aqueous/methanol, and water extracts of barley bran using various cell lines in comparison to the normal Vero cell line and to doxorubicin*.

Cell line	IC_{50} of ethanol (mg/ml)	IC_{50} of *n*-hexane (mg/ml)	IC_{50} of aqueous/ methanol (mg/ml)	IC_{50} of water (mg/ml)	IC_{50} of doxorubicin (μg /ml)
MCF-7	0.98 ± 0.22	0.77 ± 0.11	0.85± 0.08	>5	5.66 ± 0.4
EMT6/P	0.84 ± 0.2	0.62 ± 0.4	0.71 ± 0.3	2.2 ± 0.1	0.57 ± 0.4
HCT-116	0.93 ± 0.1	0.38 ± 0.1	0.67 ± 0.1	1.3 ± 0.1	10.84 ± 0.2
A549	2.8 ± 0.1	1.23 ± 0.1	2 ± 0.02	>5	101.7 ± 0.3
VERO	>5	>5	>5	>5	>200

**Data were presented in (mean ± SD).*

TABLE 8 | The SI of ethanol, n-hexane, aqueous/methanol, and water extracts against MCF-7, EMT6/P, HCT-116, and A549 cell lines.

Cell line	SI of ethanol	SI of *n*-hexane	SI of aqueous/methanol	SI of water	SI of doxorubicin
MCF-7	5.1	6.49	5.88	1	35.3
EMT6/P	5.95	8.06	7.04	2.27	350.8
HCT-116	5.38	13.16	7.46	3.85	18.5
A549	1.79	4.07	2.5	1	2

MCF-7, HCT-116, A549, and EMT6/P cell lines. SI signifies the ratio between the IC50 of the normal cell line (Vero)/IC50 of each extract. N-hexane extract was mostly effective against HCT-116 and moderately effective against other cell lines. Ethanol and aqueous/methanol extracts were moderately selective against all cancer cell lines except A549. Water extract was the least effective extract against all cancer cell lines. Doxorubicin was the safest against EMT6/P followed by MCF-7 and HCT-116. **Table 8** demonstrates the SI of the tested plant extracts.

Total Phenolic Content (TPC) of Barley Bran Different Extracts Using Folin-Ciocalteu Reagent

The Folin-Ciocalteu procedure was utilized to identify the TPC in barley bran extracts. At 630 nm, the TPC of the extracts was carried out in a concentrations range of 5–0.156 mg/ml.

At concentrations of 5, 2.5, and 1.25 mg/ml, aqueous/methanol extract showed the highest TPC with 0.44, 0.24, and 0.100 mg/ml equivalent of gallic acid, respectively. Ethanol extract results were 0.40, 0.24, and 0.16 mg/ml at the same mentioned concentrations. The third one in a raw was water extract with values of 0.33, 0.142, and 0.065 mg/ml gallic acid equivalent, and, finally, the lowest content was n-hexane with values of 0.12, 0.046, and 0.024 mg/ml (**Figure 2**).

The Effect of Barley Bran Different Extracts on the Proliferation of Splenic Lymphocytes

Experimental findings showed that most extracts induced an increase in lymphocytes cell proliferation in the presence and absence of Con A and LPS (**Figures 3A–C**). In the presence of Con-A and at a concentration of 5 mg/ml, n-hexane extract was the most effective extract, with the highest stimulation index of 3.4, followed by aqueous/methanol and ethanol with stimulation indices of 1.4 and 1.5, respectively.

In the presence of LPS, also n-hexane extract was the most effective extract with the highest stimulation index of 4, followed by aqueous/methanol, aqueous, and ethanol extracts with stimulation indices of 1.7, 1.6, and 1.5, respectively.

The same experiment was repeated without LPS and Con A. Experimental findings showed that all extracts induced an increase in lymphocytes cell proliferation. The stimulation indexes of ethanol, n-hexane, aqueous/methanol, and water extracts were 1.8, 3.6, 1.6, and 1.77, respectively, at concentration of 5 mg/ml (**Figure 3C**).

The Effect of Barley Bran Different Extracts on Phagocytosis

Phagocytic activity of peritoneal macrophages was identified by measuring the ability of NBT reduction after treatment with different extracts. We estimated the extracts' effects on phagocytic indices of the cells after 1-h incubation with yeast cells and NBT. The results revealed that peritoneal phagocytic activity was significantly increased after exposure to the extracts at concentration that ranged from 5 to 0.625 mg/ml. At concentration of 5 mg/ml, n-hexane had the highest increase with 1,108% of phagocytic activity, followed by ethanol with 784% and aqueous/methanol with 497%, while water extract had a lower activity at the same concentration with phagocytic index of 445% (**Figure 4**).

The Effect of Barley Bran Different Extracts on Pinocytosis

This test was utilized to estimate the effect of each solvent extract on the pinocytotic activity of macrophages. At a concentration that ranged within 5–0.625 mg/ml, the pinocytotic activity was enhanced under exposure to different extracts. N-hexane extract produced the highest increase in the pinocytotic activity with value of 2.9 compared with control value of 0.8. However, the pinocytotic activity of aqueous/methanol, ethanol, and water extracts was 2.3, 1.5, and 0.9, respectively (**Figure 5**).

Part Two: *In vivo* Results

Effect of Barley Bran Consumption on Tumor Development and Growth

After Balb/C mice were fed fodders having various percentages of barley bran for 15 days, tumor inoculation was performed with the EMT6/p cell line subcutaneously. After that, we waited for 15 days with the continuing of feeding until the day of the sacrifice. Caliper digital was utilized to measure the tumor sizes, and then the tumors were weighed at the end of the study.

Tumor size in mice given fodders with barley bran was significantly ($p < 0.05$) reduced compared to the control group (**Table 9**). Feeding 10, 20, and 30% barley bran with fodders showed significant inhibition of tumors where a reduction in tumor size was reported −53.4, −54.2, and −47.7%, respectively. These values are significantly lower than the negative control

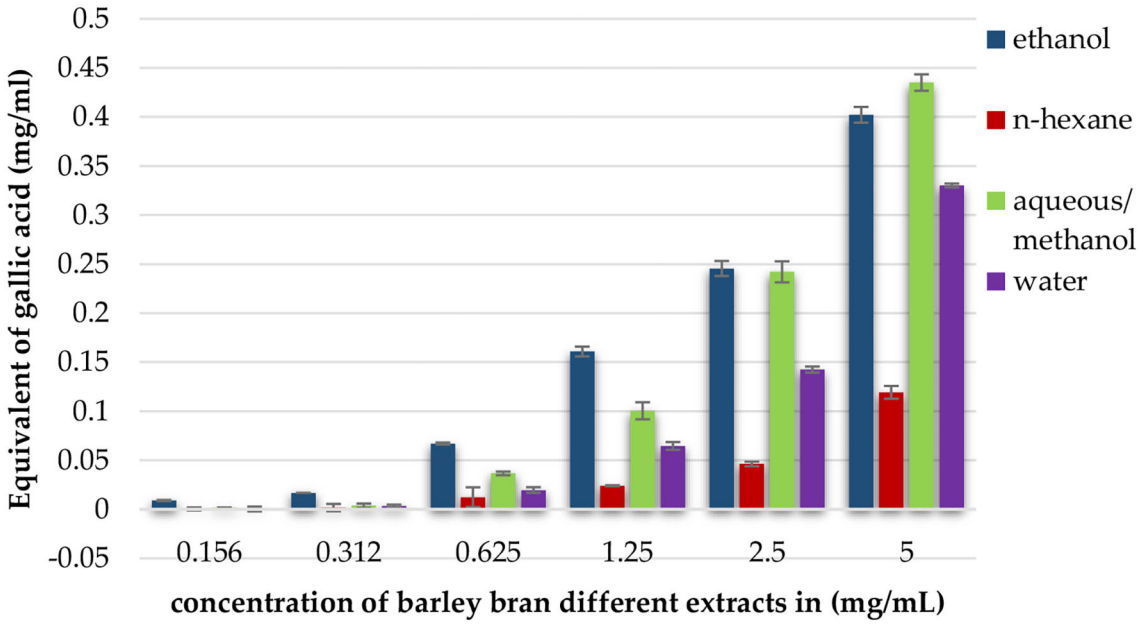


FIGURE 2 | Total phenolic content expressed as equivalent of gallic acid in mg per ml of ethanol, *n*-hexane, aqueous/methanol, and water extracts of barley bran in different concentrations.

group, which showed an increase in tumor size by 107.02% (**Figure 6**). Only one case of death was recorded for the control group. Groups-treated barley bran showed no mortality. The percentages of mice with no detectable tumor in groups Numbers 1 (10% BB), 2 (20% BB), and 3 (30% BB) were 20, 20, and 30%, respectively. However, the percentage of the mice that showed no tumors after tumor inoculation was 30%. The mice exhibited normal activity without side effects (**Figure 7**).

The Effect of Barley Bran on the Serum Levels of IFN-γ, IL-2, IL-4, and IL-10

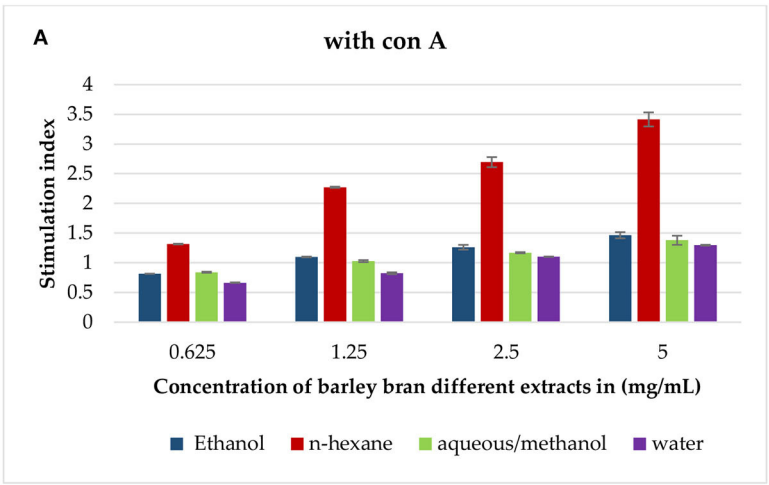
Three mice of each group were utilized to take serum samples to measure the level of cytokines and compare them with the control group. The use of barley bran resulted in change in the cytokine levels. IFN-γ, IL-2, IL-4, and IL-10 values of 30% barley bran group were 265 0, 336 0, 14 0, and 0.8 pg/ml, respectively. While IFN-γ, IL-2, IL-4, and IL-10 values of the control group were 227, 224, 68, and 14 pg/ml, respectively (**Figure 8**), meaning that the 30% barley bran group induced higher levels of IFN- γ and IL-2 and lower levels of IL-4 and IL-10 compared with the control group.

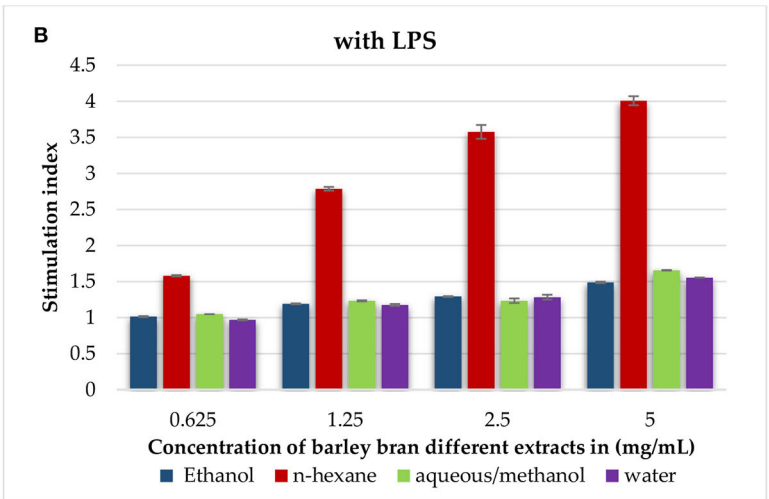
DISCUSSION

Increased emphasis has now been put on complementarity and alternative medicine (CAM) anti-cancer therapies due to the rapid rise in cancer incidence and mortality. Medicinal herbs have been used in conjunction with chemotherapy and radiotherapy as adjunctive therapy to minimize drug resistance and side effects such as osteoradionecrosis (14). Barley bran has secondary metabolites, such as saponins, tannins, phenols, flavonoids, alkaloids, terpenoids, steroids, and carbohydrates that play a crucial role in cancer prevention (14). In this study, crude extracts of barley bran were prepared and assessed for their anticancer and immunomodulatory effect. Ethanol, n-hexane, aqueous/methanol, and water extracts were produced *in vitro* by maceration and examined for their antiproliferative activity against MCF-7, HCT-116, A549, EMT6/p, and the VERO normal monkey kidney cell line. On the other hand, an *in vivo* study was performed to examine the prophylactic and antitumor effects of the barley bran on female Balb/C mice inoculated with the EMT6/p breast cancer cell line. Barley bran extracts were capable to inhibit tumor progression *in vitro* and *in vivo* experiments.

In a dose-dependent way, barley bran extracts showed potent inhibitory effects on cancer cells. Based on the registered results, n-hexane and aqueous/methanol extracts inhibited the cellular growth of all tested cancer cell lines (**Figures 1B,C**). Ethanol extract was active against all cancer cell lines; however, water extract was only active against EMT6/p and HCT-116 cell lines (**Figures 1A,D**).

As quantitative phytochemical screening tests were performed for all extracts, the phytochemical active constituents were revealed. N-hexane extract has terpenoids, steroids, flavonoids, and carbohydrates, while aqueous/methanol extract has saponins, phenols, flavonoids, alkaloids, and carbohydrates. Further analysis was applied using liquid chromatography-mass spectrometry (LC-MS) to determine the most important phytochemicals in n-hexane and aqueous/methanol extracts (**Tables 5**, **6**). These findings are consistent with previously





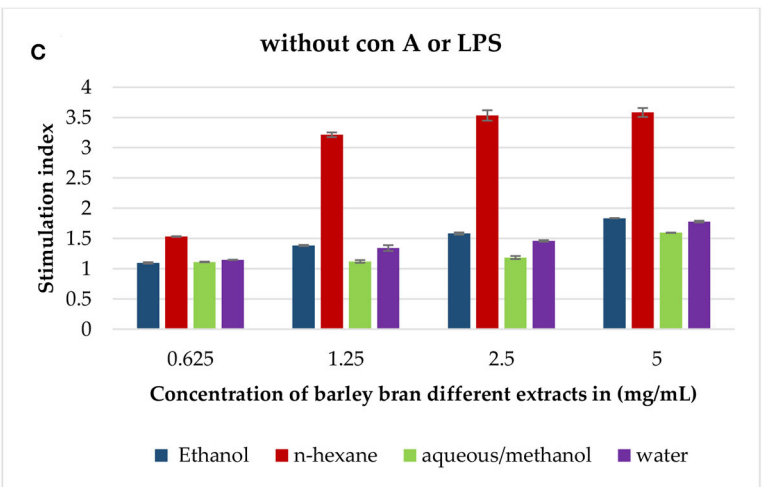


FIGURE 3 | (A) The effect of barley bran different extracts at different concentrations on the proliferation of splenic lymphocytes in the presence of Con A (5 μg/ml). **(B)** The effect of barley bran different extracts at different concentrations on the proliferation of splenic lymphocytes in the presence of LPS (4 μg/ml). **(C)** The effect of barley bran different extracts at different concentrations on the proliferation of splenic lymphocytes.

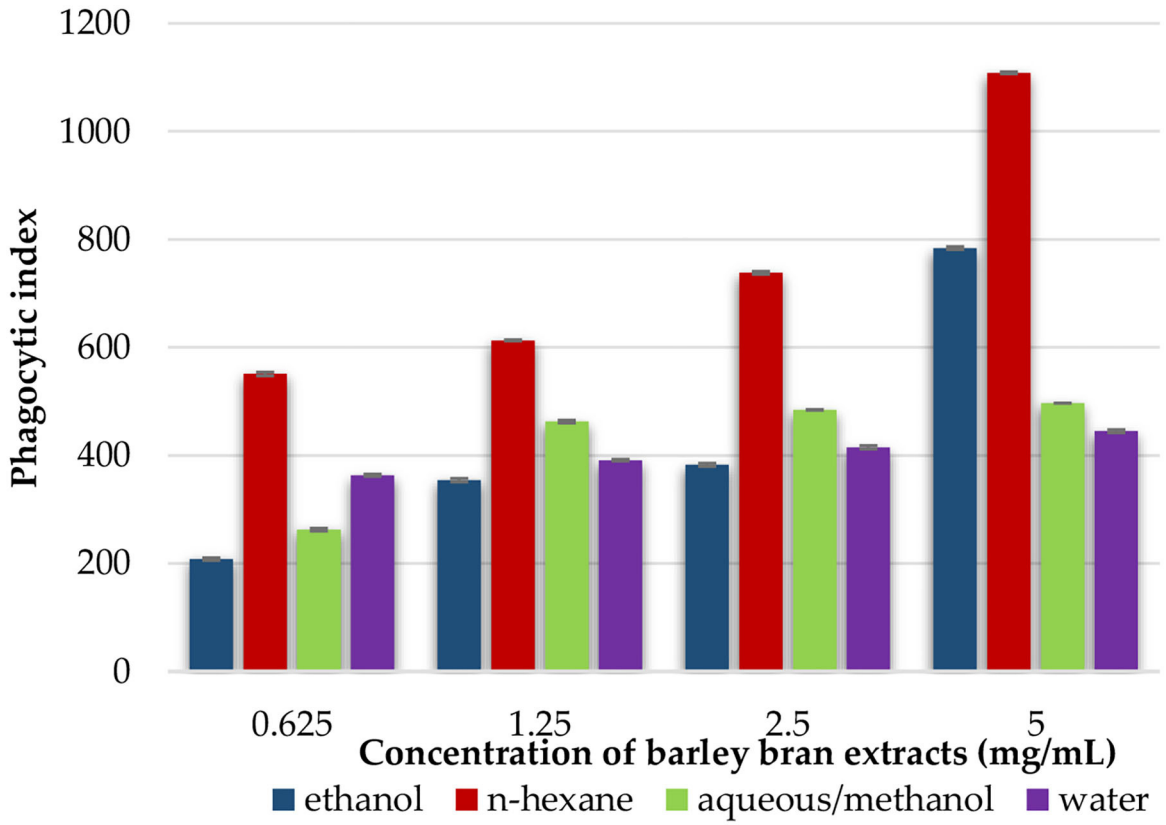


FIGURE 4 | An *in vitro* phagocytic assay using nitro blue tetrazolium (NBT) reduction test of peritoneal macrophage treated with various concentrations of barley bran extracts for 48 h.

conducted study, which revealed phenolic acids, flavonoids, lignans, tocols, and phytosterols as phytochemicals in barley (14). These phytochemicals affect cancer through the regulation of various molecular pathways involved in tumor growth and progression. Proliferation inhibition, cell cycle arrest, apoptosis induction, immune system modulation, and improvement of the antioxidant environment are the particular mechanisms of phytochemicals (26). By modulating the levels of reactive oxygen species (ROS) in cells, herbal extracts rich in phenolic compounds can control cell proliferation, survival, and apoptosis. Also, plant phenolic acids played an important role in inhibiting cancer growth in several *in vitro* observations, preclinical and epidemiological studies (27). Our qualitative phytochemical screening revealed the presence of phenolic compounds in many extracts. Reactive oxygen species (ROS) regulate cell proliferation, survival, and apoptosis by causing genetic mutations, increasing oxidative stress, and activating oncogenes (28). A previous study showed that barley seeds extracts have antioxidant activities (29), which might explain the ability of our extracts to inhibit the viability of cancer cells.

Guneidy et al. (30) showed that there were large preventive effects and small cytotoxic effects of flavonoids on various cancer cell lines, for instance, the MCF-7 cell line. Flavonoids were also effective against A549, and HCT-116. Li et al. (31) revealed that the flavonoids can reduce cell proliferation and metastasis; also, they can strengthen apoptosis. Moreover, trihydroxy-methoxyflavone showed antiproliferative activity against human leukemia cells through inducing apoptosis (cell-programmed death), and activating the MAPK pathway (32). As the four extracts contained flavonoids, the previous study confirmed the ability of all extracts to have antitumor properties.

Thoppil and Bishayee (33) showed the cytotoxic and chemopreventive effects of terpenoids on different cancer cell lines. Also, Bardon et al. (34) showed the inhibition effect of terpenoids on tumor growth. In order to demonstrate the antiproliferative activity of n-hexane against all tested cancer cell lines, Kumar et al. (35) showed that n-hexane was utilized as a main solvent in the terpenes extraction. Moreover, alkaloids are essential phytochemicals in plants. Several alkaloids isolated from natural herbs possessed anti-metastasis and anti-proliferation effects on various cancer forms, both *in vitro* and *in vivo* (36). Also, Talib (37) showed that the cellular growth of EMT6/p was inhibited by alkaloids. Our results showed that flavonoids and carbohydrates were found in both n-hexane and aqueous/methanol extracts, which confirms the potent cytotoxic effects of these two extracts against different cancer cell lines.

Barley is regarded as a good phytosterol source, even though the phytosterol level of barley is moderate compared to other

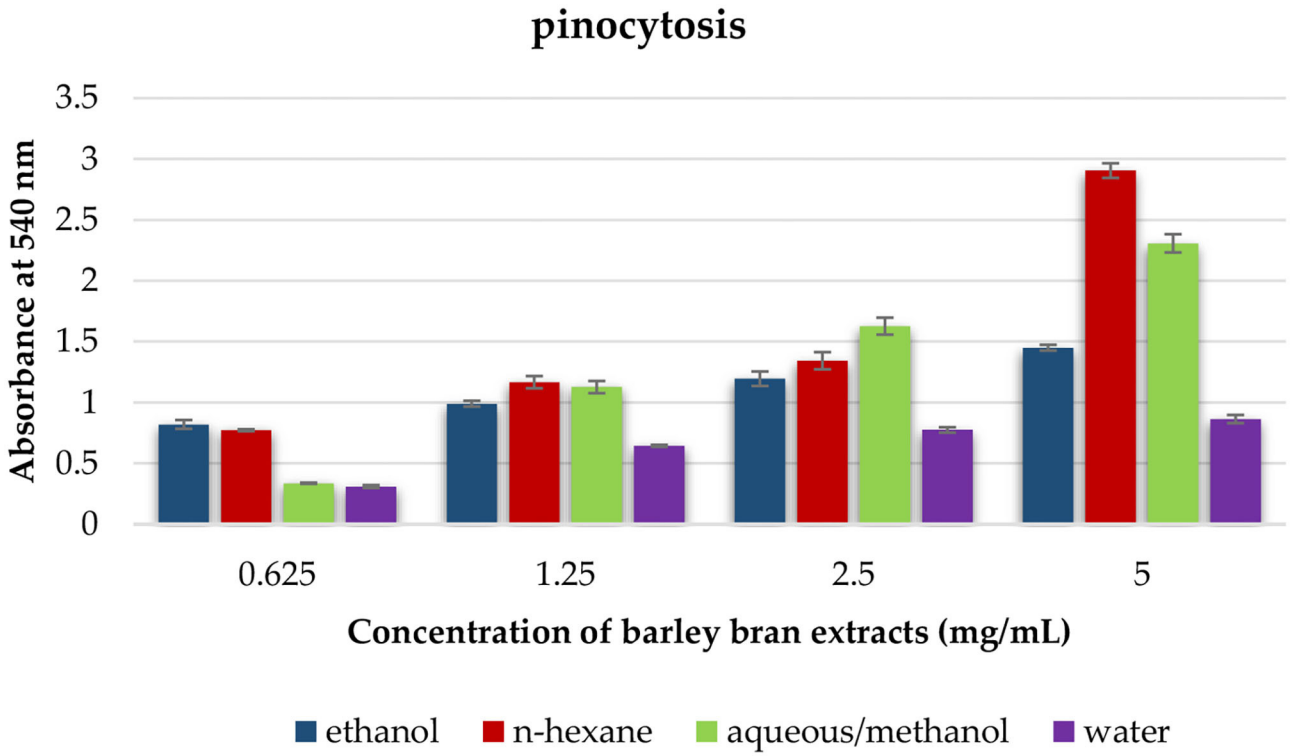


FIGURE 5 | The effect of barley bran different extracts on macrophage pinocytosis.

major grains (38). Awad and Fink (39) showed that natural dietary plant sterol intake can have a beneficial impact and may prevent cancer of the colon, prostate, and breast. Sitosterol is the most abundant type of sterol in barley, as in most grains (38). Numerous mechanisms of action against cancer were proposed for phytosterols, including activation of apoptosis, reduction of carcinogen production, and stimulation of the sphingomyelin cell cycle (40). Beta glucans are complex carbohydrates made up of polysaccharides. Beta-glucans were utilized to slow cancer growth and prevent it from spreading to other parts of the body (41). In our study, n-hexane and aqueous/methanol extracts possessed potent cytotoxic activity, which can be explained by the presence of steroids and carbohydrates, respectively.

In our study, saponins were detected in aqueous/methanol extract. Geronimo et al. (42) showed that the saponins inhibited the cellular growth of different cancer cell lines. Saponins and tannins possessed antitumor properties in MCF-7 and HCT-116 (42).

As we made Folin-Ciocalteu procedure, total phenolic content was highest in aqueous/methanol. The main phenolic groups in barley are ferulic acid, vanillic acid, syringic acid, and p-coumaric acid (14). A previous study showed that barley can act as an excellent natural diet for its anti-proliferative capacity because it has abundant content of phenolic acids (14). Anthranilic acid derivatives inhibited different cancer cell lines, including A549. A study revealed that γ-Linolenic acid exerted potent inhibitory effect on pheochromocytoma cancer cells (43). Palmitic acid was able to reduce cell viability in MCF-7 breast cancer cells by enhancing the expression of apoptosis-related proteins, including caspase-3, 9, Bax, and P53 (44). These results confirm the cytotoxicity effect of aqueous/methanol and n-hexane extracts, respectively.

Vero cells are cell lineages that were first isolated from kidney epithelial cells taken from an African green monkey (Cercopithecus aethiops). Yasumura and Kawakita developed the lineage in 1962. The original cell line was termed "Vero" after an acronym of verda reno, which means "green kidney"(45). The

TABLE 9 | The effect of barley bran on tumor size and weight in mice ($n = 10$) (mm^3: cubic millimeter).

Group	Av. initial tumor size (mm^3) ±SEM	Av. final tumor size (mm^3) ±SEM	% change in tumor size	% of mice with no detectable tumor	Av. tumor weight (g)
1: 10% BB; $n = 10$	383.4056 ± 2.47	178.646 ± 0.102	−53.4055	20%	0.190
2: 20% BB; $n = 10$	333.83345 ± 2	152.6542 ± 1.6	−54.2724	20%	0.172
3: 30% BB; $n = 10$	234.1909 ± 2.6	122.4254 ± 2	−47.7241	30%	0.082
4: Control; $n = 10$	432.62 ± 1.19	895.61 ± 1.08	107.02	10%	0.72

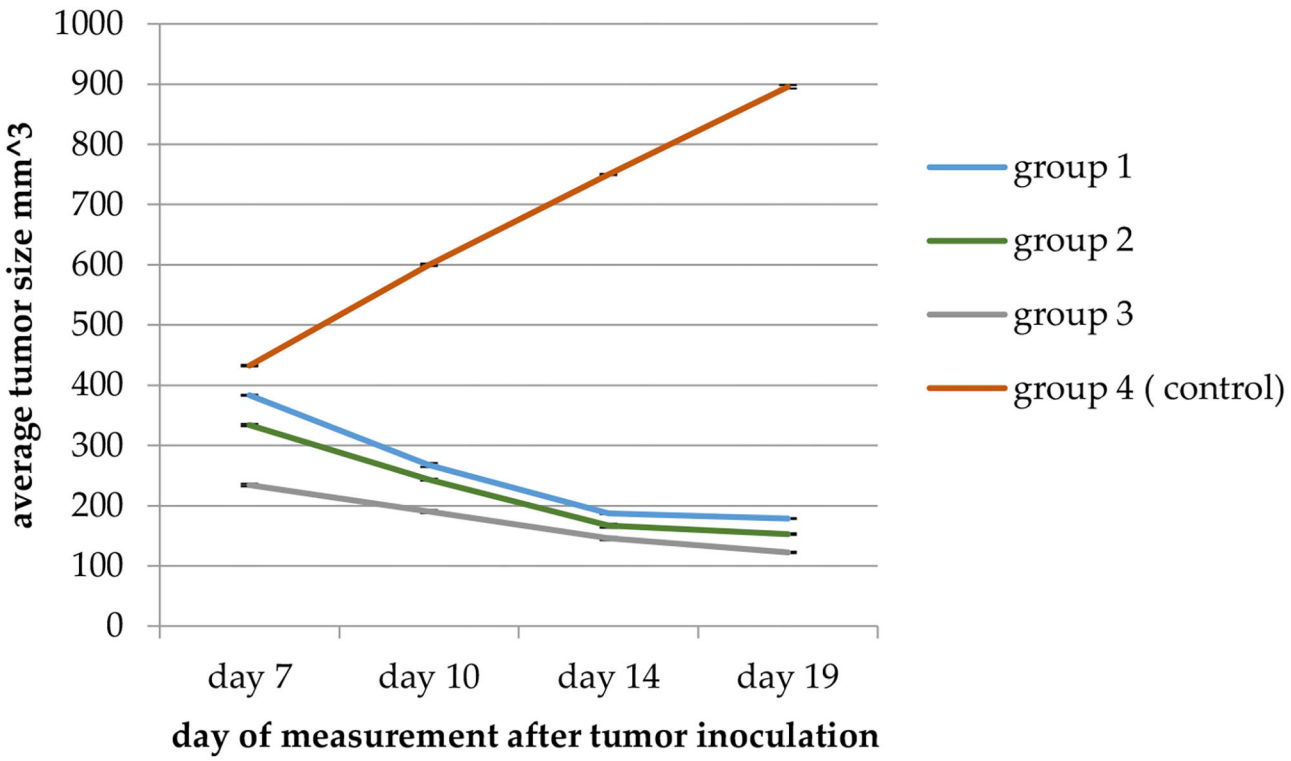


FIGURE 6 | A plot of change in average tumor size (mm^3) vs. time in (days) of measurement in the EMT6/P cell line. Average tumor size in Group 3 (mice given fodders with 30% barley bran) was significantly (p <0.05) reduced compared with the control group.

Vero cell lineage is continuous and aneuploid, which means it contains an aberrant number of chromosomes. A continuous cell lineage can be replicated through many cycles of division without becoming senescent. Vero cells have the interferon-alpha/beta receptor; thus, they respond appropriately when recombinant interferon is given to their culture media (45). Ethanol, n-hexane, aqueous/methanol, and water extracts of barley bran showed limited toxicity against the VERO cell line with IC_{50} value more than 5 mg/ml. This may demonstrate the safety of all extracts against normal cells.

The immune system is a complex network of cells and proteins that defend the body against invading microorganisms and against tumor cells. Immune system modulation can be achieved through a range of specific and non-specific approaches. Immunomodulation refers to any changes in the immune system, and it can be induction, expression, amplification, or inhibition of any part in the immune system (46). Lymphocytes are a critical part of the acquired immune system, and their ability to proliferate is seen as a measure of the extent of cell immunity (46).

According to our findings, the proliferation of both T and B lymphocytes was increased by barley bran extracts with some variation. The rising level of splenic lymphocytes upon using barley bran indicates an immune-stimulatory effect on the acquired immune system.

Either in the presence of Con A or LPS, n-hexane extract was the most effective extract with the highest stimulation index followed by aqueous/methanol (**Figures 3A,B**). Also, n-hexane extract was the most effective extract in the absence of both Con A and LPS. These findings may be clarified by the fact that most of the phytochemicals existed in plants had immune-stimulating activity (47). It was reported that methoxylated flavonoids have the ability to inhibit CYP1B1 activity and mRNA expression in human oral squamous cell carcinoma SCC-9 cells (48). Terpenoids had anticancer and immune-stimulating activities (49). A study showed that the percentage of T and B cells along with the induction of Splenocyte proliferation in both resting and LPS-stimulated cells was risen by plant-derived polyphenols (50). Saponins were utilized to increase lymphocyte proliferation, enhance cytolytic activity of natural killer cells (NK), and produce a high CD4+/CD8+ ratio (51). Furthermore, the immunostimulatory effects of carbohydrates derived from barley leaf were revealed by enhancing splenocytes proliferation and NK cytotoxic activity with a significant rise in Th1 cytokines (52). Stearic acid derivatives can influence the anti-inflammatory activity through the inhibition of NO and TNF-α. Palmitic acid, a saturated fatty acid, significantly upregulates the expression of signaling lymphocytes-activation molecule family Member 3 through the JAK/STAT5 pathway as well as increased the induction of TNF-α, IL-1β, IL-6, IFN-γ, IL17A, and IL-2 (53). γ-Linolenic acid has been proved to boost lymphocytes, which are important components of immunological response. γ-Linolenic acid may also have crucial roles in cancer treatment.

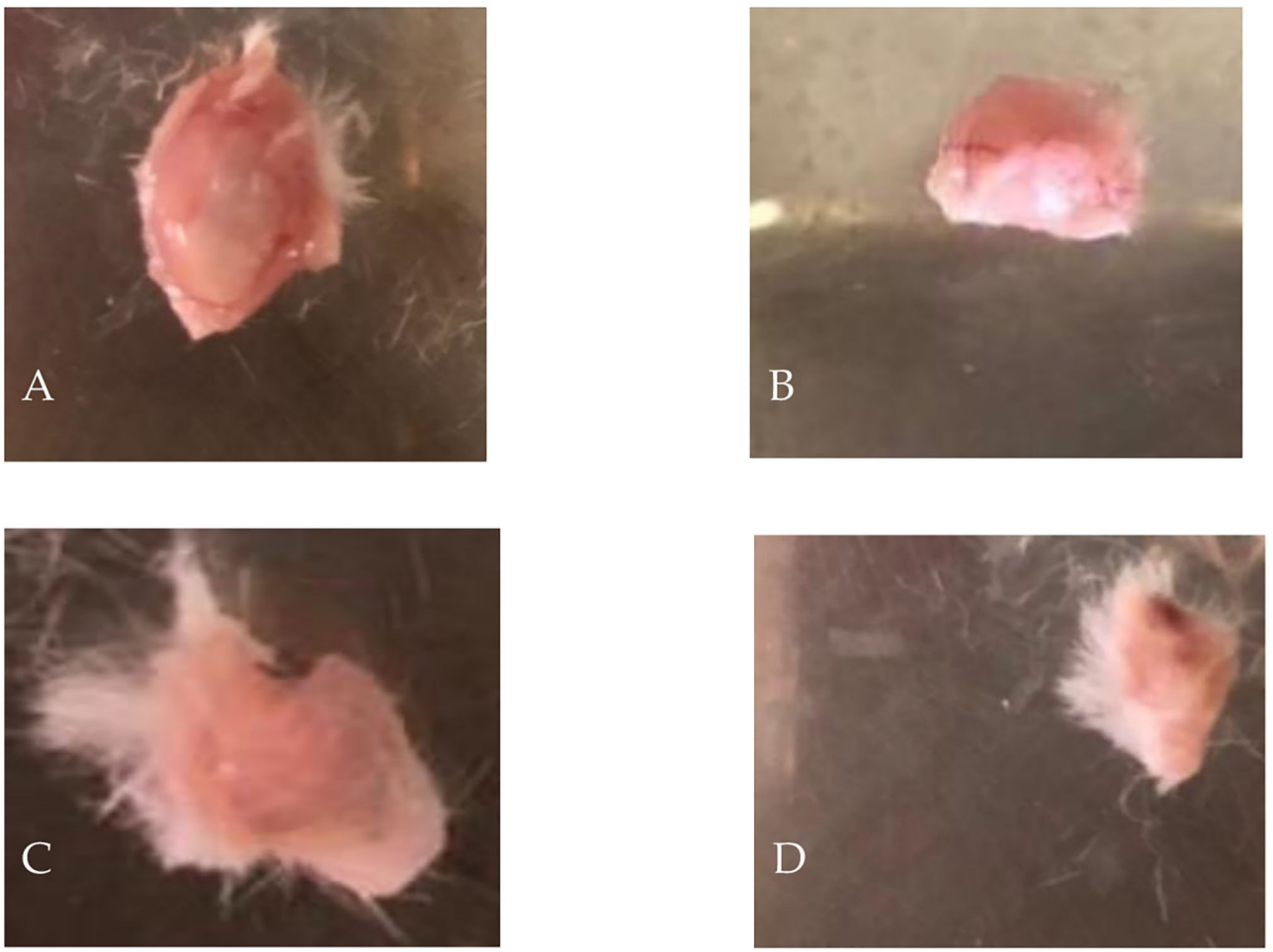


FIGURE 7 | Dissected EMT6/P tumors showing: **(A)** negative control, **(B)** given 10% of barley bran, **(C)** given 20% of barley bran, **(D)** given 30% of barley bran.

It demonstrated some improvement in immunologic status in patients with estrogen-sensitive breast cancer and bladder cancer (54). The relevance of γ-Linolenic acid stems from the fact that it is the precursor of prostaglandins, leukotrienes, and thromboxanes, which operate as mediators of inflammation and immunological processes in diseases like cancer, diabetes, arthritis, cardiovascular disease, and cellular aging (55). Other study showed that tricin, a type of trihydroxy-methoxyflavone, possessed antiangiogenic activity *in vitro*. It was able to reduce VEGF expression, inhibit HIF-1α accumulation in tumor cells, and modulate ROS generation in endothelial cells (52).

The effects of barley bran on innate immune system were evaluated by utilizing phagocytic activity test and pinocytic activity test. Endocytosis includes both phagocytosis (cell eating) and pinocytosis (cell drinking). The frontline defensive mechanism against pathogen invasion and a crucial part of the innate immune system was phagocytosis. Macrophages were recognized for their phagocytic activity and capacity to polarize into phenotypes, which are pro-inflammatory (M1) and anti-inflammatory (M2) (56). During carcinogenesis, M1-like polarization of anti-tumor macrophages was involved in the removal of more immunogenic tumor cells (57). This study showed that barley bran has the ability to increase the phagocytic activity of macrophages in a dose-dependent manner. N-hexane extract was the most active, followed by ethanol extract, while aqueous/methanol and water were less active (**Figure 5**).

The high immune-stimulating effect of n-hexane extract might be due to the high content of flavonoids. A previous study showed that oral flavonoids were utilized to enhance the activity of macrophages through converting β-glucuronidase to aglycones in macrophages (58). Immunostimulation in γ-Linolenic acid was characterized by a rise in phagocyte oxidative burst, CD4 + CD8-lymphocytes in blood, and the CD4: CD8 ratio (59). Stearic acid can stimulate the NF-κB pathway via TLR4 signaling (60). Other studies have shown that palmitic acid was able to enhance activation of TLR4 and NF-?B pathways as well as increase secretion of IL-18, TNF-α, IL-1β, and activation of TLR2 (61). Immunostimulation activity and phagocytic induction were revealed by phytosterols by increasing ROS and enhancing the production of NO (62).

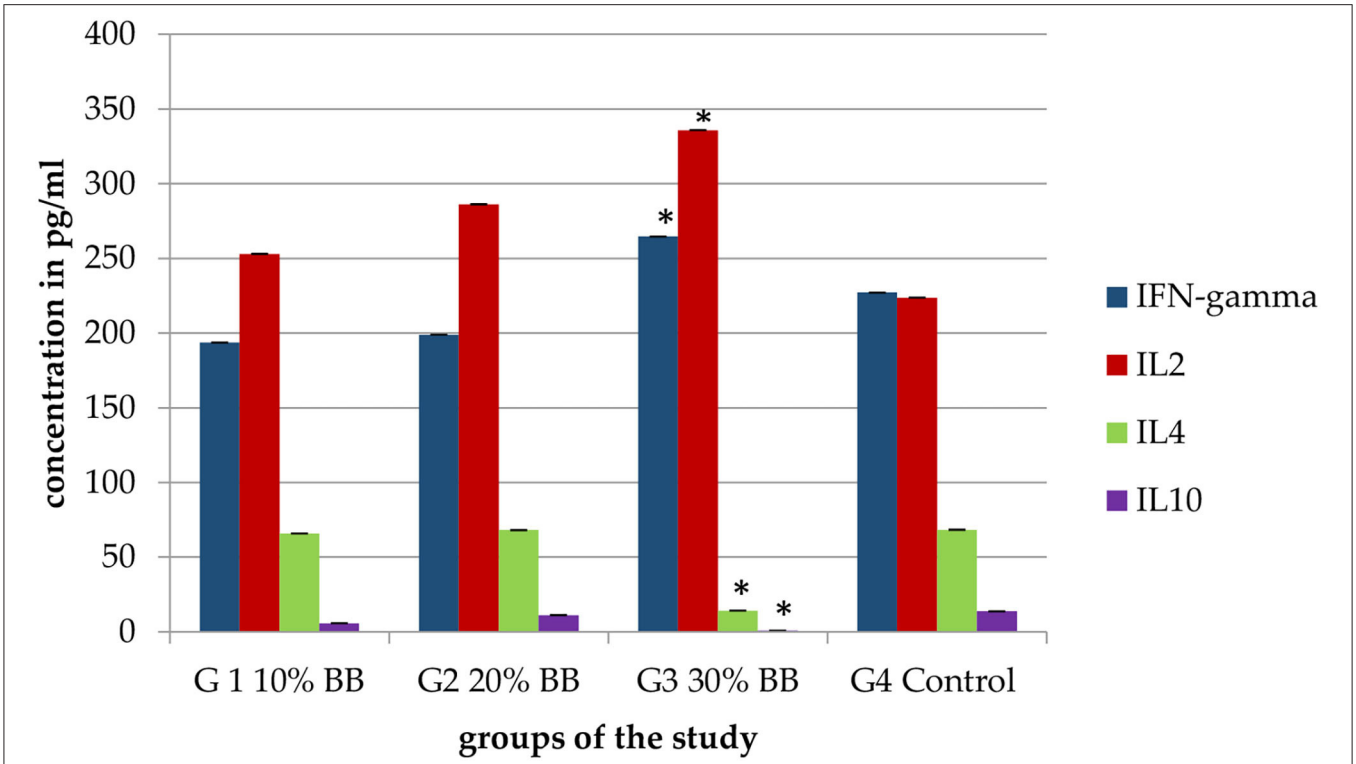


FIGURE 8 | Serum levels of IFN- γ, IL-2, IL-4, and IL-10 for the mice treated with increasing levels of barley bran. The G3 30% barley bran group significantly ($p < 0.05$) induced higher levels of IFN- γ and IL-2 and lower levels of IL-4 and IL-10 compared with the control group. The * symbol indicates the significant values.

For pinocytic activity assay, our findings revealed that the activity of pinocytosis of barley bran was increased in a dose-dependent model. N-hexane and aqueous/methanol showed high activity compared to control (**Figure 5**).

An *in vitro* study revealed that plant-derived polysaccharides could stimulate murine lymphocyte proliferation, increase murine pinocytic activity, and enhance releasing of nitric oxide (NO), IL-1β, and TNF-α in macrophages (63). Palmitic and stearic acids can activate inflammatory pathways in microglia, as evidenced by an increase in pro-inflammatory cytokine production (IL-1β and TNF-α) (60). Other study demonstrated that the phytosterols stimulated phagocytosis and pinocytosis activity (64). These facts may describe the pinocytic activity of n-hexane and aqueous/methanol extracts of barley bran.

For the *in-vivo* part, the size of breast tumor was reduced compared to the control group with the usage of 30% bran fodder. This reflects the prevention ability of barley bran usage against breast cancer. Reddy et al. (65) revealed the prevention effect of bran on colon cancer in rats when bran oil was utilized with 2% in their food, leading to a significant decrease in the incidence of cancer when it was fed before and after inoculation. Other study showed that the rats were fed 20% wheat bran fodder and a significant decrease in the size of the colon cancer foci was revealed (66). Using tramp mice as a prostate cancer model, Carter et al. (67) found that the incidence of cancer was decreased significantly due to its antioxidant effect by feeding them wheat bran diet in different concentrations.

Xiao et al. (68) listed the prevention of breast cancer and how the estrogen binding capacity of wheat bran could contribute to prevent breast cancer.

In determining the influence on cytokine's level, the 30% group showed higher concentration of IFN-gamma and IL-2 compared with the negative control group. IFN-gamma and IL-2 are Th1 cytokines, while IL-4 and IL-10 are Th2 cytokines. A balanced ratio of Th1/Th2 cytokines is observed in healthy humans. Increased concentrations of Th2 cytokines were observed in patients with different tumor types. Inhibition of Th2 cytokines and enhancement of Th1 were significantly observed in the 30% barley bran group. It revealed the influence of arabinoxylans present in the bran. Arabinoxylans had an immune boosting effect when Balb/c mice were fed enzymatically modified bran. Li et al. (69) showed that ergosterol has the ability to potentiate the immune system. Kumar et al. (70) revealed the immunomodulatory activity of terpenoids. The immunomodulatory and antitumor activities of carbohydrates were demonstrated by Gajos et al. (71). A previous study showed that the flavonoids could activate the secretion of IFN-γ and IL-2 (72). Another study revealed that the production of interferon (IFN)-g was induced by beta-glucan.

Barley contains about 17% fiber, which is one of the highest percentages of any whole cereal grain. Fiber in barley bran includes beta glucan, arabinoxylan, and lignin. Barley bran fiber has been proved to improve glycemic response, blood lipid

attenuation, intestinal enzymatic activity, dietary digestibility, and gut flora (73).

Fiber can aid in the prevention of constipation and diarrhea by generating a bulk within the digestive tract and so regulating bowel movements. A study investigated the impact of adding more barley to adult women's diets and discovered that, after 4 weeks, barley intake had a positive influence on both lipid metabolism and gastrointestinal function (74). Fiber is also necessary for a healthy bacterial balance in the digestive tract. It essentially feeds probiotic bacteria in the gut, assisting in the production of short-chain fatty acids, such as butyrate, which have anti-inflammatory properties and may aid in the treatment of symptoms associated with irritable bowel syndrome, Crohn's disease, and ulcerative colitis (74).

Lignan may offer protection against the development of cancer and heart disease by assisting the body in metabolizing bacteria and maintaining a healthy ratio of "good-to-bad" bacteria within the gut, hence reducing overall inflammation (73). Lignans, polyunsaturated fatty acids, oligosaccharides, plant sterols, and saponins are components found in barley bran that can help fight free radical damage and inflammation. These compounds have mechanistic actions that include binding to and eliminating toxic carcinogens from the body (73).

The synergistic action of barley bran components can aid in the anticancer and immunomodulatory properties of barley bran.

CONCLUSION

The present study was performed to evaluate the anticancer and immunomodulatory activities of barley bran. The outcomes revealed that the barley bran can diminish cancer cell viability and boost the immune system. The findings of this study revealed the immunomodulatory activity of barley bran by prompting splenic lymphocytes proliferation, phagocytosis, and pinocytosis. Also, when we tested the bran *in vivo*, the prevention percentage and the small-scale-produced tumors were a good simulation for the prevention effect of barley bran in reality. The presence of various phytochemicals in barley bran can explain its stimulator effect for the innate and acquired immune system.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by Research and Ethical Committee at the Faculty of Pharmacy-Applied Science Private University.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Anticancer Effect of Spices Used in Mediterranean Diet: Preventive and Therapeutic Potentials

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Cancer is one of the leading causes of death worldwide, with almost 10 million cancer-related deaths worldwide in 2020, so any investigation to prevent or cure this disease is very important. Spices have been studied widely in several countries to treat different diseases. However, studies that summarize the potential anticancer effect of spices used in Mediterranean diet are very limited. This review highlighted chemo-therapeutic and chemo-preventive effect of ginger, pepper, rosemary, turmeric, black cumin and clove. Moreover, the mechanisms of action for each one of them were figured out such as anti-angiogenesis, antioxidant, altering signaling pathways, induction of cell apoptosis, and cell cycle arrest, for several types of cancer. The most widely used spice in Mediterranean diet is black pepper (*Piper nigrum* L). Ginger and black cumin have the highest anticancer activity by targeting multiple cancer hallmarks. Apoptosis induction is the most common pathway activated by different spices in Mediterranean diet to inhibit cancer. Studies discussed in this review may help researchers to design and test new anticancer diets enriched with selected spices that have high activities.

Keywords: spices, cell apoptosis, chemo-prevention, anti-angiogenesis, ginger

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INTRODUCTION

Since ancient times spices and herbs have been extensively used as a food flavoring and traditional medicines (1). Based on history and several current studies, the Mediterranean region has been recognized across generations with a rich reserve of natural medicinal plants (2). As well, the consumption of the main components of the Mediterranean diet has shown a diverse array of health benefits due to the presence of abundant natural phytochemicals (3). Besides, it is believed that using herbs and spices in the traditional Mediterranean diet is associated with emphasizing its medicinal properties and protecting against chronic diseases, including cancer (3, 4). According to statistical analysis, the Mediterranean area exhibited a lower incidence rate of different types of cancer compared to other areas of the world (5). Several studies have reported the antioxidant, anti-inflammatory, and immunomodulatory impact of spices, which may be correlated with the prevention and treatment of cancer (1). Moreover, polyphenols are the main bioactive chemical compounds found in spices and culinary herbs (6). The recent research demonstrated the role of dietary polyphenols as powerful antioxidant and anticancer agents along with many medicinal

properties (7–10). It revealed chemo-preventive potency represented by modulation of different processes and biomarkers, such as tumor cell apoptosis, cell cycle progression, inflammation mediators, cell invasion, and metastasis (11). In literature, there are countless spice-derived secondary metabolites that exhibited potential for cancer prevention, however; they are still under research and development (12, 13). This review summarized some studies about well-known spices in the Mediterranean diet demonstrating their anticancer effects and mechanisms of action. Studies discussed in this review may provide a solid base for researcher and nutritionists to develop effective anticancer nutrition.

SPICES IN THE MEDITERRANEAN DIET: FLAVOR CHARACTERISTICS AND TRADITIONAL USE

Spices are used in different Mediterranean food recipes to impart aroma, color, and taste to food preparations and sometimes mask undesirable odors (14). Spices refer to the dried part of a plant that contains volatile oils or aromatic flavors such as buds (cloves), bark (cinnamon), root (ginger), berries (black pepper), seeds (cumin, coriander) (15, 16). Recently, measurements of dietary intake of spices are gaining much significance as various phytochemicals present in spices, have been recognized to have health-promoting benefits (17). Spices are used in traditional Mediterranean cuisines such as soups, cooked lamb roast, fish preparations, marinades, bouquet garni, baked fish, rice, salads, occasionally with egg preparations, dumplings, vinegar, jams, and marmalades (15).

Spices such as ginger (*Zingiber officinale*), that gives a refreshing pleasant aroma, biting taste, and carminative property, which make it an indispensable food ingredient in most Mediterranean food recipes (16), is used in different forms such as fresh ginger, dry ginger, ginger powder, ginger oil, and ginger paste to enhance both sweet and savory traditional Mediterranean recipes (18).

Rosemary (*Rosmarinus officinalis*), an aromatic herb that has been known from ancient times as a memory herb, a native to the Mediterranean from Spain to the Balkans and into North Africa (14, 19). At present, rosemary is widely cultivated in Spain, Morocco, Tunisia, France, Algeria, Portugal, and China (20). The fresh and dried leaves of rosemary are used frequently in traditional Mediterranean cuisine as they have a bitter astringent taste and are aromatic, dried, and powdered leaves (21). Some spices are used in small amounts because of their intense flavor, such as clove (*Syzygium aromaticum* L.), clove used as a whole or ground form or in oil form that is used in a small amount, for example, curry powder uses 2 % (mild) to 3 % (sweet) by weight of ground clove buds (15). Clove oil is one of the most important essential oils used for flavoring all kinds of food products, such as sausage, baked goods (22).

Black Cumin (*Nigella sativa L.)* is an ancient spice with a mild odor and warm, bitter taste (23). Black cumin is used as a spice in Middle Eastern cuisines. In ancient Egypt, it was used as a preservative in mummification (24). The seeds of black cumin have a pungent bitter taste and aroma and are used as a spice in Middle Eastern cuisines. The dry-roasted nigella seeds flavor curries, vegetables, and pulses. Black cumin is used in food as a flavoring additive in bread and pickles (24, 25).

The most popular and the most widely used spice in Mediterranean food is black pepper (*Piper nigrum L*) (15). Black pepper contributes toward flavor, taste the predominating ones being taste and flavor, and hence pepper is a multifunctional spice (26). Pepper plays an important role in the cuisines of China, South East Asia, Greece, Italy, and France such as meat dishes, fish preparations, soups, and pickles (27). Some spices such as turmeric (*Curcuma longa L*), is used as color agents, it is made into a yellow powder with a bitter, slightly acrid, yet sweet taste. Fresh spice is much preferred than dried spice in Spain, France, Italy, Greece, Turkey (14, 28). In Egypt as early as 3000 BC. cinnamon (*Cinnamomum cassia*) was used in the Testament of the Bible and there indications (29). Cinnamon is used as a flavoring and coloring agent of the foods. However, it gives a sweet sensation of the food that is enhanced because of the synergetic effect between the sweet taste of sugar and the sweet aroma of cinnamon (16). Moreover, cinnamon makes a tan or brown color for food and it is used in many Mediterranean food recipes such as milk, apple pie, and cinnamon buns (30). **Table 1** describes the spices classification and general characteristic.

Mediterranean Plants Used as Food Additives

There is a growing interest in the use food additives from natural sources to improve taste and appearance, preserve flavor and reduce microorganisms' growth. Because the Mediterranean area has high plant species biodiversity, many of its wild plants can be a useful source for natural food additives (31, 32). In the following paragraphs selected examples of such plants are discussed. *Carex distachya* Desf. (Cyperaceae) is an herbaceous plant that is globally distributed in different habitats. It is a steno-mediterranean species and is known with the Italian name "*carice mediterranea*. *Carex* genus is known of the presence of high content of stilbene derivatives (32). Additionally, flavonoids, including resveratrol, flavolignans, lignans and terpenes were also isolated from the *C. distachya*, as well as other unusual metabolites such as feruloyl monoglyceride macrolactones and dibenzoxazepinones. The high contact of polyphenols made this plant a potential source of natural antioxidants for their food protective effect (32, 33).

Teucrium chamaedrys L. (Lamiaceae) is a perennial evergreen euri-mediterranean species that is rhizomatous dwarf shrub. *Teucrium* species are rich in essential oils and is the most abundant source of furanic *neo*-clerodane diterpenes. Other phytochemicals present in this plant include phenylethanoid glycosides, iridoid glycosides and phenolic compounds (32). The medicinal use of *Teucrium chamaedrys* is prohibited in some countries due to its liver toxicity, however, alcoholic extracts are still permitted as flavoring agents, because they are fundamental in providing a bitter aromatic taste (32, 34). *Teucrium polium* L. (Lamiaceae) is another plant from *Teucrium* genus that has medicinal properties and is used as a natural food

TABLE 1 | Description of spices used in the Mediterranean diet along with their classification and characteristic.

Spice name	Classification of spices	Edible part(s)	Flavor characteristic	References
Ginger (*Zingiber officinale)*	Hot spices	Rhyzome	Flowery flavor and spicy taste, biting taste, and carminative property.	(16)
Black Peppers (*Piper nigrum L)*	Hot spices	Fruits (Seeds)	A colorant, flavoring, and/or as a source of pungency.	(24)
Rosemary (*Rosmarinus officinalis)*	Herbs	Leaf, terminal shoot	A bitter astringent taste and aromatic.	(20)
Tumeric (*Curcuma longa L)*	Aromatic spices	Rhizome	A colorant, flavoring and medium aromatic.	(28)
Black cumin (*Nigella sativa L)*	Aromatic spices	Fruits (Seeds)	A strong aromatic smell and warm, bitter taste.	(23)
Clove (*Syzygium aromaticum)*	Aromatic spices	Buds	A pungent, strong, and sweet with a bitter, astringent flavor	(22)
Cinnamon (*Cinnamomum cassia*)	Aromatic spices	Stem bark	A sweet and aromatic, and less bitter.	(29)

preservative due to its antioxidant and antimicrobial properties (35, 36). The plant contains phenylethanoid glycosides, *neo*-clerodane diterpenes, iridoid glycosides and flavonoids (32, 37). *Petrorhagia velutina* (Guss.) (Caryophyllaceae) is an annual sud-mediterranean herbaceous plant with a characteristic densely glandular-tomentose stem. Flavonoids C-glycosides were isolated from its leaves, in addition to cinnamoyl glucose esters and phytotoxic chlorophyll derivatives (32). Due to its antioxidant properties, *Petrorhagia velutina* can be used as a natural food preservative, by impeding oxidation, which is a mandatory step in rotting, either by aerobic or anaerobic mechanisms (38). *Arbutus unedo* (Ericaceae) is a steno-mediterranean evergreen small tree that is reported to have various phytochemicals, such as flavonoids, steroids and terpenoids (32). It has antioxidant properties, and thus can also be used as a food preservative (39). *Myrtus communis* (Myrtaceae) is an evergreen small tree that contains important essential oils. Phytochemical investigation of this plant revealed that it contains various monoterpenoids, triterpenes, flavonoids and small amounts of phenolic acids (32). The plant was demonstrated to have antioxidant and antimicrobial properties allowing it to be used as a natural food preservative without altering the nutritional characteristics of the food products (40).

In a study conducted using a number of Mediterranean spices, namely, annatto, cumin, oregano, rosemary, saffron and sweet and hot paprika, to compare the oxidative stability of refined olive oil tested by the Rancimat method with common food additives during storage at different temperatures, reported that the spice extracts have significant stabilizing effects ($P < 0.05$) (41).

ANTICANCER ACTIVITY OF SPICES FROM THE MEDITERRANEAN DIET: CHEMICAL CONSTITUENTS AND MECHANISMS OF ACTION

Ginger

Ginger (*Zingiber officinale* Roscoe) rhizome is widely used as a spice and folk medicine, affiliated to the Zingiberaceae family, belonging to Southern Asia (42, 43). It has various constituents which may vary as a reason of environmental factors, the place of origin and whether the rhizomes are fresh or dry. Its characteristic odor is due to the presence of volatile oil containing various monoterpenoids and sesquiterpenoids (44). The fresh rhizomes pungency is due to its gingerols content where most abundant one is 6-gingerol (1-[40-hydroxy30-methoxyphenyl]-5- hydroxy-3-decanoate). On the other hand, the pungency of dry rhizomes is due to the shogaols content, such as 6-shogaol, which are formed as a result of thermal degradation of gingerols (44). Additionally, ginger also contains terpenoids, alkanes, paradols and diarylheptanoids (45). The phenolic compounds of ginger including gingerols shogaols and paradols were found to exhibit antioxidant, anti-tumor and anti-inflammatory properties (43, 46, 47).

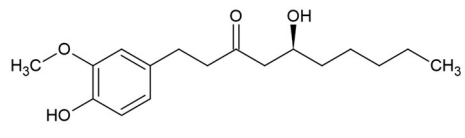


FIGURE 1 | 6-gingerol chemical structure.

6-gingerol (**Figure 1**) was identified as the main active medicinal component of ginger (45). It is usually found as yellow oil and can form a low-melting crystalline solid.

Several mechanisms of action for 6-gingerol have been discussed in many studies, including its chemo-preventive and chemo-therapeutic effects.

The activation of mitogen-activated protein kinase (MAPK) signaling pathway has a role as a possible mechanism behind the chemo-preventive and chemo-therapeutic activity of ginger via the induction of cell arrest against several types of cancer as reported in scientific literature as follows:

One of the studies has investigated the mechanism of the cytotoxic effect of 10-gingerol on human colon cancer cells via the activation of MAPK in a dose-dependent manner, this morphological changes lead to apoptosis that also could be obtained by way of increasing DNA in the sub-G1 phase of the cell cycle (48). The additional study stated the anti-proliferation effect of 6-gingerol on human skin keratinocyte cell lines as a consequence of MAPK and AP-1 signaling pathways

(49) and on mouse skin tumor cells through the activation of NF-kappa B(NF-κB), p38 MAPK, and cyclooxygenase-2 (COX-2) expression as reported in a published study (50). Interestingly; another study highlighted the suppression of oral cancer cell growth and inhibition of migration by suppressing the AKT/mTOR signaling pathway and inducing AMP-activated protein kinase (AMPK) which in turn leads to cell arrest and apoptosis (51). 6-Gingerol plays a role in fighting gastric cancer cells along with chemotherapy, particularly Cisplatin, by altering phosphatidylinositol-3-Kinase and Protein Kinase B (PI3K/AKT) signaling pathway; consequently, this will induce cell cycle arrest at the G1 phase (52). Also, 6-Gingerol leads to cell cycle arrest at the G2 phase as well, against oral and cervical carcinoma (53).

Moving to renal cells, cell-cycle G1-phase arrest could be obtained upon 6-Gingerol treatment (54). The impressive study emphasized how 6-Gingerol can induce cell arrest at the G1cell cycle phase of osteosarcoma cells, by dint of AMPK signaling activation, therefore growth abolition (55).

Furthermore, 6-Gingerol could fight human pancreatic cancer cells via the suppression and the downregulation of the ERK/NF-κ B/Snail signal transduction pathway as stated in the reference (56). Reactive oxygen species (ROS) has a role as a possible mechanism behind the chemopreventive and chemotherapeutic activity of ginger via the induction of cell arrest against several types of cancer as reported in scientific literature as follows:

One study approved the anti-tumor activity of 6-dehydrogingerdione which is one of the active extracts of ginger against breast cancer cells in humans that causes growth suppression due to the generation of ROS (57).

Interestingly, another study figured out the inhibitory effect of 6-Gingerol against lung cancer in mice via the generation of ROS (58).

Angiogenesis could be defined as the creation of totally new blood vessels from previously existing endothelium, which is a necessary process in tumor formation (59). It's worth mentioning here the anti-angiogenesis effect of 6-Gingerol via the induction of micro-vessel normalization due to the stabilization of p-VEGFR2/VE-cadherin/β-catenin/actin complex (46). Moreover, an Invitro study showed the inhibitory effect of 6-Gingerol in the suppression of endothelial cell tube formation, therefore it prevents the tumor blood supply (60).

6-gingerol has a suppression effect on the renal cell carcinoma metastasis *in vitro* and *in vivo*, this effect was due to the upregulation of yes-associated protein (YAP) ser127 phosphorylation and the downregulation of YAP levels in cell nuclei that is responsible for cancer cell migration (61).

Peppers

Capsicum is a genera of pepper, consisting of more than 31 different species including five domesticated species, *C. baccatum*, *C. annuum*, *C. pubescen*, *C. frutescens*, and *C. chinense* (62). Pepper is widely used as a food spice due to its pungency and unique flavor. Pepper contains provitamin A, vitamin E vitamin C, carotenoids and phenolic compounds including capsaicinoids, luteolin, and quercetin (62). Capsicum fruits have been used in the treatment of toothache, infections, coughs, sore throat, rheumatism and for wound healing (62). The main constituent,

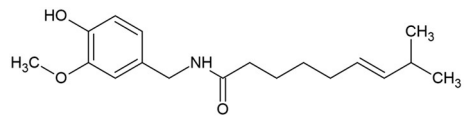


FIGURE 2 | Capsaicin chemical structure.

capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) (**Figure 2**), which is an off-white crystalline lipophilic colorless and odorless alkaloid (63), has antioxidant, anti-inflammatory, cytotoxic and antiproliferative effects.

Capsaicin has shown a chemo-therapeutic effect against several types of cancer through the initiation of cancer cell apoptosis (64). Cellular responses upon treatment with capsaicin affect mechanisms of cell death, especially through the downregulation of β-catenin which plays an important role in β-catenin-dependent signaling that is a significant event in the development of malignancies (65). In addition, upregulation of pro-apoptotic genes in other words pro-apoptotic stimuli in tumorigenic cells (66, 67). Furthermore, one study stated the anti-proliferative effect of capsicum through the suppression of FBI-1-Mediated NF-κB Pathway that led to breast cancer cell apoptosis (68).

As discussed previously the anti-angiogenesis effect plays a significant role in killing tumor cells, as it is a possible mechanism of the anti-cancer effect of capsaicin that is figured out *in vivo* and *in vitro* (69). In *in vitro* model, was through the inhibition of tube formation, while *in vivo* through the suppression of vascular endothelial growth factor (VEGF)-induced vessel formation (70, 71).

Capsaicin took part in fighting metastases of cancer, by altering signaling pathways that are important in cell migration (72), Moreover, the anti-invasive effect of capsicin could be done due to the suppression of phosphoinositide 3-kinase (PI3K) signaling cascade and RASrelated c3 botulinum toxin substrate1 (RAC1), that control cancer cell migration (73).

Rosemary

Rosmarinus officinalis L., often known as rosemary, is the scientific name for a Mediterranean plant that is grown in a variety of nations (74). Recently, rosemary extract (RE) was allowed by European Union legislation, allowing food companies to use the label "antioxidant: rosemary extract" on their products (75). Rosemary has been identified as a potential anticancer medication due to its antioxidant properties. It has the ability to act on free radicals and protect DNA, proteins, and lipids from oxidative damage (76), as later discovered, rosemary derivatives are capable of producing cytotoxicity precisely through the generation of ROS in particular conditions. The main active compounds of Rosemary are summarized in **Figure 3**. Rosemary Extract (RE) has been shown to affect intracellular antioxidant systems by activating the activation of nuclear transcription factor (Nrf) 2 target genes (77) and increasing glutathione levels, with a reduction in its reduced form (GSH) relative to its oxidized form (GSSG) (78).

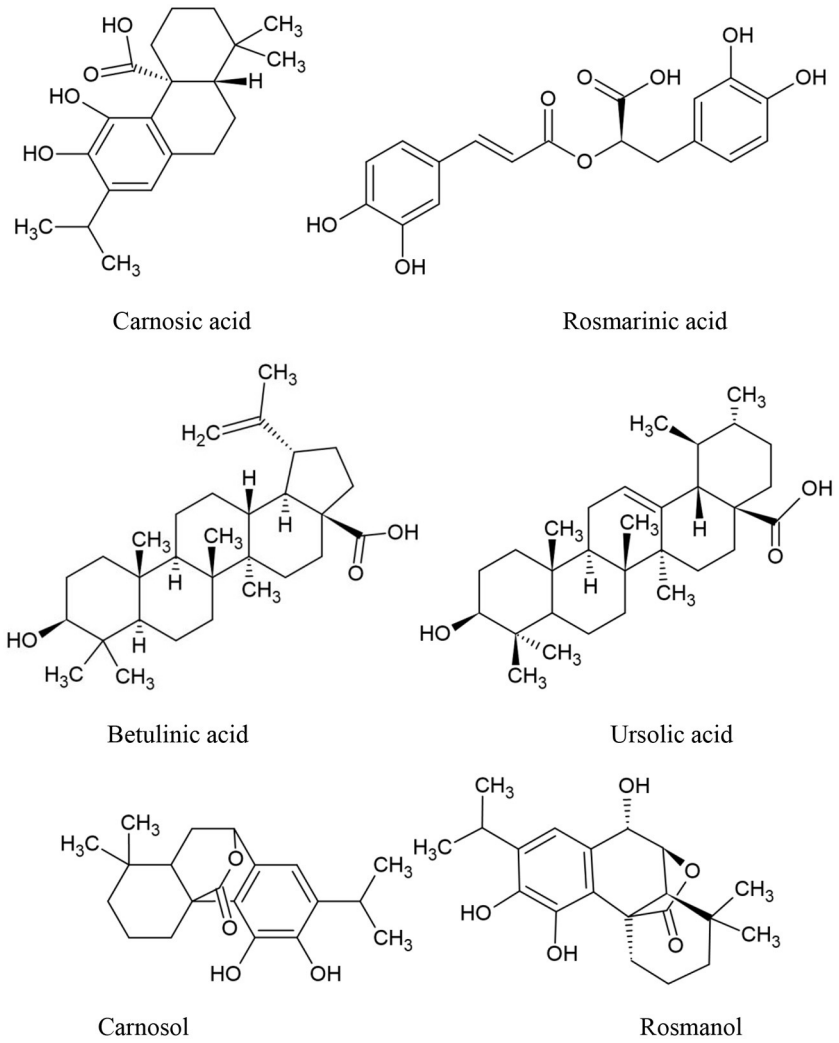


FIGURE 3 | The main components of *Rosmarinus officinalis* chemical structures.

However, some antioxidants, such as beta-carotene, vitamin E, and vitamin C, have shown mixed results in clinical research addressing their involvement in reducing the risk of cancer formation [the antioxidant impact and anticancer action has been questioned (79–85). Furthermore, Carnosic Acid (CA) and Carnosol (CS) inhibit endothelial cell differentiation, proliferation, migration, and differentiation capacity, as well as other angiogenic capabilities. Several data show that their effects on endothelium and cancer cell development may be related to the programmed cell death stimulation (86).

CA also inhibits cytokine-induced adhesion molecule production and monocyte adherence to endothelial cells via an (NF-kB -dependent mechanism (87, 88).

Histone deacetylases (HDACs), which regulate gene expression by acting on the acetyl group of histones, have abnormal expression patterns that coincide with the beginning of malignancies (89). HDAC2 has been shown to be highly expressed in tumor cells, where it inhibits the production of p53, resulting in a decrease in programmed cell death. The effect of rosmarinic acid (RA) vs. suberoylanilide hydroxamic acid (SAHA), an HDAC inhibitor utilized as an antitumoral medication, on the survival and programmed cell death of tumor cells lines, as well as HDAC production, was recently investigated. Similar to SAHA, RA inhibited cell growth and cancer spheroid formation, as well as causing tumor cell death and blocking HDAC2 expression. RA also decreased cyclins D1 and E1 as well as proliferating cell nuclear antigens, while increasing p21. Finally, a rise in p53 generated from the HDAC2 decrease regulated the protein synthesis of intrinsic mitochondrial apoptotic pathway-related genes (90).

The antineoplastic impact of rosemary could be due to a regulatory effect on the immune system. by enhancing the innate immune response; this enhanced response is attributable to cytotoxic natural killer cells and the formation of an anti-inflammatory cytokine profile, which may aid the immunological response to cancer cells (91). CS inhibited tumor growth, also

resulted in a decrease in interleukin-4 (IL-4) and IL-10 (IL-10) and an increase in interferon production (92).

Along with the molecular mechanisms discussed above, additional molecular mechanisms of rosemary have been described and linked to its anticancer effects, including hormone signaling alteration (93), and the ability to interact with a broad range of molecular targets (94, 95). Furthermore, rosemary has recently been shown to boost the expression of genes with known cancer-suppressing capabilities (96). Finally, rosemary phenolic compounds may play a role in a variety of metabolic pathways as well as basic cellular activities and macro-and micronutrient metabolism. These altered pathways may have a clinical impact on the initiation and course of cancer (97, 98). In addition, rosemary extract has been studied in combination with antitumor agents such as 5 Fu, cisplatin, doxorubicin, paclitaxel, tamoxifen, trastuzumab, and Vinblastine. Rosemary extract has a synergistic effect and plays a role in modulating gene expression for enzymes involved in the mechanism of resistance (99–102).

To summarize, while the use of rosemary and its derivatives in the treatment of neoplasms is an interesting topic of research, big and controlled studies are needed to definitively determine the substance's true influence in clinical practice. Taking into account the need to standardize the extraction procedure in order to get REs with consistent antiproliferative properties (103).

Turmeric

Turmeric (*Curcuma longa*) belongs to Zingiberaceae, which is extensively cultivated for its rhizomes. It is used as spice, preservative and coloring agent in addition to possessing many medicinal applications such as anti-inflammatory, antihyperlipidemic, and antimicrobial activities (104, 105). Turmeric is known to contain poluphenolic compounds known as curcuminoids, including curcumin (**Figure 4**), demethoxycurcumin and bisdemethoxycurcumin (104, 105).

Curcumin (**Figure 5**), the main coloring principal of *Curcuma longa*, is an odorless, yellowish crystalline lipophilic compound, offers a surprising number of health benefits, including anti-inflammatory, antioxidant, chemo-preventive, and chemo-therapeutic characteristics (106, 107).

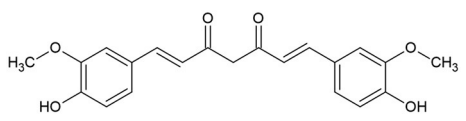


FIGURE 4 | Curcumin chemical structure.

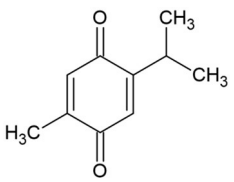


FIGURE 5 | Thymoquinone chemical structure.

The intrinsic and extrinsic routes are the two primary pathways that create apoptotic signals. The intrinsic apoptotic pathway works by stimulating the mitochondrial membrane to inhibit anti-apoptotic protein expression (108), curcumin disrupts the mitochondrial membrane potential balance leading to increased suppression of antiapoptotic proteins (109). The extrinsic apoptotic pathway works by increasing death receptors (DRs) on cells and triggering tumor necrosis factor (TNF) related to apoptosis. Curcumin also plays a role in this pathway by increasing the expression of DRs (106, 107, 110).

In addition, findings from *in vitro* and *in vivo* investigations have indicated that curcumin has a powerful cytotoxic effect on many cancer cells by inhibiting oxidative stress and angiogenesis, as well as inducing apoptosis (111).

The PI3K/AKT signaling pathway regulates VEGF expression. Curcumin therapy decreased protein expression levels of PI3K and AKT. Curcumin therapy also dramatically reduced the levels of mRNA expression of VEGF, PI3K, and AKT (112).

Curcumin's anti-inflammatory properties would almost certainly result in its anti-tumor properties, given the close link between inflammation and cancer. Curcumin has been shown to inhibit the development of numerous types of cancer by lowering the production of inflammatory mediators (113).

Increased production of pro-inflammatory molecules such as cytokines, ROS, COX-2, and transcription factors such as NF-B, AKT, activator protein 1 (AP1), and signal transducer and activator of transcription 3 (STAT3) is induced by inflammation, leading to the initiation and progression of cancer. Curcumin's anticancer property comes from its immunomodulatory ability, which it does via interacting with a variety of immunological mediators as it inhibits the transcription of TNF- and, as a result, the expression of inflammatory genes via suppressing NF-B activity. Curcumin's immunomodulatory properties, on the other hand, are directed not only at molecular targets, but also at cellular components like macrophages, dendritic cells, and T and B lymphocytes (114–118).

Curcumin's anti-cancer properties are also related to its interference with the cell cycle and reduction in cyclin-dependent kinases (CDK) expression. CDKs regulate the progression of the cell cycle (119). Curcumin also inhibits STAT3, which is involved in signaling carcinogenic pathways (120).

In the early phases of cancer growth, free radicals and hazardous compounds produced by oxidative stress play a significant role. As a result, substances with antioxidant properties may be useful in avoiding cancer. Curcumin has the ability to trap free radicals, which means it can help prevent cancer from developing. Curcumin prevents DNA damage induced by oxidative causes like ionizing radiation by reducing free radicals and active oxygen species, according to several cellular and preclinical investigations (121).

Curcumin used with chemotherapy medications like docetaxel, 5-fluorouracil, doxorubicin, and cisplatin improves the synergistic effect by altering numerous signaling pathways, inhibiting tumors including prostate, hepatic, gastric, Hodgkin lymphoma, bladder, and colorectal cancers (122).

Curcumin is thought to have anti-cancer properties by interfering with several cellular processes and activating or inhibiting the production of certain cytokines, enzymes, and growth factors. Curcumin's anti-cancer potential, however, has been limited, owing to its low water solubility. Curcumin compounds with improved efficacy and/or water solubility or stability have resulted through chemical modification of these moieties (107).

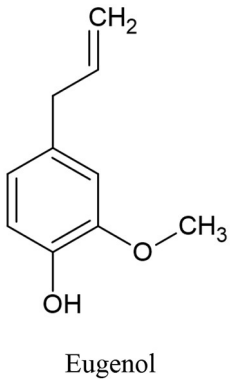
Black Cumin

Nigella sativa (*N. sativa*) is a tiny shrub with annual flowers that belongs to the Ranunculaceae family. It has white, pink, yellow, and purplish exquisite flowers with 5 to 10 petals (123). When the fruit is ripped open, it reveals a great number of black seeds known as black cumin in English, and Habbat el Baraka or Habbah Sawda in Arabic (124). Syria, Lebanon, Pakistan, India, and Afghanistan are among the Middle Eastern and Western Asian countries where the *N. sativa* plant is widely farmed. *N. sativa* are used as a spice in Indian and extensively in Middle Eastern cuisines due to its pungent bitter taste and aroma. The seeds contain many vitamins and minerals in addition to important active compounds including thymoquinone, thymohydroquinone and dithymoquinone (nigellone) (24).

The pharmacological properties of *N. sativa* are mainly due to its quinine constituents, primarily thymoquinone (**Figure 6**) because it is the most abundant monoterpene (24).

Among several therapeutic plants, *N. Sativa* has long been regarded as one of the most valued nutrient-rich herbs in history, and various published scientific studies are currently ongoing to confirm the traditional applications of this species' small seed (72).

Because of its low toxicity and numerous mechanisms of action, *N. Sativa* can be a useful tool for health improvement (125). Recent research suggests that *N. Sativa* oil and extracts contain anti-inflammatory and antimicrobial characteristics, as well as bronchodilator, hypoglycemic, immune booster, anticancer and antioxidant properties (126–130). Once the antitumor characteristics of the *N. Sativa* seed and extracts were established, the researchers investigated the antitumor properties of its major active components, such as thymoquinone and dithymoquinone (131). Black cumin's antitumor mechanism of action is as follows:



Eugenol

FIGURE 6 | Eugenol chemical structure.

Thymoquinone (TQ) antioxidant and cytotoxic effect has been studied *in vitro* and *in vivo* utilizing a variety of animal models and tumor cell lines.

One of the first publications pointing to *N. sativa*'s possible anti-cancer characteristics, An aqueous extract of *N. Sativa* seeds were found to have considerable cytotoxic effects on tumor cell lines (HepG2, MOLT4, and LL/ 2), but not on healthy, non-cancerous umbilical cord endothelial cells (132).

Both aqueous and ethanolic extracts of *N. Sativa* seeds were also found to exhibit significant cytotoxic effects on MCF-7 cells in the presence and lack of H_2O_2, apart from their anti-proliferative properties (133).

A crude methanolic extract of *N. Sativa* also induced around 50% cytotoxicity in Sarcoma180 cells (S-180 cells), Dalton's lymphoma ascites, and Ehrlich ascites carcinoma, in an *in vitro* cytotoxic study (134).

Another *in vivo* study found that 6-month oral administration of *N. Sativa* seeds protected rats from methylnitrosourea-induced oxidative stress and colon carcinogenesis due to lower production of malondialdehyde (MDA), a lipid peroxidation biomarker, and nitric oxide (NO) biomarker (135).

A few researches has investigated the possibility of *N. Sativa* having an anti-mutagenic effect against the directly acting mutagen N-methyl-N0 -nitro-N-nitrosoguanidine (MNNG).

Due to dramatically reduced chromosomal abnormalities in primary rat hepatocytes, an ethanolic extract of *N. Sativa* exerted an inhibitory effect against MNNG mutagenicity. MNNG's anti-mutagenic actions were assigned to the stimulation of detoxifying enzymes that break down MNNG, chemical contact with or uptake of MNNG (or its electrophilic degradation products), increased DNA replication fidelity and enhanced DNA repair (136).

Several studies examined the impact of N. Sativa oil on the fibrinolytic capability of HT1080 human fibrosarcoma cell lines, which is a marker of malignant tumors.

In cell cultures, *N. Sativa* oil produced a dose-dependent downregulation of major fibrinolytic products such as urokinase-type plasminogen activator (u-PA), tissue-type plasminogen activator (tPA), and plasminogen activator inhibitor type 1. The capacity of N. Sativa to prevent local tumor invasion and metastasis is highlighted in this study (137).

In many studies, several research groups have postulated that increasing NK cytotoxic activity against cancer cells is a mechanism underlying *N. sativa*'s anti-cancer properties (138, 139).

The ability of *N. Sativa* to alter the activity of key enzymes has been primarily related to the key mechanisms underlying the reported anti-cancer properties of the plant (140, 141).

The inducible nitric oxide synthase (iNOS) pathway is one mechanism that has been linked to tumorigenesis. NO is an endogenous radical that is synthesized by iNOS or another NOS isoforms throughout physiological events such as inflammation and has been linked to tumor growth. In a recent study,

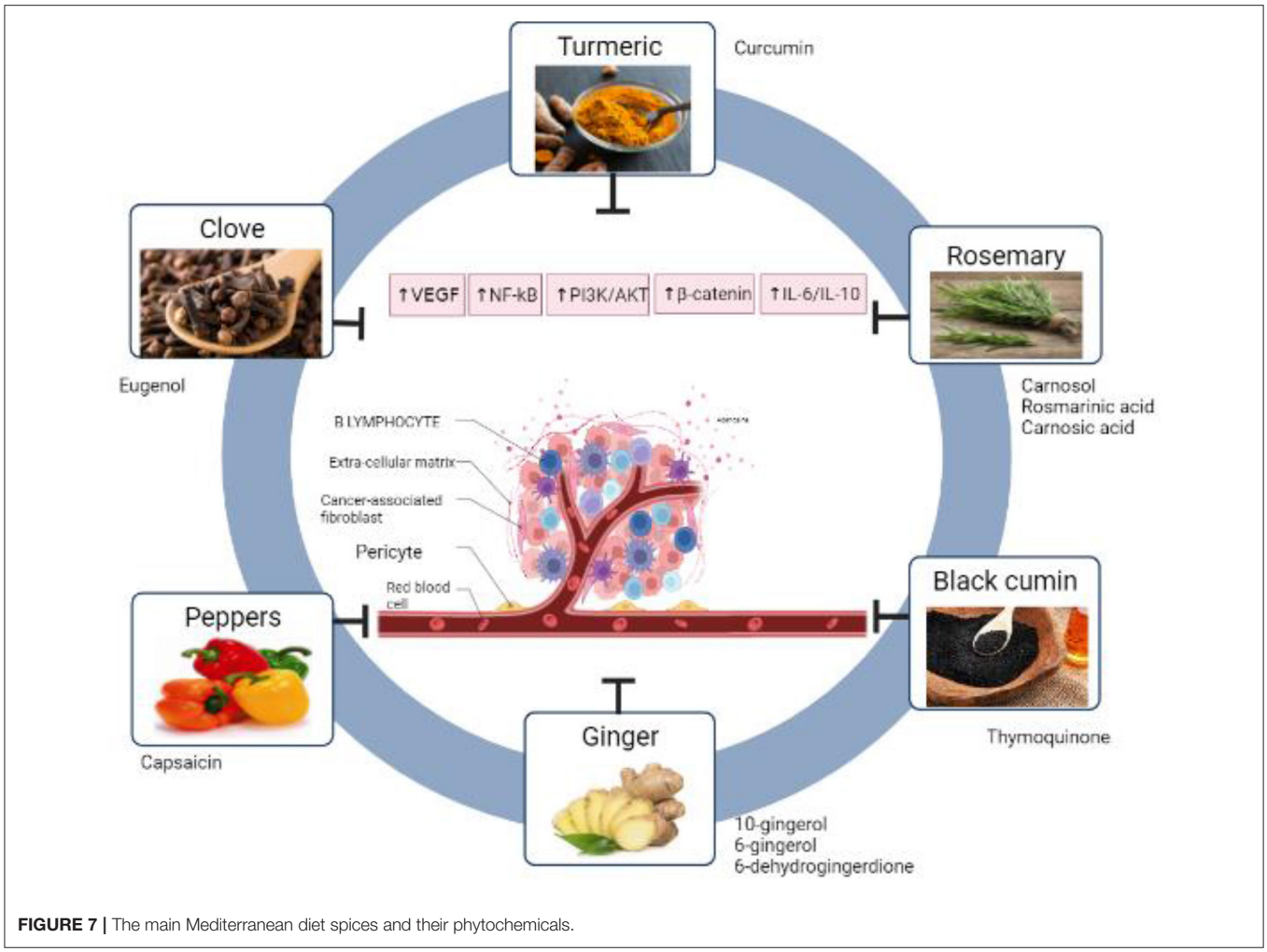


FIGURE 7 | The main Mediterranean diet spices and their phytochemicals.

they investigated how an ethanolic extract of N. Sativa would modify the iNOS pathway in rats with hepatocarcinogenesis induced by diethylnitrosamine (DENA). The serum levels of alpha-fetoprotein (AFP), NO, IL-6, and TNF-α factors whose production was considerably bolstered after treatment with DENA, were dramatically reduced after oral administration of N. Sativa ethanolic extract (142).

A study published recently found that a methanolic extract of *N. Sativa* seeds caused apoptosis in MCF7 cells in a dose- and time-dependent manner. In MCF7 cells, the methanolic extract of N. Sativa resulted in a significant increase in the expression of apoptotic factors such as caspase-3, caspase-8, caspase-9, and the p53 tumor protein, implying that *N. sativa*'s anti-cancer activity is mediated through the p53 and caspase signaling pathways (143).

Thymoquinone, the active phytochemical of *Nigella sativa*, exhibited an anticancer effect toward different cancer cells. It has suppressed the expression of janus Kinase 2 (Jak2) and STAT3, as well as upregulated the ROS level, and promoted apoptosis in human melanoma cells (144). Guler et al. reported the molecular anticancer activity of TQ in glioma cells. It has mediated apoptosis via inhibiting pSTAT3, hindering matrix metalloproteinases (MMP) and GSH levels, increasing iROS generation (145). Another study revealed the cytotoxic effect of TQ in Neuro-2a cells. The caspase-3 induction, KIAP protein reduction, and uprising of BAX/Bcl2 ratio have been observed upon the treatment with TQ (146, 147). Several studies demonstrated the antitumor mechanisms of action of thymoquinone, including its effect on the main cancer biomarkers and cell growth (148, 149). Hence, TQ can suppress NF-Kb, IL-8, PI3K/AKT, and MAPK as well as prevent cell migration by reducing the expression of N-cadherin gene (149–151).

Clove

Cloves, *Syzygium aromaticum* L, dried buds, have long been used as a spice and in traditional Chinese and Indian medicine. Cloves include a diverse variety of bioactive components. Sesquiterpenes, volatile oil (eugenol), caryophyllene, tannins, and gum are among the major chemical constituents of cloves (152, 153).

Clove oil is an effective antibacterial, analgesic, expectorant, antioxidant, and antispasmodic. Eugenol (**Figure 7**) is one of clove oil components that is responsible for its characteristic

TABLE 2 | Anticancer activity of the main Mediterranean diet spices and their mechanisms of action.

Type of spices	Active ingredients	Model of experiment		Anticancer mechanism of action	References
Ginger	10-gingerol	Human colon cancer cells (HCT-116)	*In vitro*	Reduced MAPK Increased DNA accumulation in the sub-G1 phase	(48)
	6-gingerol	Human keratinocyte cell line (HaCaT)	*In vitro*	Suppressed cell growth by reducing MAPK and AP-1 signaling pathways	(49)
		Mouse skin cells (ICR mice)	*In vivo*	Inhibited NF-kB, p38, and COX-2 expression	(50)
		Oral cancer cells	*In vitro*	Induced cell apoptosis and cell cycle G2/M phase arrest Activated AMPK and suppressed AKT/mTOR signaling pathway	(51)
		Gastric cancer cells (HGC-27 and MGC-803)	*In vitro*	Inhibited cell proliferation, migration and invasion via modulating of PI3/AKT signaling pathway	(52)
		Oral and cervical carcinoma cells (SCC4, KB and HeLa)	*In vitro*	Enhanced apoptosis and cell cycle arrest	(53)
		Renal carcinoma cells (ACHN, 786-O, and 769-P)	*In vitro* *In vivo*	Induced cell cycle arrest via modulation of AKT-GSK 3β-cyclin D1 pathway	(54)
		Osteosarcoma cells	*In vitro*	Suppressed AMPK signaling	(55)
		Human pancreatic cells (PANC-1)	*In vitro*	Downregulation of the ERK/NF- κ B/Snail signal transduction pathway	(56)
		Lung cancer cells (A549)	*In vitro* *In vivo*	Inhibited cell growth via decreasing of USP14 expression	(58)
		Rat colonic adenocarcinoma	*In vivo* *In vitro*	Inhibited cell proliferation and angiogenic potential of endothelial cell tubule formation	(60)
		Renal carcinoma cells (786-O and ACHN) BALB/C nude mice	*In vitro* *In vivo*	Suppressed cell migration through downregulation of YAP level	(61)
	6-dehydrogingerdione	Human breast cancer cells (MDA-MB-231 and MCF-7)	*In vitro*	Induced cell apoptosis through oxygen species/c-Jun N-terminal kinase pathway	(57)
Peppers	Capsaicin	Human colorectal cells (HCT-116, SW480, and LoVo)	*In vitro*	Enhanced cell apoptosis by suppression transcriptional activity of β-catenin	(65)
		Human breast cancer cells (MDA-MB-231 and MCF-7)	*In vitro*	Suppressed cell proliferation and induced apoptosis by downregulation of FBI-mediated NF-kB pathway	(68)
		Human multiple myeloma cell lines (U266 and MM.1S) Male athymic nu/nu mice	*In vitro* *In vivo*	Inhibited the interleukin-6-induced STAT3 activation Suppressed tumor growth in mice	(70, 71)
		Non-small cell lung carcinoma cells (A549)	*In vitro*	Reduced cells angiogenesis by downregulation VEGF expression	(70, 71)
		Transgenic adenocarcinoma in mouse prostate model	*In vivo*	Reduced tumor growth and metastasis	(72)
Rosemary	Rosmarinic acid	Prostate cancer cell lines (PC-3 and DU145)	*In vitro*	Induced cell apoptosis through inhibition of HDAC2 expression	(90)
	Carnosol	BALB/C WEHI-164 fibrosarcoma model	*In vivo*	Inhibited tumor growth Decreased IL-4 and IL-10 Increased IFN production	(92)
Turmeric	Curcumin	Human epidermal keratinocytes	*In vitro*	Activated apoptosis by suppressing AP1 transcription dependent and Bcl-xL level	(109)
		Gastric and colon cancer cells (KATO-III and HCT-116)	*In vitro*	Induced apoptosis via upregulation of capase-3, PARP, and caspase-8 Reduced Bcl-xL level	(106, 107, 110)
		Hepatocellular carcinoma (H22HCC) Nude male mice	*In vitro* *In vivo*	Inhibited cell proliferation and induced apoptosis by decreasing VEGF expression and PI3K/AKT signaling	(112)
Black cumin	*N. sativa* extracts	Breast cancer cells (MCF-7)	*In vitro*	Reduced cells proliferation and enhanced apoptosis	(133)
		Sarcomal180 cells, Dalton's lymphoma ascites, Ehrlich ascites carcinoma	*In vitro* *In vivo*	Induced around 50% cytotoxicity Reduced tumor growth	(134)

(Continued)

TABLE 2 | Continued

Type of spices	Active ingredients	Model of experiment		Anticancer mechanism of action	References
		Hepatocellular carcinoma rats model	*In vivo*	Reduction of tumor growth via suppression of iNOS pathway and decreasing TNF-α and IL-6 levels	(142)
		Breast cancer cells (MCF-7)	*In vitro*	Induced apoptosis via increasing caspase-3, caspase-8, caspase-9, and p53 expression	(143)
	N. sativa oil	Human fibrosarcoma cell line (HT1080)	*In vitro*	Inhibited local tumor invasion and metastasis by downregulation u-PA, tPA, and PAI-1	(137)
	Thymoquinone	Human melanoma cells (SK-MEL-28) Xenograft mouse model	*In vitro* *In vivo*	Induced apoptosis by decrease the expression of Bcl-2, Bcl-xL,D cyclines, STAT3, and survivin Suppressed tumor growth in xenograft mouse model	(144)
		C6 glioma cells rats model	*In vivo*	Mediated apoptosis via inhibiting pSTAT3, hindering MMP, GSH levels, and increasing iROS generation	(145)
		Mouse neuroblastoma cells (Neuro-2a)	*In vitro*	Inhibited cell growth through caspase-3 induction, KIAP protein reduction, and uprising of Bax/Bcl2 ratio	(146)
		Breast cancer cells	*In vitro*	Inhibited PI3K/AKT1 pathway	(151)
Clove	Eugenol	Skin tumor in male Swiss albino mice model	*In vivo*	Decreased the activation of NF-kB	(157, 163)
		Human cervical cancer cells (HeLa)	*In vitro*	Induced apoptosis via downregulation of Bcl-2, COX-2, and IL-1β	(157, 163)
		Human melanoma cells Female B6D2F1 mice bearing B16 melanomas	*In vitro* *In vivo*	Suppressed tumor growth through inhibition of E2F1 transcriptional activity Tumor size decreased almost 40% compared to the control group	(164)
		Human promyelocytic leukemia (HL-60)	*In vitro*	Induced cell apoptosis through upregulation of Bax, caspase-3, caspase-9, and cytochrome c Decreased Bcl-2, and ROS expression	(165)
		Human prostate cancer cells (PC-3and DU 145)	*In vitro*	Produced cytotoxicity and caused a rise in the G2/M phase	(166)

odor, is a colorless to pale yellow oily liquid and has been found in a few anticancer formulations (154).

Clove's antitumor mechanism of action as follows:

The capability to inhibit oxidative stress has been defined as a protective effect against cancer formation (carcinogenesis or tumorigenesis); however, whenever cancer has formed, the antioxidant effect can contribute to cancer's development, whereas the pro-oxidant effect can evoke cancer cell death through several signaling pathways (155, 156).

Notably, eugenol has been identified as an agent having a dual effect, antioxidant, and pro-oxidant, with beneficial effects in both cancer prevention and treatment (157–159).

With eugenol antioxidant activity, as assessed by diverse models, It has a strong 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging ability when it reacts with DPPH (160–162).

Eugenol also exhibits ferric ion (Fe3+) reducing ability and electron donor properties, allowing it to neutralize free radicals by producing stable products (162).

Furthermore, in many studies eugenol has been shown to reduce microsomal lipid peroxidation as well as iron and OH radical-induced lipid peroxidation in rat liver mitochondria. The production of thiobarbituric acid-reactive compounds was used to evaluate the antioxidant effect (160, 161).

Some inflammatory markers, such as inducible iNOS and COX-2 expression, as well as the levels of pro-inflammatory cytokines IL-6, TNF-α, and prostaglandin E2 (PGE2), were reduced in dimethylbenz[a]anthracen (DMBA)-exposed animals after treatment with eugenol, showing its anti-carcinogenic effect. Furthermore, in mouse skin with otetradecanoylphorbol-13-acetate-induced inflammation, eugenol was observed to decrease the activation of NF-B (157, 163).

According to certain research, eugenol can induce cytotoxicity at concentrations in the μM range. In the μM range, eugenol suppresses melanoma cell proliferation by causing cell cycle arrest in the S phase, followed by cell apoptosis (164).

In one study, HL-60 (human promyelocytic leukemia), HepG2 (human hepatocellular carcinoma), U-937 (human histiocytic lymphoma), 3LL (Lewis mouse lung carcinoma), and SNU-C5 (human colon carcinoma) lines are also inhibited by eugenol in the μM range. Also, DNA fragmentation, loss of mitochondrial transmembrane potential, Bax translocation, Bcl-2 reduction, cytochrome c release, and caspase-9 and−3 activation are all observed in cells treated with eugenol in the μM range, implying that eugenol causes cell apoptosis (165).

In another study, Eugenol in the μM range produced cytotoxicity and caused a rise in the G2/M phase in LNCaP (androgen-responsive human prostate cancer) and PC-3

(androgen-independent human prostate carcinoma) cell lines (166) (**Figure 1**). Demonstrate the six spices that have mentioned in this review with their main phytochemicals (**Table 2**). Summarize the anticancer activity of the main Mediterranean diet spices and their mechanisms of action.

CONCLUSION

The clue in this review suggested that spices could be part of your daily diet that may lower cancer risk and affect tumor manner of acting. This review only scratches the surface of the overall impact of spices because roughly speaking there are 180 spices widely being used for several purposes. The proof goes on those numerous processes, involving proliferation, apoptosis, angiogenesis, signaling pathways, transduction, cell cycle phases, and immunocompetence could be affected by one or more of the previously mentioned spices, which in turn is reflected on the tumor activity. The Mediterranean diet is rich source of numerous spices. Compared with other diets, it includes multiple spices instead of focusing on single one. The presence of a cocktail of spices in single diet increases the chance of possible synergistic effect that may enhance the anticancer effect of standard therapies. The most common spice in the Mediterranean diet is black pepper (*Piper nigrum* L). Apoptosis induction is the most common anticancer pathway activated by different spices in the Mediterranean diet. Ginger and black cumin have the highest anticancer activities by targeting multiple cancer hallmarks. Further studies are needed to design anticancer diets containing the correct combination of spices.

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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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Gaz Alafi: A Traditional Dessert in the Middle East With Anticancer, Immunomodulatory, and Antimicrobial Activities

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Background: From the earliest times, manna has been widely used as a tasty local sweet or folk medicine. The type of manna being investigated in the present study is called Gaz-alafi, a mixture of insect and *Quercus brantii* leaves secretions from oak forests in the north of Iraq and west of Iran.

Methods: Aqueous and ethanol extracts were prepared as decoction. Various phytochemical tests were conducted to analyze manna composition, including total phenolic contents using the Folin-Ciocalteu method and LC-MS. Gallic acid and catechin were detected in both extracts, in addition to tiliroside presence in ethanol extract, which added more value to the phenolic content of ethanol extract. Cytotoxic activities of Gaz alafi were evaluated against breast cancer cell lines and compared to normal cell lines and doxorubicin using the MTT assay. Antimicrobial properties were assessed against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Candida albicans* using the dilution method of the micro-titer plate. Serum levels of IFN-γ, interleukin-2 (IL-2), interleukin-4 (IL-4), and interleukin-10 (IL-10) were measured using ELISA. The effect of extracts on splenocyte proliferation was evaluated using the lymphocytes proliferation assay. Macrophage function was evaluated using the nitro blue tetrazolium assay, whereas pinocytosis was evaluated using the neutral red uptake assay. Ten days after tumor inoculation, changes in tumor size, survival rates, levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine were measured.

Results: The growth of cancer cells was inhibited by Gaz alafi ethanol extract. An alteration in IFN- γ, IL-2, and IL-4 levels toward antiproliferation immune response were reported for both extracts. The aqueous extract efficiently stimulated lymphocyte proliferation, phagocytosis, and pinocytosis, followed by the ethanol extracts with moderate activity. After treating the mice with ethanol extracts, a significant reduction in tumor size and several undetected tumors were recorded.

Conclusions: Gaz alafi extracts (aqueous and ethanol) are promising sources for anticancer and immunostimulatory agents. Further studies are needed to fully identify the chemical composition of Gaz alafi extracts.

Keywords: antiproliferation, antimicrobial, immunomodulatory, functional food, *in vivo*

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INTRODUCTION

Cancer is considered as one of the most serious public health problems worldwide. Its progression and mortality quickly grow, causing more than eight million deaths annually (1, 2). Female breast cancer has exceeded lung cancer as the most diagnosed cancer, with an estimated 2.3 million new cases (11.7% of total cases). In fact, it has been the leading cause of death for females in present days (3, 4).

Nowadays, biological molecules, drugs, and immune-mediated therapies are being used to treat cancer. Until now, the scientists has not reached the excepted therapy level that reduces the mortality rate and decreases the prolonged survival time for metastatic disease (1). The side effects and toxicity are significant drawbacks in conventional radiotherapy and chemotherapy, encouraging scientists to find alternative cancer therapies to enhance the efficiency of current treatments and reduce toxicity and side effects (5).

Natural products are considered as an attractive source for alternative anticancer therapies, which could play a crucial role in the treatment of commonly occurring cancers worldwide by targeting proliferating tumor cells. The therapeutic effects of these products could be mediated by multiple pathways, such as indirect effects on the immune systems by increasing (immunostimulatory) or decreasing (immunosuppressive) the immune response (6). Another way to target the cancer is by affecting one of its causes, such as the free radicals responsible for various ailments such as cell aging and are considered a triggering factor in cancer (7).

World Health Organization (WHO) estimated that 80% of world inhabitants depend on plant-derived traditional medicines in health care, and, in 2050, the global market of natural products is expected to grow and reach $5 trillion (8). Phytochemicals used as chemo prevention are of great interest and are considered inexpensive, acceptable, readily applicable, and accessible for cancer control and management. Natural products offer protective and therapeutic actions with relatively low toxicity compared to synthetic anti-cancer drugs, which cause nonspecific killing of cells. Currently, many phytochemicals are in the preclinical or clinical trials for cancer chemoprevention. These natural products could be found in vegetables, fruits, plant extracts, and herbs. Although their mechanism of action is unclear, the consumption of fruits and vegetables reduces carcinogenesis in a wide variety of types of cancers (9, 10). In addition to the anticancer effects of the natural products, they are considered as an essential ingredient of functional foods and dietary supplements that can support the body systems against various diseases, such as bacterial infections, cardiovascular, gastrointestinal, and inflammatory conditions (11).

For centuries, in the north of Iraq and Iran, a popular sweetmeat has been made with a substance collected from the plants. The plant produces the exudate in response to an attack of insects. The sweet exudate that results from this infection is commonly referred to as *gaz*. manna (Gaz alafi). It is known from the earliest times and is widely used as a tasty local sweet or, in folk medicine, as laxatives. It is also used in the traditional medicine of Iran as a sedative, antipyretic, and analgesic and as a treatment of chickenpox, rubella, and related itching (12). The same study showed that the Gaz alafi contains the highest concentration of iron and minerals, essential elements for human well-being, compared with other types of manna. They found that it can be used by those who have ulcerative colitis, bleeding disorders, leukemia, immune system disruption, or by those who suffered from blood loss. Taking manna in a diet could benefit persons with low white blood cells or those with lowered immunity conditions. Also, it can provide energy for growth and improve the quality of life (12–14).

There is more than one source of manna. It could be produced naturally from plants without any apparent stimulus or artificially by human-made wounds, used in the commercial production of manna sweets. Another source is the animal origin and is only produced indirectly from plants, although often confused with direct exudations from the plants themselves (15, 16). It is a stiff, resinous, sweet natural product that appears on the leaves of some *Quercus* species, including (*Q. brantii* Lindi.) from the family Fagaceae. In this study, the *Q. brantii* dry leaves with manna exudate was used. The excretion on the leaves was obtained from two insects known as (*Thelaxes suberi* Del.) and (*Tuberculoides annulatus* Hart) from the Aphidoidea family.

To the best of our knowledge, no research has been done demonstrating manna's anticancer and immunomodulatory effects. The present study will investigate these activities using different extracts prepared from Gaz alafi obtained from *Q. brantii*. In addition to the *in vitro* and *in vivo* experiments, chemical analysis of the manna extracts and antimicrobial evaluation were tested.

MATERIALS AND METHODS

Plant Samples and Preparation

One kilogram of Gaz alafi (as a solid mass of *Q. brantii* leaves with manna exudate) was purchased from an Iraqi farmer in the Penjwen district of Sulaymaniyah (north of Iraq) in September 2020. The dry leaves of *Q. brantii* contained manna exudate. Since the origin of this material was Sulaymaniyah, this sample will be referred to throughout the research, with the local name "Gaz alafi" indicated that the studied material is not purely the leaves of *Q. brantii*. The species were identified by Mr. Nizar Obaidat (Ministry of Agriculture, Jordan). Images of the manna and the plant are found in the **Supplementary Figure 1**.

Two different extracts were prepared with distilled water (DW) and 70% ethanol using the ratio of plant to solvents at 1:10 w/v by boiling the samples separately for 3–5 min. Extracts were kept on the bench overnight at room temperature (RT) and then filtered. Ethanol extract was concentrated using a rotary evaporator, and the aqueous extract was dried completely using a lyophilizer, and then both extracts were kept at −20°C until used (17). Upon the extraction of 100 g, 70% ethanol extracts were concentrated to yield 58 g (58%) dry material, while aqueous extract yielded 4 g (4%) only.

Total Phenolic Content

The total phenolic content (TPC) in Gaz alafi extracts was determined according to the Folin-Ciocalteu (F-C) procedure

described in the literature (18). A 200 μL of 1 mg/ml from each extract was diluted with 10-ml distilled water (DW) in 25 volumetric flasks, and then 200 μL of Folin-Ciocalteu reagent was added. After 5 min, 800 μL of 20% sodium carbonate was added, and the volume was adjusted to 5 ml with DW. The resulting greenish-blue solution was incubated at room temperature for 1 h in a dark place. The absorbance was measured at 750 nm. The absorbance values were then read from the standard curve obtained from the same procedure on the gallic acid standard (**Supplementary Figure 6**) (19). Results were represented as mg GAE/g dry weight of extracts.

Liquid Chromatography-Mass Spectrometry

A Bruker Daltonik Impact II ESI-Q-TOF System with a Bruker Daltonik Elute UPLC system (Bremen, Germany) was used to screen the compounds of interest in the extracts. The extract samples were dissolved in 2 ml DMSO and made up to 50 ml using acetonitrile. Then, the samples were centrifuged at 4,000 rpm for 2 min and transferred to autosampler where 3 μL was injected (59 standards were used to identify ms/z and the retention time).

After chromatographic separation, high-resolution Bruker TOF MS was utilized to determine m/z and the exact retention duration of each analyte. The Ion Source Apollo II ion Funnel electrospray source was used to power this instrument. The capillary voltage was 2,500 V, the nebulizer gas pressure was 2 bar, the dry gas (nitrogen) flow rate was 8 L/min, and the dry temperature was 200°C. The mass resolution was 50,000 FSR, and the mass accuracy was one ppm (Full Sensitivity Resolution). The repetition rate of the TOF was up to 20 kHz. Chromatographic separation was achieved using an Elute UHPLC connected to a Bruker Impact II QTOFMS.

TABLE 1 | *In vivo* experiment intraperitoneally (IP) injection of treatment (ethanol extract) and control groups.

Groups	Injection
Treatment	6 mg/kg/day
Control	100 μL Tween 20 and 100 μL (PBS) daily

Antiproliferation Study

Animals

This research was carried out following accepted ethical standards. The Research and Ethical Committee approved all experimental protocols at the Faculty of Pharmacy, Applied Science Private University (Approval No: 2015-PHA-05). A total of 54 healthy female Balb/C mice, weighing between 21 and 25 grams and aged 6 to 8 weeks, were used in this study. The mice were housed in well-ventilated rooms with room temperature (25°C), 50–60% humidity, and alternate dark and light cycles every 12 h. They were kept in cages with bedding made of wood shavings, a special water bottle, and food.

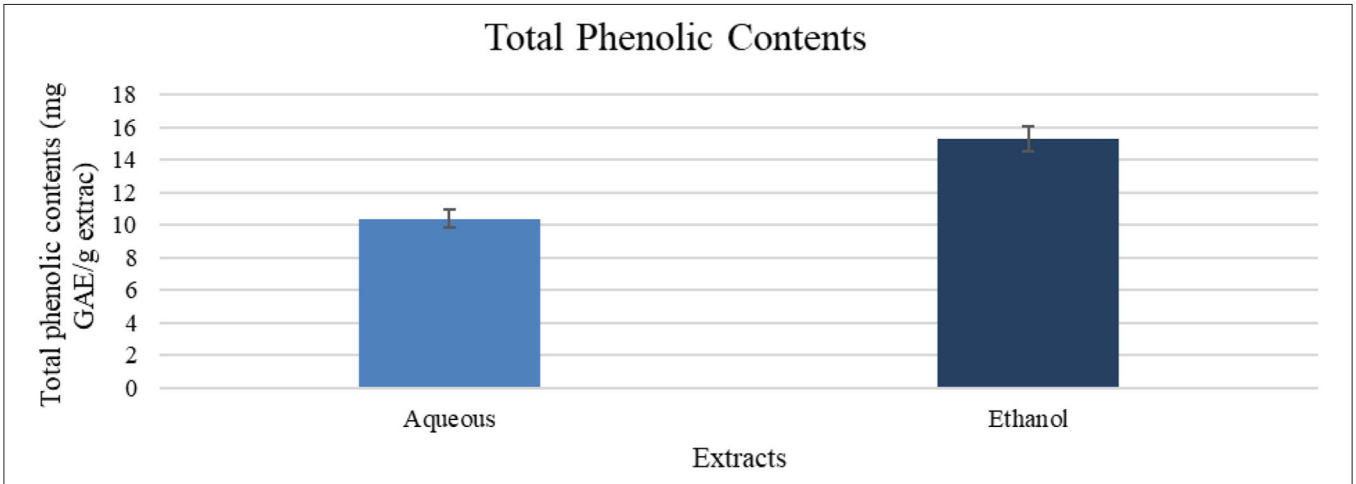

FIGURE 1 | Total phenolic contents (mg GAE/g of extract). Results are expressed as means (bars) ± SEM (lines), $n = 3$.

TABLE 2 | The components of Gaz alafi extracts using LC-MS qualitative system.

Name	Molecular Formula	Molecular Weight	Retention Time [min]	Amount (Aqueous)	Amount (Ethanol)	% Of the Identified Compounds (Aqueous)	% Of the Identified Compounds (Ethanol)
Gallic Acid	$C_7H_6O_5$	170.02	1.11	0.0344 mg/kg	0.024 mg/kg	65.2	58.03
Catechin	$C_{15}H_{14}O_6$	290.08	2.81	0.0258 mg/kg	0.0354 mg/kg	34.79	15.79
Tiliroside	$C_{30}H_{26}O_{13}$	594.14	9	-	0.0035 mg/kg	-	26.19

Cell Lines and Cell Culture Conditions

Four different breast cancer cell lines (T47D, MCF-7, MDA-MB231, and EMT-6) and Human Gingival Fibroblast (HGF) were used in this study. The cells were cultured using suitable tissue culture media supplemented with L-glutamine, serum, and antibiotics. A humidified atmosphere of 5% CO_2 at 37°C was applied to incubate different cell lines (20). T47D and MCF-7 cell lines were cultured in a complete RPMI 1,640 medium. The EMT6 cell line was cultured in complete Minimum Essential Medium (MEM), while Dulbecco's Modified Eagle Medium (DMEM) was used for the MDA-MB231 cell line.

Cytotoxicity and Antiproliferative Activity Assay

Cell viability was measured using MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] assay kit (Bioworld, UK). The cells were dispensed (100 μL/well) into each well of 96-well tissue culture plates at a density of 15,000 cells/well. After a 24-h incubation period, the medium in each well was removed, and the cells were treated in triplicate with varying doses of the extracts (initially dissolved in DMSO with final concentration not exceeding 1%). Doxorubicin (5–0.015 mg/ml) was used as a positive control. After 48-h incubation, media were withdrawn from each well, rinsed with phosphate buffer saline (PBS), and replaced with fresh media, followed by adding 20 μL of thiazolyl blue tetrazolium bromide solution, with a 3-h incubation period. One hundred microliters of DMSO was added to each well to terminate the reaction. The cells treated with 1 DMSO were used as negative controls. Microsoft Excel software was used to apply further calculations that estimate the percentage of survival cells and calculate the IC50 values (20), where OD is optical density:

$$(\textbf{Percentage of cell viability}\ (\%)\ = \frac{(\textbf{OD of treated cell})}{(\textbf{OD of control cell})} \times \textbf{100})$$

Acute Toxicity Test of Gaz Alafi Ethanol Extract

A pilot study was conducted on a small group of mice to select the dose ranges for actual LD_{50} (median lethal dose). Gaz alafi ethanol extracts were dissolved in PBS and 5% tween 20. Four female mice (6 weeks old, 20–23-g weight) were injected intraperitoneally (IP), with a plant extract dose (2 g/kg), which was obtained from the literature (21). The mice were observed for 24 h for any mortality. The next-day dose was adjusted by increasing 1.5 times if tolerated or decreasing 75 times if it showed toxicity. The maximum non-lethal and minimum lethal doses were used as lower and upper limits to calculate LD_{50} doses. After the pilot study, five groups (n = 6) of mice were injected IP with concentrations of (1.7, 3, 4.5, 6.5, and 10 g/kg). The sixth group was used as a negative control and was injected with PBS. The mice were monitored for 24 h for mortality and general behavior. The concentration that causes 50% mortality was recorded as LD_{50}. The actual LD_{50} was determined using the arithmetical method of Karber (22).

Antitumor Effect of Gaz Alafi in Mice Model Experiment

Subcutaneous injection was used to inoculate 100 μL (150,000 cells) of EMT-6/P cells in the abdominal area of female Balb/C mice. Ten days after tumor inoculation, the mice were subjected to daily intraperitoneal (IP) injections with 6 mg/kg/day ethanol extract for 10 days (**Table 1**). After 10 days of treatment, tumor volumes were measured using a digital caliper to define the length and width. Tumor volumes were calculated using the following equation (23):

$$V = \frac{L \times W^2}{2}$$

where: V, L, and W are the volume, length, and width of the tumor, respectively.

Evaluation of Liver and Kidney Function in Treated Mice

Aspartate transaminase (AST), alanine transaminase (ALT), and creatinine in serum samples were measured using commercially available kits, following kit instructions (BioSystems, Barcelona, Spain). Serum levels of liver enzymes AST and ALT were investigated for Gaz alafi, the treated mice, the untreated mice, and the normal mice without tumor. Serum samples were collected, and the reagent was mixed according to the protocols to prepare working reagents. The working reagents were incubated at 37°C, which is the optimal reaction temperature. In cuvette, 50 μL of each sample was mixed with 1 ml of working reagent, incubated for 1 min, and the initial absorbance was recorded. Absorbance readings were recorded also after 0, 1, 2, and 3 min. The spectrophotometer was set to read absorbance at 340 nm. Creatinine serum levels were investigated for the same groups. The reagents were mixed and incubated at 37°C. In a cuvette, 100 μL of each sample was mixed with 1 ml of working reagent, Absorbance readings were recorded after 30 s and 90 s. The spectrophotometer was set to read absorbance at 500 nm. Working reagents in all the tests were used as blanks.

Immunomodulatory Assay

Preparation of Murine Splenocytes

Balb/C mice were sacrificed, and the spleens were removed aseptically. The spleen cells were passed through the mesh of a tissue grinder. The cell suspension was washed for 10 min using RPMI-1640 media and then resuspended in 1 mol/L NH_4Cl to eliminate red blood cells (RBC). The cells were centrifuged after 10 min and resuspended in RPMI-1640 media. After that, splenocytes were cleansed, counted, and used in various tests (24).

Determination of Cytokines Levels in Activated Lymphocytes

The serum levels of IFN-γ, IL-2, IL-4, and IL-10 were measured using a Mouse T helper Th1/Th2 ELISA kit (Invitrogen by theme fisher scientific, Australia). Serum samples from treatment and control groups were prepared from mice blood samples. Measuring cytokine levels was based on the kit instructions. Standard curves for the tests are found in the **Supplementary Figures S2–S5**.

Lymphocyte's Proliferation Assay

Splenocytes suspension was made (2×10^6 cell/ml) in RPMI-1640 media (supplemented with 50-U/ml penicillin, 50-U/ml

TABLE 3 | The effects of Gaz alafi extracts on the proliferation of breast cancer cell lines and Fibroblast normal cell line represented as IC_{50} (mg/ml) as mean ± SEM, $n = 3$.

Extracts	T47D	MCF-7	EMT-6	MDA-MB231	Fibroblast
Aqueous	0.80 ± 0.08	6.07 ± 0.40	2.5 ± 0.01	4.90 ± 0.05	>20
Ethanol	<0.15	1.36 ± 0.02	1.16 ± 0.18	3.02 ± 0.09	>20
Doxorubcin	0.00032 ± 0.04	0.005 ± 0.40	0.00057 ± 0.22	0.00082 ± 0.03	>0.2

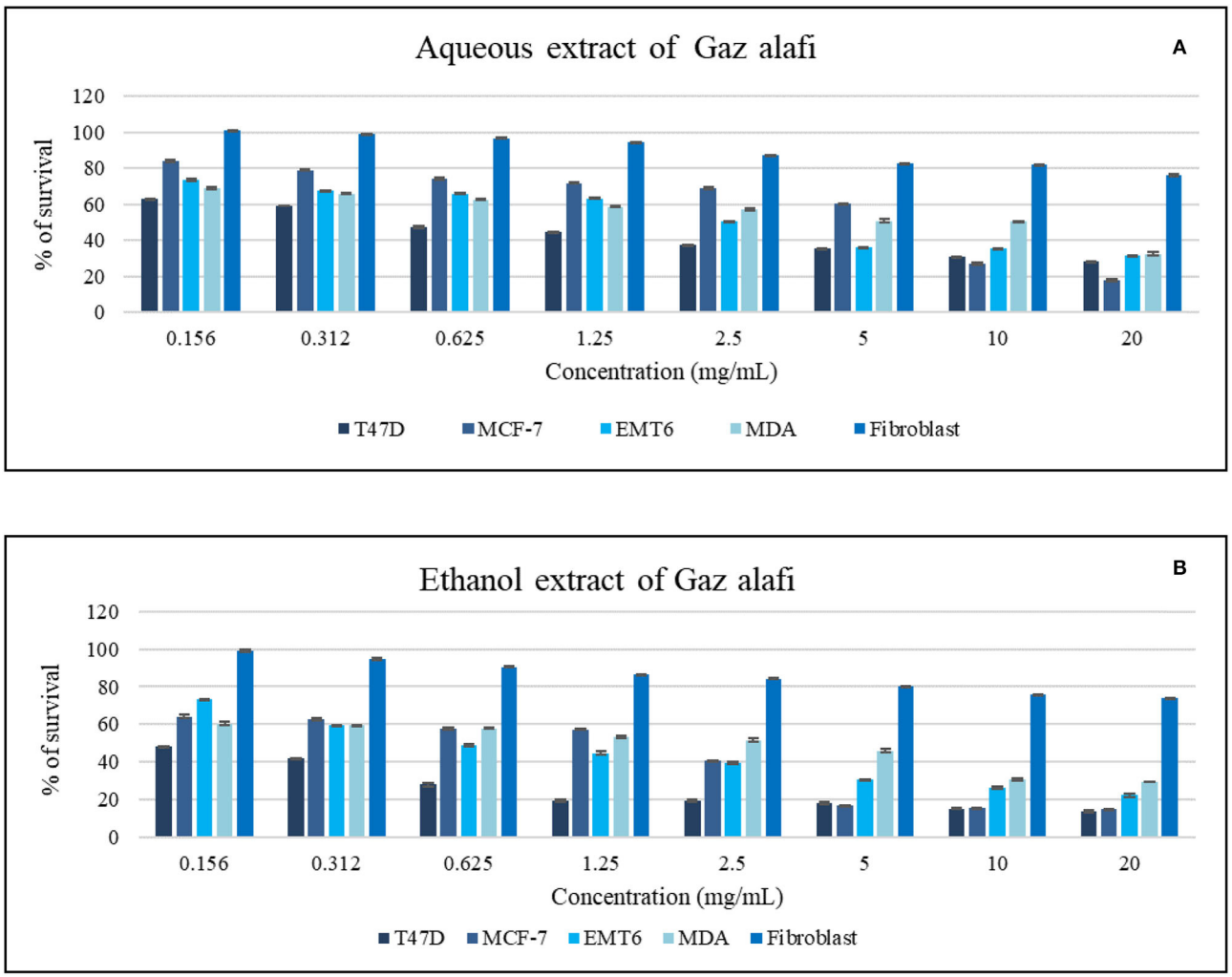

FIGURE 2 | Antiproliferative activity of aqueous **(A)** and ethanol **(B)** extracts of Gaz alafi on breast cancer and fibroblast cell lines using a concentration range of (0.156-20 mg/ml). Results were represented as mean (bars) ± SEM (lines), ($n = 3$).

streptomycins, and 10% FBS), and then it was seeded into a 96-well culture plate, containing either 5 µg/ml Con A (Concanavalin A) or 4-µg/ml LPS (lipopolysaccharide). Then, 100 µL of (5–20 mg/ml) of the extracts was added (in triplicate). The plate was incubated for 48 h under 5% CO_2 and a humidified atmosphere of 95% air at 37°C temperature. Later, 10 µL of (5 mg/ml) MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide] solution from an assay kit (Bioworld, UK) was added to each well and incubated for 4 h, followed by the addition of 100-µL DMSO. The absorbance was measured at 550 nm using an ELISA microplate reader. Results were expressed as a percentage of proliferation (%) compared to the control cells. The same procedure was repeated without Con A and LPS (25, 26).

Macrophage Isolation

Peritoneal macrophages (PEM) were isolated from peritoneal mice cavities. The cervical dislocation method was applied to euthanize the mice, followed by abdominal cavities exposure, dispensing 5-ml ice-cold PBS, and gentle massaging, and then

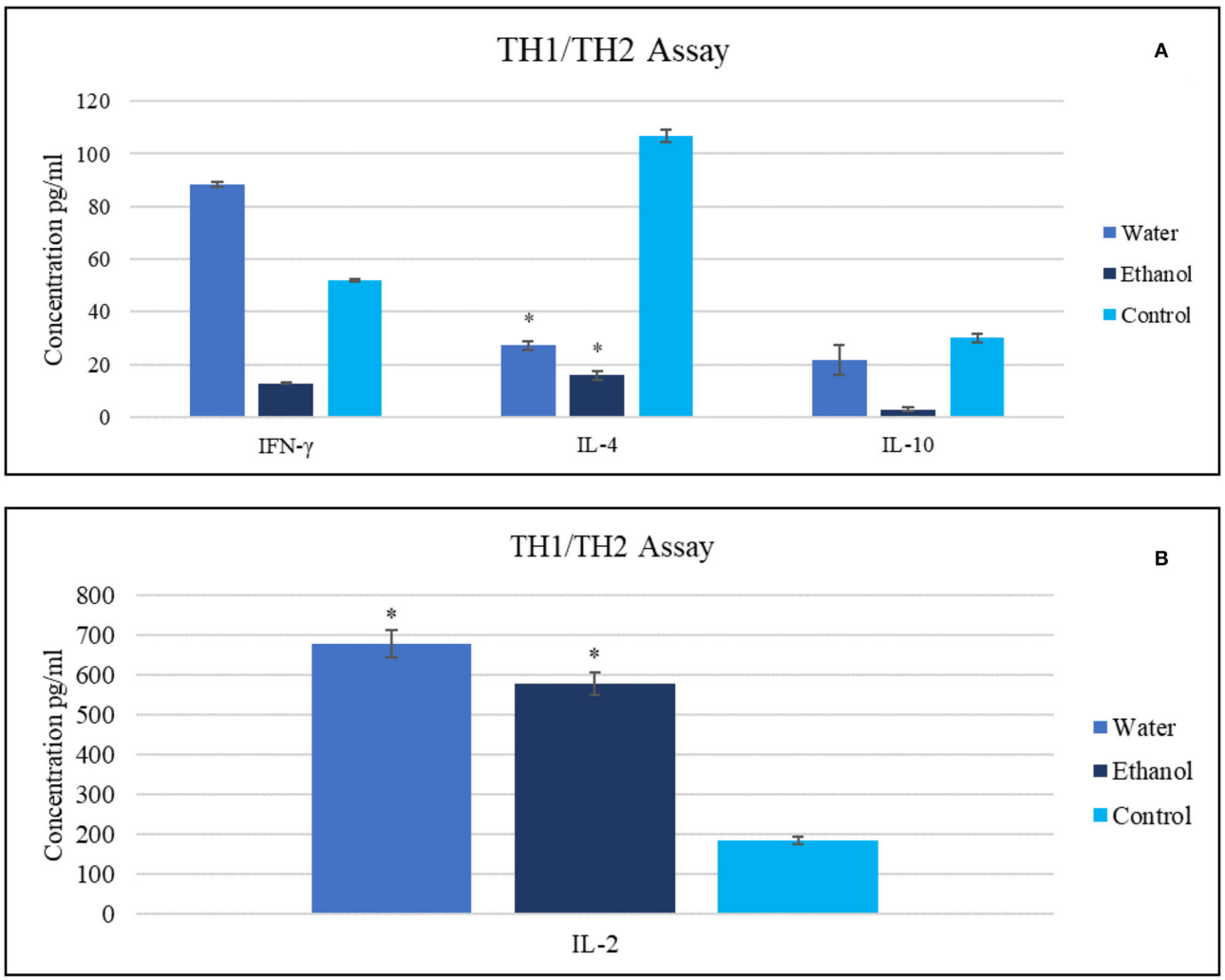

FIGURE 3 | The impact of Gaz alafi extracts (20 mg/ml) on **(A)** IFN- γ, IL-10, IL-4, and **(B)** IL-2 levels. Each treatment was performed in duplicate. The highest levels of IFN-γ and IL-2 were detected after aqueous therapy. IFN, interferon; IL, interleukin. $^{*}P < 0.05$ (statistically significant).

fluid was withdrawn. This method was performed five times. After centrifuging, pellets were suspended in an RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 U/ml) (all chemicals were supplied from Sigma, Chennai) and let to adhere for 3 h at 37°C in an 5% CO_2-humidified incubator. These cells were used in the tests outlined below (27).

Phagocytic Activity Assay

The nitro blue tetrazolium (NBT) reduction assay was carried out according to the method of Rainard (28). PEM (5 x 10^6 cells/well) was cultured with (5–20 mg/ml) extracts concentrations for 48 h at 37 °C. Next, 20 μL yeast suspension (5 × 10^6 cells/ml in PBS) and 20 μL NBT (1.5 mg/ml in PBS) were added to the wells. The control wells contained 20 μL PBS and 20 μL DMSO, followed by incubation for 60 min at 37 °C, air-drying, and addition of 120 μL (2-M KOH) and 140 μL (DMSO). The absorbance was measured for the turquoise blue solution at 570 nm using the plate reader. The measurement of NBT reduction was estimated as below (25, 29), where OD is (optical density):

$$\textbf{Phagocytic index} = \frac{(\textbf{OD sample} - \textbf{OD control})}{(\textbf{OD control})} \times \textbf{100}$$

Pinocytic Activity Assay

The neutral red uptake method was used to determine the effect on macrophage function. Peritoneal mice macrophages were collected and cultured for 48 h with extracts concentrations of (5–20 mg/ml), employing a 96-well plate, followed by the addition of 100 μL of neutral red solution (7.5 mg/L in PBS), incubation for 2 h, and washing with PBS. Then, 100 μL of cell lysis solution (ethanol plus 0.01% acetic acid, ratio of 1:1) were added to each well. Afterward, the cells were incubated overnight. The optical density was determined at 540 nm. Absolute OD values representing dye uptake have been used to measure pinocytic activity (24, 25).

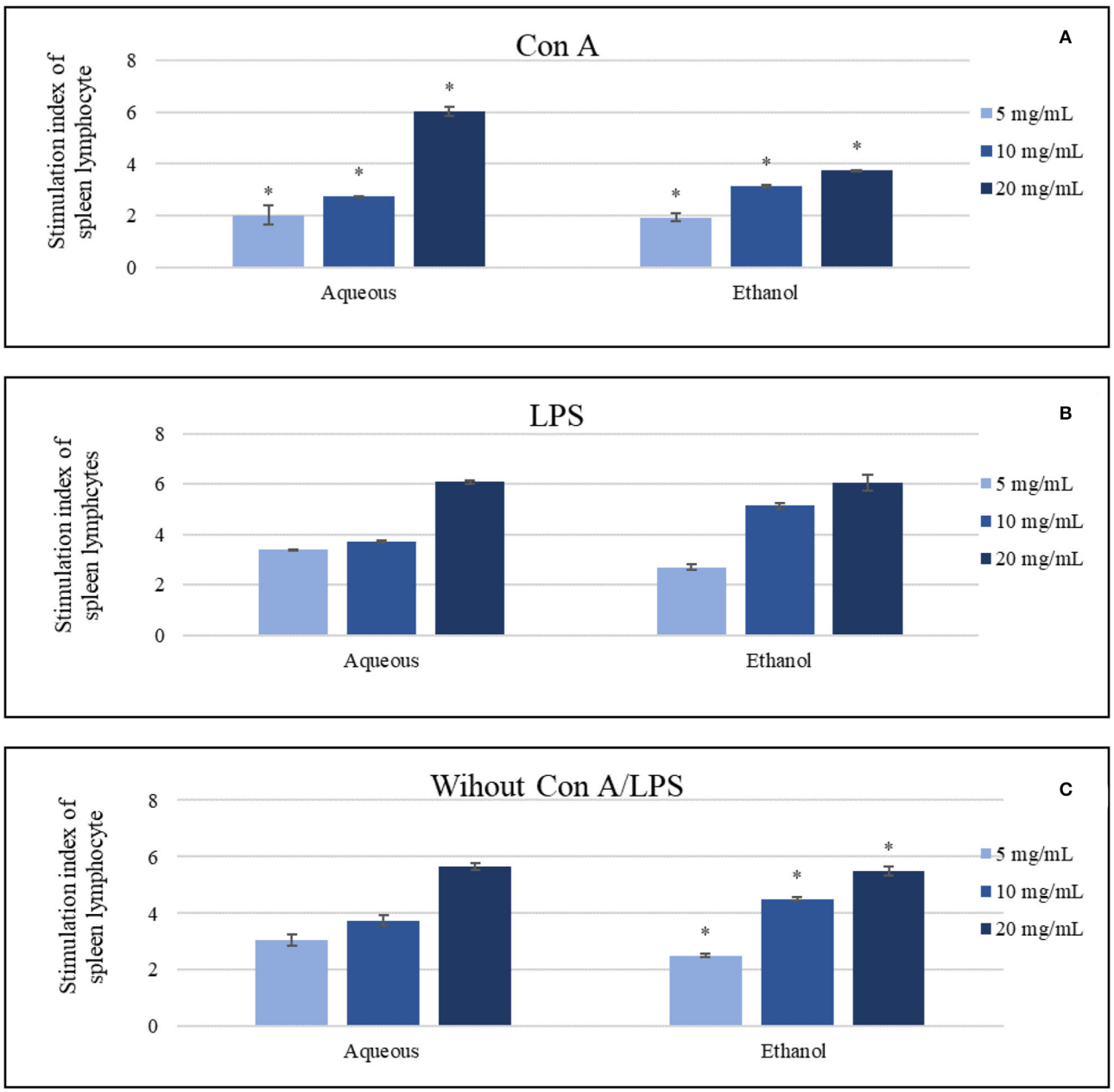

FIGURE 4 | The impact of the aqueous and ethanol extracts (5-20 mg/ml) on the splenic lymphocytes in comparing to negative control, which was 1, **(A)** in the presence of concanavalin A, **(B)** in the presence of lipopolysaccharide, **(C)** in the absence of both Con A and LPS. Results were represented as mean (bars) ± SEM (lines), $^{*}P < 0.05$ (statistically significant).

Antibacterial Assay

The antibacterial and antifungal potential was studied using the Gaz alafi extracts dissolved in DMSO (dimethyl sulphoxide). The bacterial strains used in this study were gram-negative bacteria, including *Escherichia coli* (ATCC®25922™), *Pseudomonas aeruginosa* (ATCC®27853™), gram-positive bacteria, including *Bacillus subtilis* (ATCC®6633™), *Staphylococcus aureus* (ATCC®6538™), and the yeast *Candida albicans* (ATCC®90028™). The dilution method of the micro-titer plate was used to determine the minimal inhibitory concentration (MIC). Each extract (100 mg) was dissolved in 40-μL DMSO, and Muller Hinton broth (MHB) was added up to l ml. Positive controls of gentamycin and amphotericin B (for fungus) were prepared at concentrations of (7.8–1,000 μg/ml). The plates were inoculated with bacterial suspension (100 μL/well) and incubated at 37°C for 24 h. Then, the turbidity was measured using an ELISA absorbance microplate reader at 620 nm for bacteria and 530 nm for the fungus. The MIC was determined

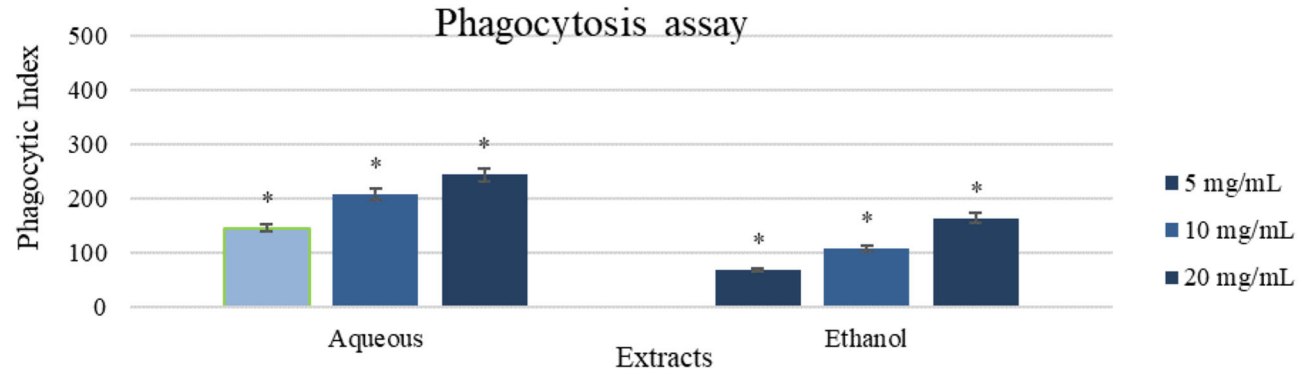

FIGURE 5 | A phagocytic assay of peritoneal macrophage treated with the aqueous and ethanol extracts (5-20 mg/ml). Results were represented as mean (bars) ± SEM (lines), ($n = 3$). $^{*}P < 0.05$ (statistically significant).

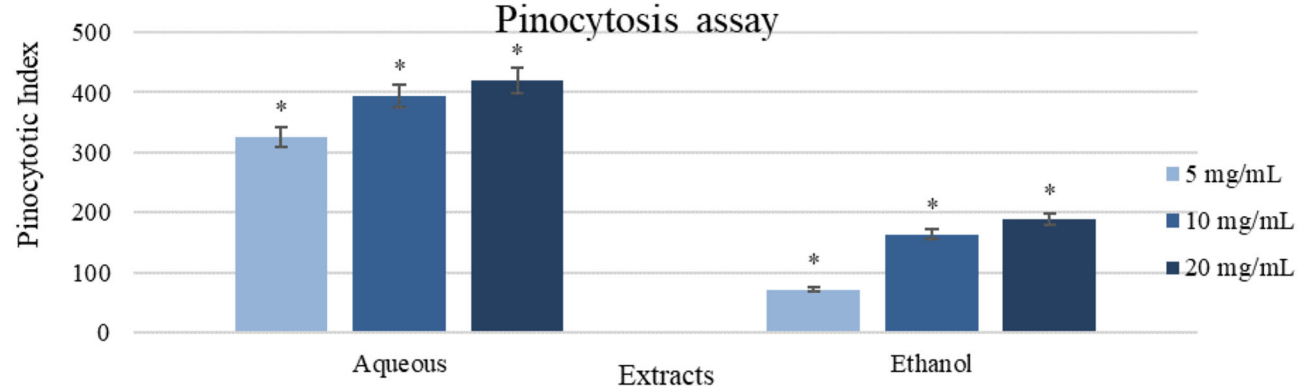

FIGURE 6 | A pinocytosis assay using the neutral red method on peritoneal macrophages treated with different concentrations of the extracts (5-20 mg/ml). Results were represented as mean (bars) ± SEM (lines), ($n = 3$). $^{*}P < 0.05$ (statistically significant).

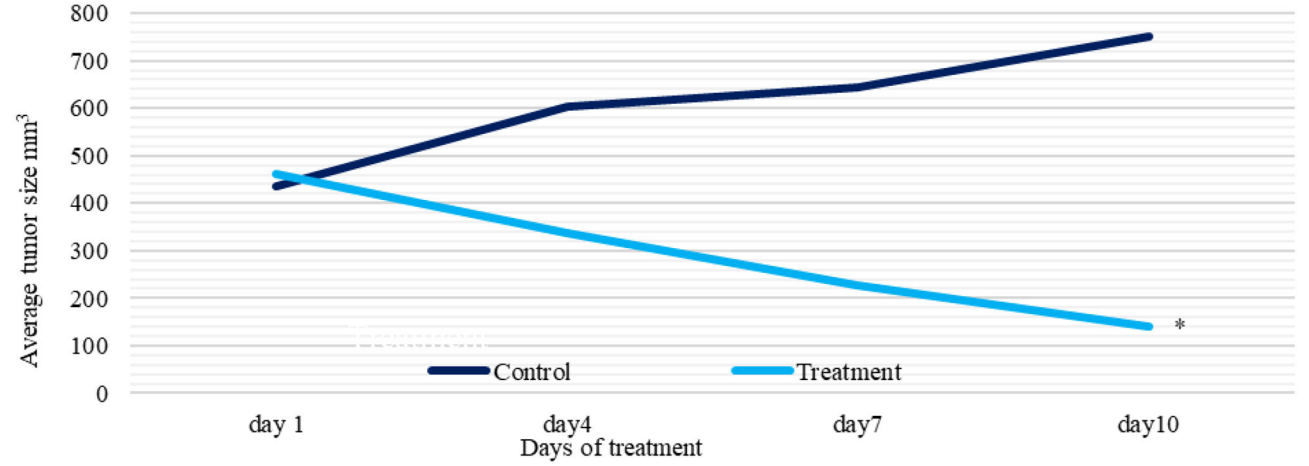

FIGURE 7 | A plot of change in average tumor size (mm 3) vs. time (days) of treatment and control groups. Tumor sizes were measured with 3-day intervals. $^{*}P < 0.05$ (statistically significant).

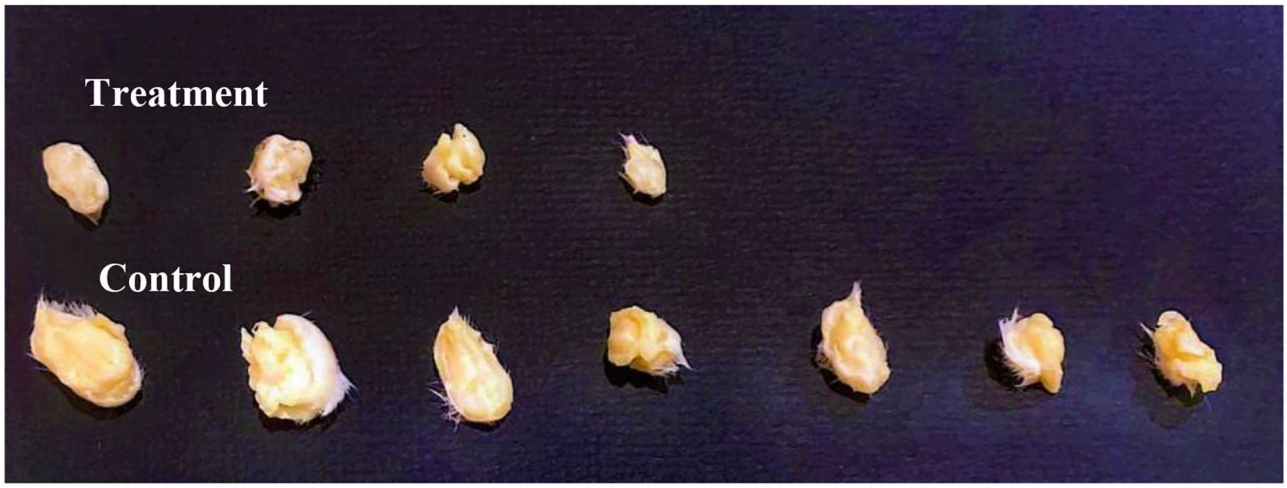

FIGURE 8 | Comparison between group tumor sizes at Day 10, $N = 7$. Ethanol extract treatment resulted in lower tumor sizes and three undetected tumors compared to the control group.

TABLE 4 | Effect of Gaz alafi ethanol extract treatment on tumor size, weight, tumors detection, and deaths percentages, in the EMT-6/P cell line, where (mm^3) is a cubic millimeter ($n = 7$).

Groups	Av. Initial tumor size (mm^3)±SEM	Av. Final tumor size (mm^3)±SEM	Change in tumor size (%)	Undetected tumors (%)	Death (%)	Av. Final tumor Weight of (mg)±SEM
Treatment	461 ± 32.80	140 ± 49.81	−70	43	0	123 ± 45.96
Control	463 ± 48.44	750 ± 143.5	72	0	14	532 ± 103.13

as the least concentration required to microbial bacterial growth (30–32).

Statistical Analysis

Using the SPSS statistical package version (version 21), data in this study were presented as the mean ± SEM (standard error of mean) of independent trials. SPSS one-way ANOVA was used to establish the statistical significance between the groups. IC_{50} estimates were determined using non-linear regression analysis. P-value of (< 0.05) was considered significant.

RESULTS

Determination of Total Phenolic Contents

Aqueous and ethanol extracts showed the phenolics content equivalent to gallic acid, with a value of 10.4 and 15.3 mg GAE/g extract, respectively, as shown in **Figure 1**.

Liquid Chromatography-Mass Spectrometry

Qualitative analysis of the aqueous extract using LC-MS compared to the standards available for this study (see **Supplementary Figure S9**) revealed the presence of two compounds in the aqueous extract, including high amount of gallic acid (65.2%), followed by catechin (34.79%). Both compounds were detected in the ethanol extract with a percentage of 58.03 and 15.79%. In addition to a third compound, tiliroside (26.19%), which is a glycosidic flavonoid. These results are semiquantitative and showed the percentage among the detected compounds. The three detected compounds were further analyzed to calculate their concentration in the extract (**Table 2**). Other compounds were below the limit of quantitation (LOQ) value, and these cannot be quantified. The detected compounds are shown in **Table 2**. The detailed LC-MS chromatograms were found in **Supplementary Figures S7**, **S8**.

In vitro Antiproliferative Assay

The effects of Gaz alafi extracts on the proliferation of several breast cancer cell lines and fibroblast normal cell lines are shown in **Table 3**. Both aqueous and ethanolic extracts exhibited the cytotoxicity against T47D cells, with IC_{50} values of 0.80 and $<$ 0.15 mg/ml, respectively. However, higher activity was observed for the ethanolic extract on the other investigated cell lines (MCF-7, MDA-MB231, EMT-6) (**Figure 2**). Regarding normal cell lines (fibroblast), no toxicity was observed for Gaz alafi extracts. Results from literature showed IC_{50} values of doxorubicin against MCF-7 were 0.68 ± 0.04 μg/ml (33), 3.16 μM for MDA-MB231, and 8.53 μM for T47D (34).

Various extract and doxorubicin concentrations were tested. The activity was noticed against T47D cells at the lowest concentration (0.156 mg/ml). In contrast, in the EMT6 cell line case, the effect started around 0.625 mg/ml. On the other hand, higher concentrations of aqueous extract were needed to have antiproliferative activity as shown in **Figure 2**.

TABLE 5 | Serum ALT, AST levels (IU/L) Serum creatinine levels in (mg/dL) for different groups (treatment, negative control treated with the only vehicle, and normal untreated mice).

Groups	AST (IU/L) ±SEM	ALT (IU/L) ±SEM	Creatinine (mg/dL) ±SEM
Treatment	34 ± 0.12*	42 ± 0.96*	0.25 ± 0.008*
Control	56 ± 0.23	98 ± 0.31	1.10 ± 0.02
Normal	36 ± 0.55	63 ± 1.9	0.80 ± 0.05

$P > 0.05$ (statistically insignificant).

TABLE 6 | Minimum inhibitory concentration (MIC) in mg/ml of Gaz alafi extracts.

The tested microorganism	Gaz alafi extracts		Positive control
	Aqueous	Ethanol	Gentamicin* & Amphotericin B **
E. coli	50 ± 0.17	25 ± 0.17	0.11 ± 0.02
B. subtilis	50 ± 0.04	25 ± 0.05	0.01 ± 0.03
P. aeruginosa	50 ± 0.08	25 ± 0.13	0.062 ± 0.008
C. albicans	25 ± 0.10	25 ± 0.16	0.76 ± 0.007
S. aureus	50 ± 0.15	6.25 ± 0.02	0.15 ± 0.06

The results were represented as mean ± SEM, (n = 3).
*From the literature: *MIC value for gentamicin against S. aureus is 0.0125 mg/ml and 0.00625 mg/ml against E. coli (35). **MIC value for amphotericin B, ranging from 0.0005 to 0.002 mg/ml (36).*

Immunomodulatory Assay

The Evaluation of Cytokines Levels

At a dose of 20 mg/ml, treatment with ethanol extract caused IFN-γ, IL-2, and IL-10 serum levels to decrease to 13, 16, and 3 pg/ml, respectively. The same treatment caused IL-2 serum levels boosting (578 pg/ml) compared with untreated tumor-bearing mice that exhibited values of 185, 107, 30, and 52 pg/ml for IL-2, IL-4, IL-10, and IFN-γ, respectively.

In contrast, different results were obtained for mice treated with aqueous extract, with an increase in IFN-γ and IL-2 serum levels to 88 and 680 pg/ml, respectively. This treatment also decreased the IL-4 level (27 pg/ml) and reduced the IL-10 level (22 pg/ml) (**Figure 3**).

Lymphocyte's Proliferation Assays

Aqueous extract was the most effective extract at a concentration of (20 mg/ml) compared to the control ($p < 0.05$) in the presence and absence of mitogens. Ethanol extract revealed active but slightly lower results than the aqueous extract. The stimulation index of aqueous extract (20 mg/ml) was around 6.04 and 6.08 in Con A and LPS-stimulated cells, respectively, where ethanol showed 3.74 and 6.06 at the same tests. In contrast, the same indexes of aqueous and ethanol extracts (20 mg/ml) in the absence of mitogens were 5.65 and 5.5, respectively (**Figure 4**). Other treating concentrations exhibited various effects.

Phagocytosis Assay

The aqueous extract (20 mg/ml) revealed the greatest inducement of peritoneal phagocytic activity (Phagocytic index of 243) compared to the control (which was zero), followed by the ethanol extract (20 mg/ml) (Phagocytic index of 164). Treated cells exhibited a concentration-dependent behavior as shown in **Figure 5**.

Pinocytosis Assay

Aqueous extract (20 mg/ml) showed a high impact with pinocytic index value of (421.75 ± 21.09) as shown in **Figure 6**. Oppositely, ethanol extract (20 mg/ml) was less active in comparison with the aqueous extract (187.5 ± 9.38), and the result of the control was zero.

In vivo Antiproliferative Assay

Acute Toxicity Test (LD_{50} Determination)

A small group of mice was used in a pilot experiment to determine the actual LD_{50} (median lethal dose). No toxicity and no mice death were found, starting from 2g/kg dose, reaching 10 g/kg using the Karber technique (22). The treatment dose (6 g/kg) was decided based on the extract solubility.

Antitumor Effects on EMT6/P Cells Implanted in Mice

Tumors were inoculated in Balb/C females; after 10 days, tumor sizes were measured. Ethanol extract was injected intraperitoneally (IP). The treatment group received 6 g/kg/day. The control group represented the vehicle-treated mice with tumor. Observations revealed a significant reduction in the treatment group tumor sizes (70%) ($p = 0.003$). While, in the control, tumor growth was increased by (72%), and one mouse died. The treatment group caused undetected tumors by (43%), with no recorded deaths (**Figures 7**, **8** and **Table 4**).

Evaluation of Liver and Kidney Functions

Liver enzymes serum levels were evaluated for the treated and control groups; also, for the normal mice that did not have any tumors (as a reference for normal liver function). The

treated group exhibited insignificant ($p > 0.05$) differences and within the range in serum ALT and AST levels compared to the normal untreated mice. Then again, the treated group showed insignificant ($p > 0.05$) differences in mean serum creatinine levels, which were lower but within the normal range compared to the normal untreated mice (**Table 5**).

Antimicrobial Assay

Ethanol extract was more effective against all tested bacteria strains and yeast than the aqueous extract. *S. aureus* was highly affected by ethanol extract with MICs of 6.25 mg/ml compared to the aqueous extract, which was 50 mg/ml. *E. coli, B. subtilis*, and *P. aeruginosa* had the same MICs for the aqueous and ethanol extracts (50 mg/ml and 25 mg/ml, respectively), while *C. albicans* was affected by both extracts with the same MICs of 25 mg/ml **Table 6**.

DISCUSSION

Gaz-Alafi (Manna) is a byproduct of insect activities on host plants, locally collected to use as candy or herbal therapy. This material is called Man-alsma in Arabic culture. Although this material or, maybe, similar has a special position among the other foods since it is mentioned in the three holy books, Taura, Bible, and Quran, the awareness of this food is very rare. The term "Manna" might include all the plant secretions. The specific origin of the manna extracted in the current study as a firm mass of *Q. brantii* leaves with manna exudate is the Penjwen district of Sulaymaniyah (north of Iraq) where the leaves are collected and boiled in water and then mixed with eggs to make a popular dessert (37). To the best of our knowledge, the present study is the first to evaluate Gaz-Alafi throughout immunomodulatory and antiproliferative evaluation *in vitro* and *in vivo* using water and ethanol extracts.

Phytochemical compounds give essential information about plant physiology and biochemical pathways. Middle East medicinal plants are considered attractive sources for these agents as they are widely used in traditional medicine (38, 39). The secondary metabolites in these plants are promising sources of pharmacologically active agents (40). Secondary metabolites are structurally diverse chemical compounds that are produced by plants or other living organisms. They have unusual and varied chemical structures and form a heterogeneous collection of biologically active molecules with multiple modes of action.

In general, plants' secondary metabolites, especially those obtained from edible plants and plants used traditionally, are relatively safer than synthetic products because most of them are biodegradable and have no severe reported side effects (40). Among plant-derived bioactive molecules, polyphenols are a large and heterogeneous set of secondary metabolites that include stilbenes, flavonoids, lignans, benzoic acid derivatives, and cinnamic acids, which have at least one hydroxylated aromatic ring in their structure (41). Polyphenol compounds are used as a basis of many current pharmaceuticals (42). The consumption of specific types of food rich in polyphenols is positively associated with health. For example, many edible plants showed activities as antioxidant, anti-inflammatory, immunomodulatory, and anticancer (43, 44).

Previous studies have reported the occurrence of secondary metabolites, such as glycosides, terpenoids, phenolic compounds, fatty acids, sterols, and tannins in the *Quercus* species (45). Phenolic acids, such as gallic, chlorogenic, ρ-hydroxybenzoic, vanillic, syringic, caffeic, ρ-coumaric- acids, and flavonoids, such as rutin, quercetin, naringenin, hesperetin, and kaempferol, are common in these species (39, 46, 47). Also, tannins are considered to be one of the major secondary metabolites found in the galls of *Quercus species*, such as flavan-3-ol monomers, catechins, epicatechins, gallocatechin, and epigallocatechin, alongside with gallic acid, syringic acid, ellagic acid, β-sitosterol, amentoflavone, hexamethyl ether, isocryptomerin, methyl betulate, and hexagalloyl glucose, and their derivatives (14, 48).

In the *in vitro* antiproliferative assay, decent activity on breast cancer was reported without toxic effect on fibroblast cells. The ethanol extract showed the most effective, inhibiting cancer cells at concentrations as low as 15 mg/ml, mainly on T47D and EMT-6 cells. Nevertheless, the aqueous extract was also efficient against some cell lines at varied concentrations.

Higher phenolic contents could explain the observed antiproliferative activity for ethanol extract than the aqueous extract resulted in the TPC assay, mainly gallic acid (GA) presence in both extracts. In general, the genus Quercus has high potency against inflammation and proliferation of cancer cells. The main antiproliferative compounds identified in the *Quercus* species are gallic acid, ellagic acid, kaempferol, quercetin, and myricetin (48, 49). These compounds are polyphenols that are quite efficient antiproliferative and cytotoxic agents against some types of cancer cells, with no significant toxicity toward healthy cells (50). They possess high antioxidant activity by donating electrons to the ROS to stabilize them and inhibiting enzymes that form free oxygen and nitrogen radicals, such as NOS, peroxidase, lipooxygenase, and xanthine oxidase so they can reduce the risk of developing cancer (51). A study in Iran evaluated the gallic acid (from 5 to 200 μg/ml) *in vitro* antiproliferative activity against MCF-7 cells. The IC_{50} for gallic acid was 18.5 μg/ml compared with tamoxifen, which has an IC_{50} of 19.5 μg/ml. This study showed that MCF-7 cells could be treated with GA by several effects associated with cell death, such as morphological changes and decreasing cancerous cells. In addition to that, this compound had considerable effects on the expression of apoptosis genes, such as P53, P21, and Mcl-1 (52).

On the other hand, gallic acid can directly increase the expression of p53, which is also known as TP53. P53 is a protein that functions as a tumor suppressor and has a role in the initiation of the apoptosis process (53). GA can cause overexpression in P53 in different types of cancers, such as breast cancer, which enhances cell apoptosis and leads to G0/G1 arrest in the cell cycle without affecting normal cells (52). A recent study has been done by Wang and Bao (54) to evaluate GA effect on lung cancer-bearing mice. The results showed the average volume and weight of tumors in the mice were reduced by treatment with GA. Also, the viability of lung cancer cells was decreased, and the apoptotic rate was increased in a dose-dependent behavior. GA

also has a potential effect on increasing the level of antioxidant enzymes, which serve as the primary line of defense against injuries caused by free radicals, so GA can be used to treat tumors initiated from oxidative stress (55).

Among the detected compounds is Catechin (flavanols), which could have played a role in the anti-proliferation response. Catechin has an antioxidant ability, which enhances the cytotoxic effect of plant extract through scavenging ROS, chelating metal ions, affecting antioxidant and pro-oxidant enzymes balance as the similarity between catechins, and ATP in the structures could result in competitive binding to the enzymes' ATP-binding sites (56, 57). Catechin was found to inhibit the growth of cancer cells (58). Catechin has been shown to inhibit tumor growth, carcinogenesis, tumor angiogenesis, and cancer cell invasion by suppressing the induction of proangiogenic factors (59, 60). Catechin in the green tea inhibited tumor growth and suppressed specific mouse mammary carcinoma 4T1 cells (61). Also, it reduces tumor angiogenesis in estrogen receptor-negative breast cancer (62). The studies suggested that the cytotoxic effects on the cancer cells result from the antioxidant properties (63). Also, catechins induced apoptosis, cell-cycle arrest, inhibited NF-κB, and cyclooxygenase-2 (COX) overexpression *in vitro*, and animal models play a significant role in preventing cancer (64, 65). Catechin could modulate apoptosis by altering the expression of the apoptotic-related genes (66–68), such as inducing the bcl-xL and bcl-2 expression, reducing Bax expression (69–71), or upregulation of the caspases-3 and caspases-10, Fas, NF-κB p105, and p53 (72, 73).

Tiliroside is a glycosyl-oxyflavone derivative of kaempferol, which was detected in the ethanol extract using LC-MS. This compound is considered uncommon due to the small amount obtained naturally (74, 75). Tiliroside demonstrates antioxidant capability due to its scavenging activity, ability to inhibit xanthine oxidase, and increase the SOD level (76). Ideally, natural compounds with antioxidant activity could be used as alternatives to synthetic cancer therapy (77). Tiliroside is a compound with an antioxidant activity that originates at the level of IGF-IR and HIF-1 alpha-signaling [76]. It cannot be considered an effective agent for cancer therapy. Studies reported that tiliroside was inactive on several cancer cell lines (78). On the other hand, per-acetylated tiliroside (a derivative of tiliroside) was significantly more potent as a specific antiproliferative agent than many other tested compounds (such as quercetin, kaempferol, and rutin), including tiliroside itself (78).

This study assessed the innate and acquired immune response alteration caused by the Gaz alafi extracts. In the lymphocyte proliferation assay, both extracts enhanced the proliferation in the presence and absence of LPS and Con-A. The aqueous extract showed slightly higher response in comparison with the ethanolic extract. Previous studies on cancer patients have revealed higher T helper-2-acquired immune response (79), where increasing T helper-1 immune response could be resulted in anticancer immune reaction (80). IL-4 levels are associated with higher Th2 response, while, for Th1, IL-2 and INF-γ are responsible for the activation (24). In contrast, healthy people had an equilibrium between both Th1/Th2 cytokines. The current study demonstrated that aqueous extract could increase IL-2 and INF-γ levels. Besides, as well, ethanol extract was responsible for increased IL-2 levels, which could indicate a shift toward anticancer immune response (Th1 antitumor response). On the other hand, macrophage phagocytosis and pinocytosis, which are important for antitumor immune response initiating by presenting the antigen to activate the T-lymphocytes (81), both were found increased after both extract supplementations. Aqueous extract showed higher activity, and ethanol extract showed some promising results in both assays in comparison with the control.

The existing active compounds could be responsible for the immune-stimulating properties of Gaz alafi. Shruthi et al. (82) established the immunomodulating effects of gallic acid against cyclophosphamide and cisplatin-induced immunosuppression in Swiss albino mice. The study showed that gallic acid at a 100-mg/kg dose counteracted the immune suppression induced by these two anticancer drugs. This effect was due to an increase in the proliferation of total leucocyte and lymphocyte counts, leading to an increase in the immune response of the host. Gallic acid can also stimulate the innate immune response by dose dependently enhancing the phagocytes and lymphocytes. It can be used as an adjuvant with immunosuppressive drugs to reduce their side effects on the immune system (82). Lymphocyte-proliferating effect could also be due to catechin, as several studies established the catechin immunomodulatory activity by acting on cellular and humoral levels (83, 84). On the other hand, the investigations have demonstrated that tiliroside can considerably inhibit the NO, TNF-α, and IL-12 production (85). Also, researchers have found that tiliroside could significantly inhibit the production of TNF-α, iNOS, IL-8, and IL-6 (86–88).

This study revealed that the ethanol extract of Gaz alafi, which was used to treat mice implanted with breast cancer caused a significant reduction in tumor size (70%) and several undetected tumors (43%). These outcomes could be a consequence of effective anticancer agents' existence, such as gallic acid and catechin. These agents could synergistically inhibit cancerous cells proliferation *via* immune system or apoptosis stimulation. Generally, this study revealed promising *in vitro* and *in vivo* antiproliferation properties from Gaz alafi extracts, particularly through their identified phytochemicals, which had numerous antitumor mechanisms, including cell proliferation inhibition, apoptosis induction, and regulation of acquired and innate immune responses.

The ethanol extract of Gaz alafi revealed considerable antibacterial activity against Gram-positive and Gram-negative bacteria (**Table 3**). The extracts and selected antibiotic (Gentamicin) showed varying inhibitory activities on various bacterial strains, with a general trend that Gram-negative bacteria were more resistant than Gram-positive strains. Based on the minimum inhibitory concentration (MIC) data, the ethanol extract activity was highest (6.25 mg/ml) against *S. aureus*. In contrast, the activity against other bacteria *E. coli, B. subtilis,* and *P. aeruginosa* had almost the same MIC value (25 mg/ml). MICs for the aqueous extract were almost the same (50 mg/ml) against all assessed bacteria. Moreover, the antifungal activity of Gaz alafi extracts against *C. albicans* showed similar MIC values of (25 mg/ml) for both tested extracts. In contrast,

Amphotericin B (an antifungal agent) displayed an MIC value of (0.76 ± 0.007).

Limited data are available to report the antimicrobial activity of Gaz alafi. In a previous study, Nebigil assessed *Q. branti* L. different seed extracts and fractions against four bacterial strains, including *E. coli* and *S. aureus*. Minimum inhibitory concentrations for total extract and water/methanol fraction were found to be 2.5 mg/ml, 3 mg/ml for *E. coli*, and 1.25 and 3 mg/ml for *S. aureus* (89). Another study suggested that the gall extract of *Q. brantii* L. probably acts through its anti-inflammatory and antimicrobial properties to affect colitis's biochemical and pathological parameters (14).

The observed antibacterial activity in the current study could be attributed to the activity of the major compounds (gallic acid, catechin, tiliroside), either singly or synergistically. Tannins have been traditionally used as antimicrobial and antiseptic agents. Their mode of action may depend on the inactivation of microbial adhesins, enzymes, and cell envelope transport proteins so that tannins can be toxic to bacteria, fungi, and yeasts. Quercus's tannic and gallic acids are responsible for this antimicrobial activity (90, 91). Gallic acid is produced through the hydrolytic breakdown of tannic acid using a glycoprotein esterase, namely tannase (92). A study was done by Lima et al. (93) to evaluate the effect of GA on enhancing the activity of antibiotics. The results showed that GA has a synergistic effect, with two types of antibiotics against *S. aureus*. GA reduced the MIC from 156.3 to 49.21 μg/ml when associated with Norfloxacin and from 49.21 to 2.44 μg/ml when associated with Gentamicin against *S.aureus*. Oppositely, when GA was tested against *P. aeruginosa*, results revealed insignificant MIC value. GAs antimicrobial activity depends on several mechanisms, including the inhibition of extracellular microbial enzymes required for microbial growth, direct action on microbial metabolism, and an anti-adhesion mechanism. These findings could explain the high effect of Gaz alafi against *S. aureus*.

On the other hand, the catechins exhibit only modest antibacterial activity. However, extensive chemical changes to the structure could significantly enhance antibacterial effectiveness and stability *in vivo*. Nevertheless, naturally occurring catechins have a range of activities on bacteria, other than the ability to relate to bactericidal or bacteriostatic effects, such as adjusting antibiotic sensitivity and altering the factors that control bacterial virulency expression (94). Furthermore, tiliroside did not exert remarkable activity against most pathogenic bacteria or fungi, such as various strains of *Staphylococcus aureus*, *P. aeruginosa*, *C. albicans* (95, 96). Although it has poor bacteriostatic and bactericidal activity, tiliroside could find a place in the bacterial infection's treatment. Notable research was also performed on strain SA-1199B of *S. aureus*, and concluded that adding 64 μg/ml of tiliroside decreased the MIC of the tested antibiotics by (2–128) times (97). Also, computational studies observations suggested that tiliroside can probably inhibit penicillin-binding proteins 2a (PBP2a) and 4 (PBP4), produced by methicillin-resistant *S. aureus* (98).

CONCLUSION

For the first time, Gaz alafi obtained from *Q. brantii* dry leaves revealed functional food benefits, involving promising anticancer and immunomodulatory properties fulfilled in the present study. Boosting the immunity responses *via* altering innate and acquired immune systems toward the cancer suppression and the existence of biologically active components like gallic acid, which has several antiproliferation property, was indicated by the present study. One limitation of this study is the low number of detected compounds in the extract. Further analysis using additional reference standards and pure compounds isolation with NMR evaluation could result in total composition identification in the future.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by the Research and Ethical Committee at Applied Science Private University.

AUTHOR CONTRIBUTIONS

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.900506/full#supplementary-material

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Combination of Ashwagandha Water Extract and Intermittent Fasting as a Therapy to Overcome Cisplatin Resistance in Breast Cancer: An *in vitro* and *in vivo* Study

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Breast cancer is considered a universal public health dilemma in women. Due to the high toxicity and low selectivity of conventional anticancer therapies, there is a growing trend of using plant-derived natural products in cancer prevention and therapy. Ashwagandha (*Withania somnifera*, WS) has been used in the Mediterranean region and Ayurvedic medicine for millennia as a functional food and a medicinal plant with anticancer activity. Besides, intermittent fasting (IF) has been engaged recently in cancer treatment. Hence, the combination of WS and IF provides possible solutions to treat cancer and reduce chemoresistance when combined with chemotherapy. In this study, WS root (WSR), IF, and cisplatin were tested on cisplatin-sensitive (EMT6/P) and cisplatin-resistant (EMT6/CPR) mouse mammary cell lines. The phytochemical content of the WSR extract was analyzed using liquid chromatography–mass spectrometry (LC-MS) analysis. Antiproliferative and apoptotic effects were assessed for WSR extract, cisplatin, and their combination *in vitro* using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (MTT) and caspase-3 assays. An *in vivo* study was used to assess the effect of WSR extract, IF, cisplatin, and their combinations in mice inculcated with EMT6/P and EMT6/CPR cells. The safety profile was also investigated using liver enzymes and creatinine assays. *In vitro,* WSR extract and cisplatin had a synergistic effect in both cell lines. The same combination induced an apoptotic effect higher than the single treatment in both cell lines. *In vivo*, several combinations of WSR extract, IF, or cisplatin caused significant tumor size reduction and improved the cure rate in mice implanted with EMT6/P and EMT6/CPR cell lines. IF-treated groups showed a significant reduction in serum glucose and an elevation in β-hydroxybutyrate (BHB) levels. In the safety profile, WSR extract, IF, and their combinations were safe. Overall, the combination of WSR extract and IF provides a promising solution for breast cancer treatment besides cisplatin by reducing the proliferation of cancer cells through induction of apoptosis. Moreover, they minimize cisplatin toxicity to the liver and kidney.

Keywords: breast cancer, nutritional intervention, Ashwagandha, intermittent fasting, multidrug resistance, cisplatin, apoptosis, Warburg effect

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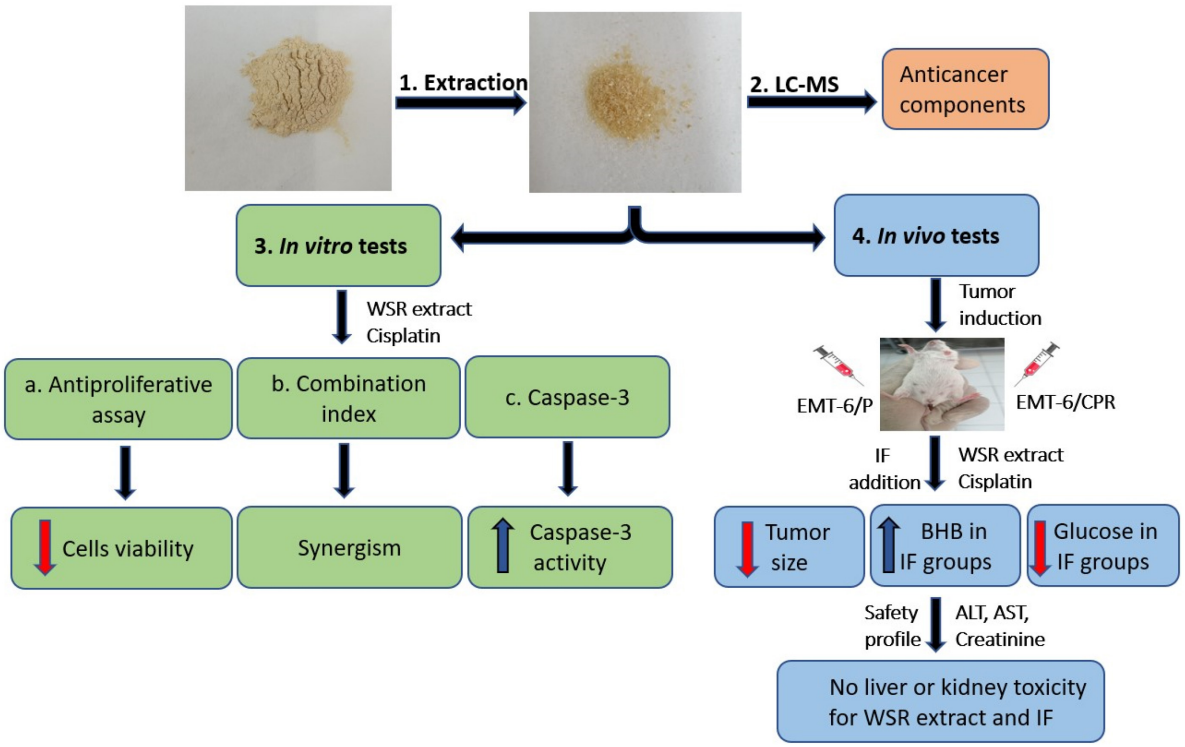

GRAPHICAL ABSTRACT | Summary of the effect of Ashwagandha and intermittent fasting on cisplatin resistance breast cancer *in vitro* and *in vivo*. Created by Sajidah Jawarneh.

INTRODUCTION

Breast cancer is a major public health problem because it is the second cause of death and the most common cancer in women worldwide. Resistance to cancer treatments plays a major role in breast cancer issue exacerbation (1). As an example, cisplatin is uniformly used in cancer treatment; nevertheless, its use is limited because of serious side effects and resistance (2). Resistance is divided into primary drug resistance and multidrug resistance (MDR) (3). To clarify, MDR is the resistance of cancerous cells to various anticancer drugs with different structures and mechanisms of action. Chemoresistance has several molecular mechanisms, such as deregulation of apoptosis, deregulated autophagy, enhanced DNA damage repair, and p53 inactivation (4).

Apoptosis is the natural mechanism for programmed cell death. Apoptosis has two major pathways: extrinsic and intrinsic. In the extrinsic pathway, the death legends (e.g., TNF and Fas-L) activate the formation of a death-inducing signaling complex (DISC). This results in the formation of caspase-8 and −10 followed by executioner caspases-3, −6, and −7 activation (5). In the intrinsic pathway, different apoptotic stimuli upregulate BCL-2 homology domain 3 (BH3)-only proteins, which activate BCL-2 homology domain 3 (BH3) and BCL-2 homology domain 3 (BH3), releasing cytochrome-c. Then, cytochrome-c facilitates the conversion of procaspase-9 to caspase-9, which can activate the executioner caspases-3 and −7. The latter start to hold up proteins leading to cell death (6). Therefore, overexpression of oncogenes mediates the inhibition of apoptosis and that leads to the suppression of p53, enhancement of antiapoptotic proteins, such as B cell CLL/lymphoma-2 (Bcl-2), and downregulation of pro-apoptotic proteins like caspases, Bcl-XL/Bcl-2-associated death promoter (Bad), and BAX/BAK. Therefore, several researchers have targeted caspases to overcome resistance to chemotherapy (5).

Nutrition interventions have valuable effects in terms of cancer prevention and treatment (7). For example, Ashwagandha has been used as an indispensable plant in the Mediterranean region and in Ayurvedic medicine for millennia (8) as a functional food due to its immense nutritional value with various biological effects like cancer (9). Comparatively, intermittent fasting (IF) has been engaged in the newly developing treatment approaches because of its benefit in fighting cancer (10).

Ashwagandha (*Withania somnifera*, WS) belongs to the family Solanaceae and has been used as an antitumor, anti-inflammatory, antidiabetic, antistress, hepatoprotective, and nephroprotective agent (11). WS root (WSR) has many active constituents, such as alkaloids, flavonoids, withanolides (e.g., withaferin A and withanone), and succinic acid (11, 12). WSR extract can promote apoptosis in breast cancer through caspase-3 activation and downregulation of the antiapoptotic protein Bcl-2 (13). Additionally, it enhances the efficacy of both chemotherapy and radiotherapy (14).

There is growing attention to harnessing IF to minimize tumor growth and improve cancer treatment efficacy. IF mimics Ramadan fasting in Islamic countries, which is applied in many of the Mediterranean region's countries (15). It reduces glucose levels and affects glucose metabolism (glycolysis) inside the cell by reversing the Warburg effect (16). IF depends on calorie restriction or fasting over an extended period (e.g., 16–48 h). This time is enough to activate ketogenesis increasing ketones [e.g., β-hydroxybutyrate (BHB) and acetoacetate], mitochondrial stress resistance, antioxidant defense, and autophagy (17). Currently, IF is used as an adjunct therapy for cancer along with chemotherapy. When used for short terms, it enhanced the chemotherapeutic effect. Moreover, short terms use of fasting showed elevation in oxidative stress and DNA damage causing induction of apoptosis (10).

In this perspective, the Warburg effect (aerobic glycolysis) represents an essential hallmark of cancer, since cancer cells have accelerated glycolysis and exaggerated lactate production, even under fully oxygenated conditions. This ends with oxidative stress reduction and consequently, resistance to diverse factors including apoptosis and cytotoxic drugs. In the bargain, lactate production produces an acidic environment, which is compatible with proliferation and metastasis (18).

A combination of herbal extract with fasting and a triple combination of herbal extract, fasting, and chemotherapy has not been evaluated in the literature to treat cancer. Accordingly, this study was designed to test the effect of WSR extract with IF with cisplatin as a new combination therapy to overcome cisplatin-resistant breast cancer in a mouse model.

MATERIALS AND METHODS

Extract Preparation

Powdered WSR was purchased from Zokiva Nutritionals, US. The extract preparation was performed following the protocol previously optimized by Kumar et al. (19). Briefly, 5 gm of the powdered root was infused in 250 ml (1:50 w/v) of freshly boiled double distilled water (DDW) for 25 min. After that, the infusion was left to cool to room temperature and centrifuged (12,000 rpm or 8050 X g, 15 min). The supernatants were recentrifuged (12,000 rpm, 10 min) (19). The supernatants were then dried in the oven at 50°C (20). The yield of the dried aqueous extract was 1.4 g (**Figure 1**).

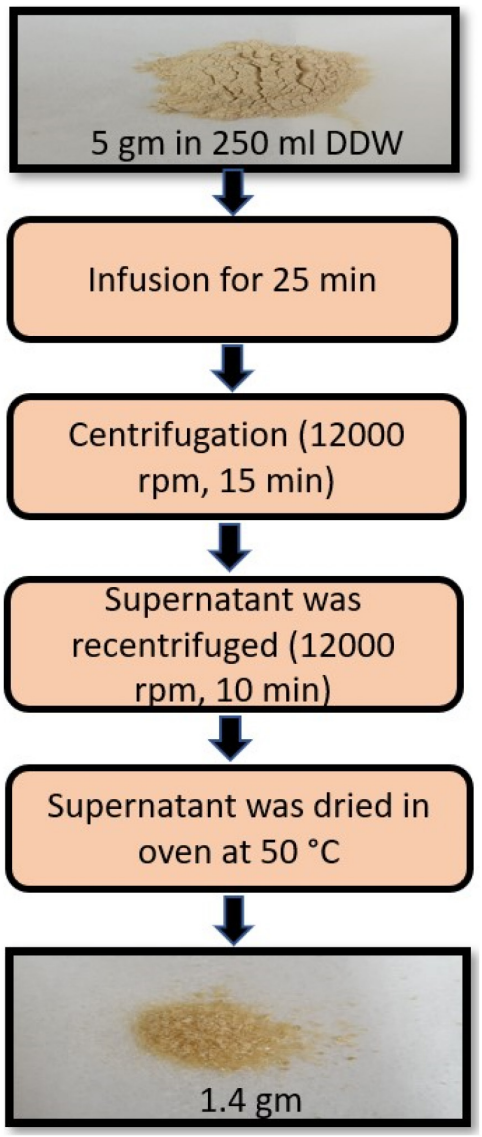

FIGURE 1 | Scheme for extraction of water-soluble material from the *Withania somnifera* root (WSR).

Liquid Chromatography–Mass Spectrometry Analysis

The preparation of the sample was carried out by dissolving 20 mg of WSR extract in 2 ml DDW and then completed with acetonitrile to 50 ml. The sample was centrifuged at 4,000 rpm for 2 min. The autosampler was then loaded with 1 ml of the sample and the injection volume was 3 μl. The instrument was utilized by Ion Source Apollo II ion funnel electrospray source with the following characteristics [dry gas flow 8 l/min; capillary voltage: 2500 v; nebulizer gas: 2 bar; dry temperature: 200°C; mass accuracy: < 1 ppm; mass resolution: 50,000 FRS (Full Sensitivity Resolution); the time-of-flight (TOF) repetition rate: up to 20 kHz]. The separation was accomplished *via* a Burker solo 2-C-18 ultra-high performance liquid chromatography (UHPLC) column (100 mm × 2.1 mm × 2 μm) at a flow rate of 0.51 ml/min and a column temperature of 40°C. All standards were used for the identification of m/z and the retention time. The analysis was performed by Burker Daltonik (Bremen, Germany) impact II ESI-Q-TOF system provided with Burker Dalotonik Elute UPLC system (Bremen, Germany) used for screening compounds of interest.

In vitro Experiments

Cell Lines and Culture Conditions

Two mouse mammary cell lines were used in this study: the parent (EMT6/P) and cisplatin resistance (EMT6/CPR) cell lines

were purchased from the European Collection of Authenticated Cell Cultures (ECACC; Salisbury, United Kingdom). Cells were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, 0.1% gentamicin, 1% penicillin-streptomycin solution, and 0.1% non-essential amino acids. Perfect cell culture conditions were provided for cell growth using complete tissue culture media (MEM). All cell lines were incubated in a carbon dioxide (CO2) incubator at 37°C, with 5% CO2, and 95% humidity.

Antiproliferative Assay (MTT)

The antiproliferative activity was detected using MTT (the tetrazolium salt, 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; Sigma, Saint Lucia, United States). The mouse mammary cell lines (EMT6/P and EMT6/CPR) were cultured overnight. Cells were collected using the trypsinization technique, and the exponentially growing cells were counted using the trypan blue exclusion method. After that, the cells were seeded at 10,000 cells/well in 96-well tissue culture flat-bottom microplates for 24 h incubation. After seeding, both the cell lines were exposed to different concentrations of WSR extract (0.39–50 mg/ml) for 48 h. They were also exposed to cisplatin (0.8–100 μ M) for 48 h.

In combination treatment, EMT6/P cells were exposed to increasing concentrations of WSR extract (0.01–1.5 mg/ml) with a fixed dose of cisplatin (10 μM) in EMT6/P. In the resistant cell line, cells were exposed to the extract with different concentrations (0.02–2 mg/ml), with a fixed concentration of cisplatin (27 μM). The reduced MTT was assayed at 550 nm using a microplate reader (Biotek, Winooski, VT, United States). Percentage cell survival was calculated for all treatments and compared with untreated cells.

The combination index (CI) was calculated for the WSR extract and cisplatin combination using the previous equation (21), and the resistance fold (RF) was calculated using the following formula (22):

$$CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2 + \alpha(D)_1(D)_2/(Dx)_1(Dx)_2$$

Where:
$(Dx)_1$ = The half-maximal inhibitory concentration (IC_{50}) of WSR extract alone
$(D)_1$ = IC_{50} of WSR extract in combination with cisplatin
$(Dx)_2$ = IC_{50} of cisplatin alone
$(D)_2$ = IC_{50} of cisplatin in combination with WSR extract

α = 0 for mutually exclusive or 1 for mutually nonexclusive interaction. Depending on the literature review, both WSR extract and cisplatin exert their anticancer effect by different mechanisms of action. Hence, we applied the mutually nonexclusive model, where α = 1.

CI values are explained according to the following:
CI > 1.3 = antagonism,
CI = 1.1–1.3 = moderate antagonism,
CI = 0.9–1.1 = additive effect,
CI = 0.8–0.9 = slight synergism,
CI = 0.6–0.8 = moderate synergism,
CI = 0.4–0.6 = synergism,
CI = 0.2–0.4 = strong synergism

$$RF = IC_{50}\ of\ resistant\ cells/IC_{50}\ of\ parental\ cells$$

Measuring Apoptosis Induction in Cultured Cells

The caspase-3 assay was used to determine the apoptotic effect of WSR extract and cisplatin in parent and drug resistance cell lines. EMT6/P flasks were treated with IC_{50} concentrations of WSR extract (2.9 mg/ml), cisplatin (positive control; 20 μM), and combination treatment of the extract and cisplatin (0.54 mg/ml + 10 μM, respectively). EMT6/CPR flasks were treated with IC_{50} concentrations of the extract (3.8 mg/ml), cisplatin (positive control; 54 μM), and combination treatment of the extract and cisplatin (0.66 mg/ml + 27 μM, respectively). MEM was used as the negative control. The flasks were then incubated for 48 h. After treatment, cells were collected, washed, and lysed using lysis buffer. Caspase-3 activity was measured using the procedure provided in the standard kit (Caspase-3 Assay Kit, My BioSource, United States).

In vivo Experiments

Mice

Forty-two female Balb/C mice weighing 21–25 g (4–6 weeks old) were used in this study. Mice were supplied by the animal house in the Applied Science Private University, Amman, Jordan. All protocols of animal experiments were validated by the Research and Ethical Committee of Applied Science University with Standard ethical guidelines. The animals were kept in separate cages with bedding of wooden shavings. The provided conditions in the animal house included stable temperature at 25°C, 50–60% humidity, continuous air ventilation, and alternating light/dark cycles of 12 h.

Tumor Inoculation

Exponentially growing EMT6/P and EMT6/CPR cells were harvested by trypsinization and were washed and resuspended in MEM, at a density of 1.5×10^6 cells/ml. After that, viability was detected using the trypan blue exclusion method. A tumor induction dose of 1.5×10^5 cells in 0.1 ml medium was injected into the abdominal area of each female BALB/C mouse subcutaneously and maintained for 14 days to grow and form new tumors.

Mice Groups, Treatment, and Antitumor Activity

Each mouse was inoculated with EMT6/P on the right side and EMT6/CPR on the left side. The treatments were started 14 days after tumor inoculation. Forty-two tumor-bearing mice were used in this investigation, and the mice were divided into seven groups (N = 6 for each group; **Figure 2A**). Group 1 was used as a negative control and was exposed to intraperitoneal injection of the vehicle (phosphate-buffered saline, PBS) of 0.1 ml daily. Group 2 was treated with cisplatin (5 mg/kg/week) by intraperitoneal injections (23). Group 3 was treated with a daily dose of WSR extract (100 mg/kg/d; 24) by gavage. Group 4 was exposed to a daily 18 h of IF and 6 h of

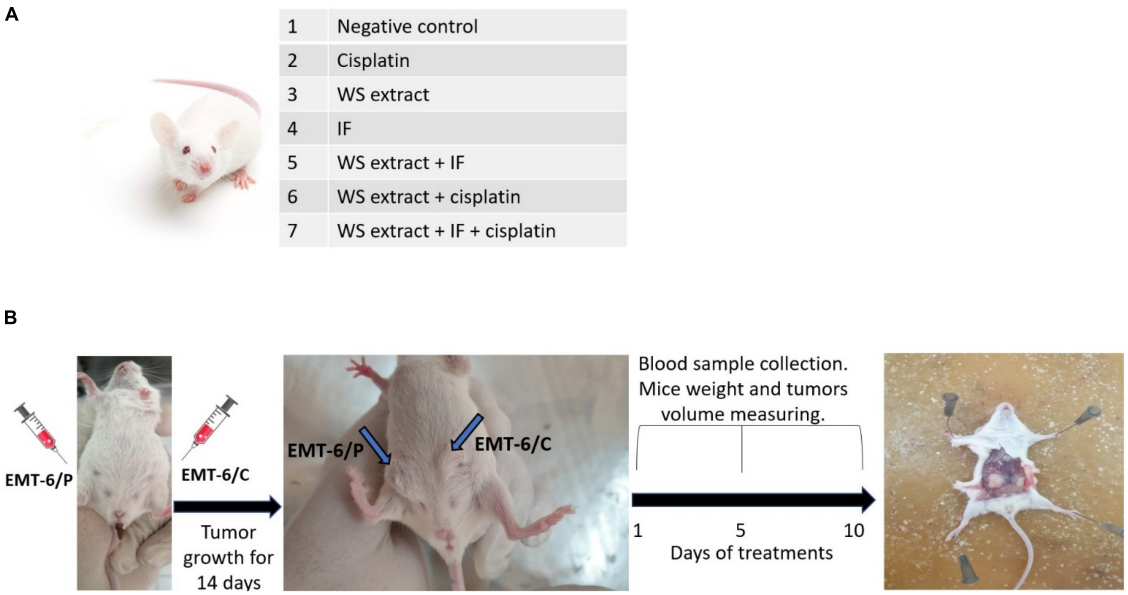

FIGURE 2 | *In vivo* mice study. **(A)** Groups of cisplatin-sensitive (EMT6/P) and cisplatin-resistant (EMT6/CPR) inoculated mice. Treatments were: WSR extract (100 mg/kg/d) by gavage, a daily 18 h of intermittent fasting (IF) and 6 h of eating or/and cisplatin (5 mg/kg/week) by intraperitoneal injections for 10 days. **(B)** Scheme for mice experiment (n = 6).

eating (*ad libitum* nutrition). Group 5 was treated with WSR extract and IF combination. Group 6 was treated with WSR extract and cisplatin combination. Group 7 was treated with the triple combination therapy of WSR extract, IF, and cisplatin. The treatment lasted for 10 days. During treatment, blood samples, mouse weight, and tumor volumes were taken at three time-points over the treatments on days 1, 5, and 10 (**Figure 2B**).

Tumor dimensions were measured using a digital caliper. The following formula was used to calculate the tumor volumes (22):

$$Tumor\ volume = L \times W^2 \times 0.5$$

where L = length of the longest aspect of the tumor,

W = length of the tumor aspect perpendicular to L.

Finally, mice were killed by cervical dislocation. The tumors were removed, weighed, and stored in 10% formalin to preserve their morphology.

Evaluation of Serum β-Hydroxybutyrate and Serum Glucose Levels

Blood levels of glucose and BHB were assessed on days: 1, 5, and 10, and compared with normal-untreated mice bearing no tumor. Blood glucose levels were measured using the Accu-Chek blood glucose monitoring system (Roche, Basel, Switzerland). BHB Assay Kit (Sigma, United States) was used to measure the levels of BHB in the serum.

Evaluation of Liver and Kidney Function in Treated Mice

The level of toxicity exerted by different treatments on the liver and kidney was assessed. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine were evaluated for WSR extract, IF, cisplatin, and their combinations in addition to the negative control and normal-untreated group. After a serum sample collection, ALT and AST were tested using ALT/GPT kit, AST/GOT kit, and a creatinine assay kit purchased from (BioSystems, Barcelona, Spain).

Statistical Analysis

Statistical analyses were performed using SPSS (Statistical Package for the Social Science, Chicago, IL, United States 25). All values were expressed as mean ± SEM. IC_{50} values were statistically analyzed using nonlinear regression. Statistical significance among the mice groups was determined using a one-way analysis of variance (ANOVA; *post hoc* test: Tukey). A probability level less than 0.05 ($p < 0.05$) represented a significant difference among groups. *In vivo*, six mice per group were used and statistics were conducted using $n = 3$ or $n = 6$ in the different tests.

RESULTS

Liquid Chromatography-Mass Spectrometry Analysis of WS Root Water Extract

According to Liquid chromatography-mass spectrometry (LC-MS) analysis, WSR water extract contained succinic acid (68.52%) as a major compound, and other compounds were found in different concentrations, such as anthranilic acid (16.87%), gallic acid (7.52%), chlorogenic acid (2.81%), and 3,5-dimethoxy-4-hydroxy acetophenone (1.29%; **Figure 3**). The

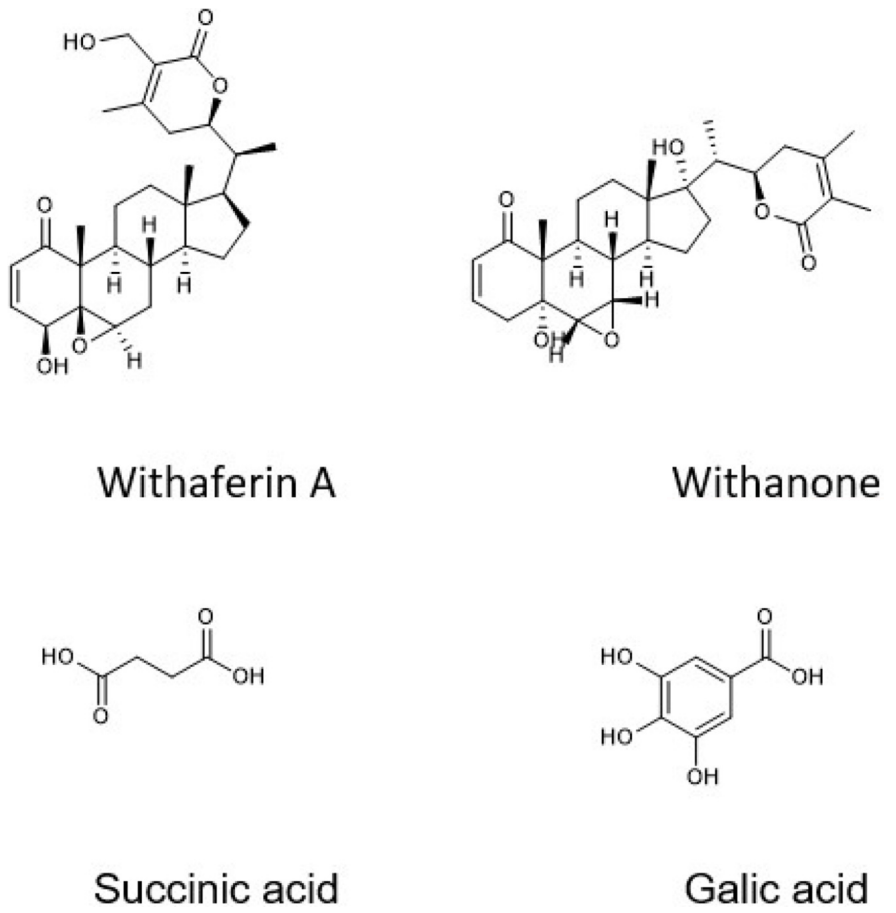

FIGURE 3 | Chemical structure for selected compounds in the water extract of WSR.

rest of the compounds were less than 1%, such as vanillic acid, protocatechuic aldehyde, caffeic acid, ferulic acid (trans), apiin, and salicylic acid, rutin, spiraeoside, kaempferol, and isorhamnetin (**Table 1**).

In vitro Results

Antiproliferative Effect of WS Root Extract, Cisplatin, and Their Combination

MTT assay was conducted to evaluate the antiproliferative activity of WSR extract, cisplatin, and their combination on cisplatin-sensitive (EMT6/P) and cisplatin-resistant (EMT6/CPR) cell lines. Single treatment of WSR extract or cisplatin attenuated cell proliferation compared with the vehicle control in a concentration dependant pattern as observed in **Figures 4A,B**. Our results revealed that EMT6/CPR cells presented higher survival rates compared to EMT6/P cells when exposed to the same concentrations of WSR extract or cisplatin. Additionally, EMT6/P and EMT6/CPR cell lines were treated with different concentrations of WSR extract and a fixed concentration of cisplatin. The results showed that this combination significantly concentration-dependently inhibited cell viability (**Figures 4C,D**).

The analysis of the CI showed that the combination treatment had a moderate synergistic effect employed on EMT6/P and EMT6/CPR cells (CI = 0.78 and 0.76, respectively; **Table 2**). The mean IC_{50} values are reported in **Table 2**, and the IC_{50} values of the extract were 2.9 $\pm$ 0.18 and 3.8 $\pm$ 0.09 mg/ml in EMT6/P and EMT6/CPR, respectively, with an RF of 1.31, which means that EMT6/CPR cells were 1.31 times more resistant to the extract than EMT6/P. Oppositely, IC_{50} of cisplatin was found to be 20 $\pm$ 0.5 μM in the EMT6/P cell line and 54 $\pm$ 0.08 μM in the EMT6/CPR cell line, which means that EMT6/CPR cells were 2.7 times more resistant to cisplatin in contrast to EMT6/P cells. Thus, a higher concentration of WSR extract or cisplatin is needed to kill 50% of the EMT6/CPR cell line. On the other hand, in the combination, IC_{50} was 0.54 $\pm$ 0.011 mg/ml WSR extract and 10 μM cisplatin in EMT6/P cells and 0.66 $\pm$ 0.05 mg/ml WSR extract with 27 μM cisplatin in EMT6/CPR cells. As observed, EMT6/P cells were more susceptible to the combination at lower doses than EMT6/CPR being a resistant cell line that has mechanisms to resist the applied combination. The RF of the combination (1.22) is lower than the RF of either WSR extract or cisplatin single treatment (1.31 and 2.7, respectively), which indicates that the WSR extract sensitized the resistant cells to cisplatin.

TABLE 1 | Liquid chromatography-mass spectrometer (LC-MS) analysis of *Withania somnifera* root (WSR) water extract.

No.	Compound	Molecular formula	RT (min)	Amount (%)
1	Succinic acid	C4H6O4	0.98	68.52
2	Gallic acid	C7H6O5	1.04	7.52
3	Protocatechuic aldehyde	C7H6O3	2.09	0.01
4	Chlorogenic acid	C16H18O9	2.88	2.81
5	Vanillic acid	C8H8O4	3.2	0.18
6	Caffeic Acid	C9H8O4	3.27	0.28
7	Anthranilic acid	C7H7NO2	4.07	16.87
8	Apiin	C26H28O14	5.11	0.01
9	Ferulic acid (trans)	C10H10O4	5.13	0.40
10	Rutin	C27H30O16	5.58	0.40
11	3,5-Dimethoxy-4-hydroxy acetophenone	C10H12O4	5.63	1.29
12	Salicylic acid	C7H6O3	5.78	0.01
13	Spiraeoside	C21H20O12	5.78	0.81
14	Kaempferol	C15H10O6	10.13	0.07
15	Isorhamnetin	C16H12O7	10.51	0.32

Apoptotic Activity of WS Root Extract, Cisplatin, and Their Combination

Caspase-3 activity was performed using a caspase-3 assay kit to evaluate the apoptotic effect of WSR extract, cisplatin, and their combination in EMT6/P and EMT6/CPR cell lines. Results of the analysis in EMT6/P cells indicated a significant difference between WSR extract, cisplatin, and their combination compared to control ($p < 0.05$) and between the treatment groups themselves ($p < 0.05$). The combination exhibited 2.98 folds increase in caspase-3 activity compared to the control (**Figure 5A**). In contrast, the detected results in EMT6/CPR revealed the existence of a significant difference between WSR extract and its combination with cisplatin compared with the control group. As shown in **Figure 5B**, WSR extract single treatment achieved 1.39 folds increase in caspase-3 activity compared to the control ($p < 0.05$). Furthermore, the combination of WSR extract and cisplatin achieved 2.31 folds increase in caspase-3 activity compared to the control group with a significant difference ($p < 0.001$) compared to the cisplatin-treated group, which showed an insignificant response to cisplatin ($p = 0.286$).

In vivo Results

Antitumor Effect of WS Root Extract, Intermittent Fasting, Cisplatin, and Their Combinations

Based on the results of the *in vitro* assay, WSR extract was selected in addition to IF, cisplatin, and their combinations for further evaluation to assess the antitumor activity in Balb/C female mice. According to the results in **Table 3**, the treated groups showed a significant reduction ($p < 0.05$) in tumor size compared with the negative control, which registered an increase in tumor size of 88.87%. Noteworthy, the triple combination of cisplatin, WSR extract, and IF recorded the highest percentage in the size reduction (100%) and curable rate (100%). Besides, the combination of WSR extract and IF recorded the lowest percentage of size reduction (60.52%) among the combination treatments, along with a curable rate of 50%. The combination of WSR extract and cisplatin registered a reduction in tumor size of 81.12% with a curable rate of 50%. As observed, the same treatments were applied to EMT6/CPR cells. Tumor size was reduced remarkably ($p < 0.05$) for all treated groups as opposed to the negative control, which showed an increase in tumor size of 60.02% from the initial tumor size. Interestingly, combination treatments had higher tumor size reduction than single treatments. Regarding triple treatment, it registered a complete reduction in tumor size (100%); therefore, there were no mice with detectable tumors (100%). On the flip side, the WSR extract and cisplatin combination exhibited a higher reduction in tumor size than the WSR extract and IF combination (69.49 and 53.36%, consequently), besides the same curable rate (66.66%).

Generally, single treatment revealed a lower reduction in tumor size than combination treatments. The analysis showed that EMT6/P complied with the positive control (cisplatin) more than the WSR extract and IF as cisplatin reduced the tumor by 74.21% against 50% of mice with no detectable tumor. WSR extract and IF displayed approximately the same reduction in tumor size (51.97 and 52.75%, respectively) and the same curable rate (33.33%). In EMT6/CPR, the cells responded to WSR extract in a way better than the other single treatments by 40.49% lowering in tumor size and 50% curable rate. Fasting recorded a higher reduction in tumor size compared to cisplatin, with values of 37.22 and 18.80%, respectively, along with the same curable rate of 33.33%. **Figures 6A,B** illustrate the difference between treatments in tumor volume reduction at three time-points during treatments. The change in tumor size can be ascertained from **Figures 6C,D**, which show the final average volumes of the dissected tumors.

Effect of Treatments on Mice's Average Weight

Concerning mice body weight, all treated groups registered weight loss except the WSR extract-treated group (25), and WSR/IF-treated group registered a significant weight gain compared with the IF-treated group, $p < 0.001$. Additionally, the control group recorded an increase in body weight (8.21%). Despite the effect of WSR extract on weight gain, cisplatin caused a non-significant weight loss compared to the control (8.20%) when administered either alone (−2.29%), along with WSR extract (−5.56%), or with WSR extract and IF (−8.71%; **Figure 7**).

Effect of the Treatments on Glucose and β-Hydroxybutyrate Levels

The subsequent analysis of glucose levels showed that treatments with IF had the lowest level of glucose either as a single treatment or in combination ($p < 0.001$). Generally, WSR extract-treated groups had lower glucose levels than the single treatment cisplatin or the control, and when we compared IF alone or along with WSR extract, nevertheless, the reduction was insignificant (**Figure 8A**). Additionally, we were interested to

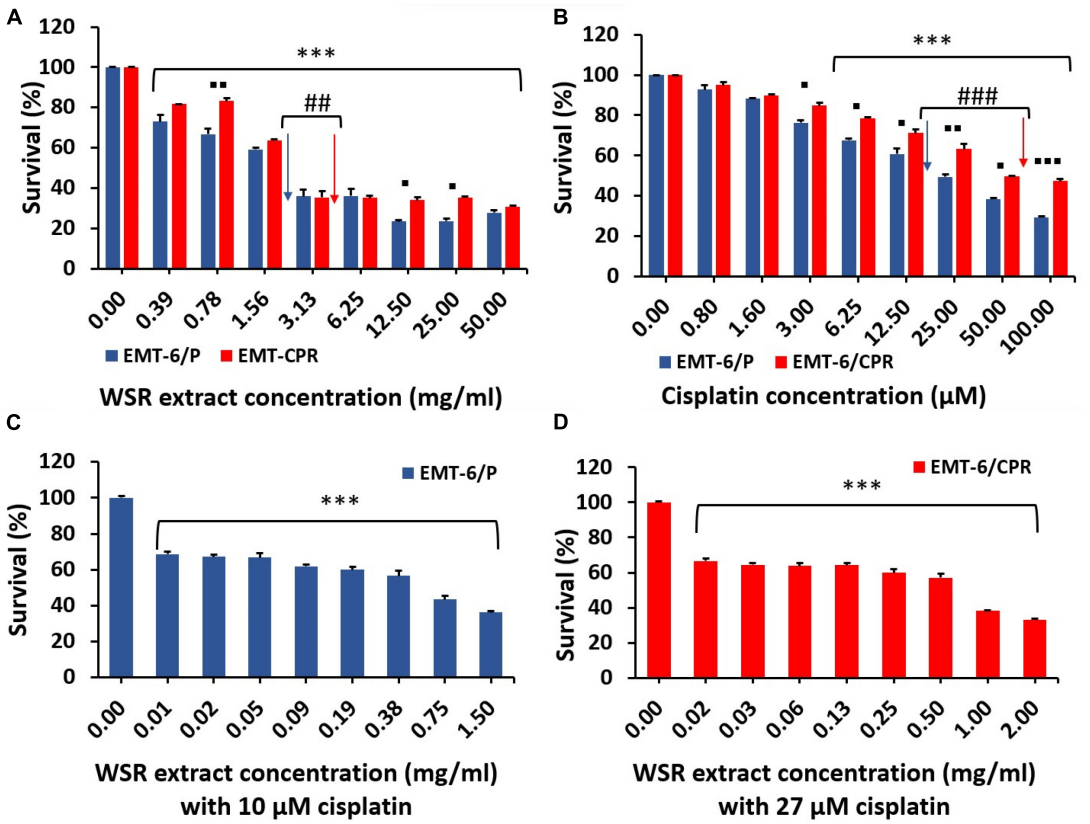

FIGURE 4 | Antiproliferative effect of WSR extract, cisplatin, and their combination against EMT6/P and EMT6/CPR cell lines. **(A)** Treatment of both cell lines with an increasing concentration of WSR extract. **(B)** Treatment of both cell lines with an increasing concentration of cisplatin. **(C)** Antiproliferative activity of various concentrations of WSR extract with 10 μM cisplatin against EMT6/P cell lines. **(D)** Antiproliferative activity of various concentrations of WSR extract with 27 μM cisplatin against EMT6/CPR cell lines. Data are presented as mean ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P = 0.001$, $^{***}P < 0.001$. $^{\#\#}P = 0.001$, $^{\#\#\#}P < 0.001$. ■$P < 0.05$, ■■$P = 0.001$, ■■■$P < 0.001$. (*: treatments groups compared with the control (0.00 μM), ■: EMT6/P survival compared with EMT6/CPR survival, #: survival of EMT6/P at IC_{50} compared with the survival of EMT6/CPR at IC_{50} concentration).

TABLE 2 | IC_{50} values for the extract and cisplatin in cisplatin-sensitive (EMT6/P) and cisplatin-resistant (EMT6/CPR) cell lines along with the combination index, related interpretation, and resistance fold.

Cell line	IC_{50} of WSR extract (mg/mL)	IC_{50} of cisplatin (μM)	WSR extract IC_{50} in combination (mg/mL)	Cisplatin IC_{50} in combination (μM)	CI	Interpretation
EMT6/P	2.9 ± 0.18	20 ± 0.5	0.54 ± 0.011	10	0.78	Moderate synergism
EMT6/CPR	3.8 ± 0.09	54 ± 0.08	0.66 ± 0.05	27	0.76	Moderate synergism
RF	1.31	2.7	1.22	2.7		

Data are presented as mean ± SEM (n = 3). CI, combination index; RF, resistance fold.

evaluate the comparative profiles between the treatment groups concerning their effect on the level of BHB. As expected, IF and its combination resulted in the highest values of BHB with a significant difference ($p < 0.05$) from the others (**Figure 8B**).

Safety Profile

Alanine aminotransferase and AST assays were performed as they are considered markers for liver toxicity. Serum levels of the liver enzymes were measured for all treated groups with WS extract, IF, cisplatin, their combinations, the negative (untreated) control, and tumor nonbearing mice, which did not bear any tumors as a reference for liver function. The current study found that the levels of serum ALT are within the normal range for all treated groups compared with the normal-untreated mice, however, cisplatin-treated group recorded significantly higher ALT value. In turn, the cisplatin group recorded 63.88 IU/L of ALT, which is 1.77 times higher than the normal group, although combination groups that included cisplatin revealed better results with lower values of ALT levels (45.33 and 25.83 IU/L for cisplatin with WSR

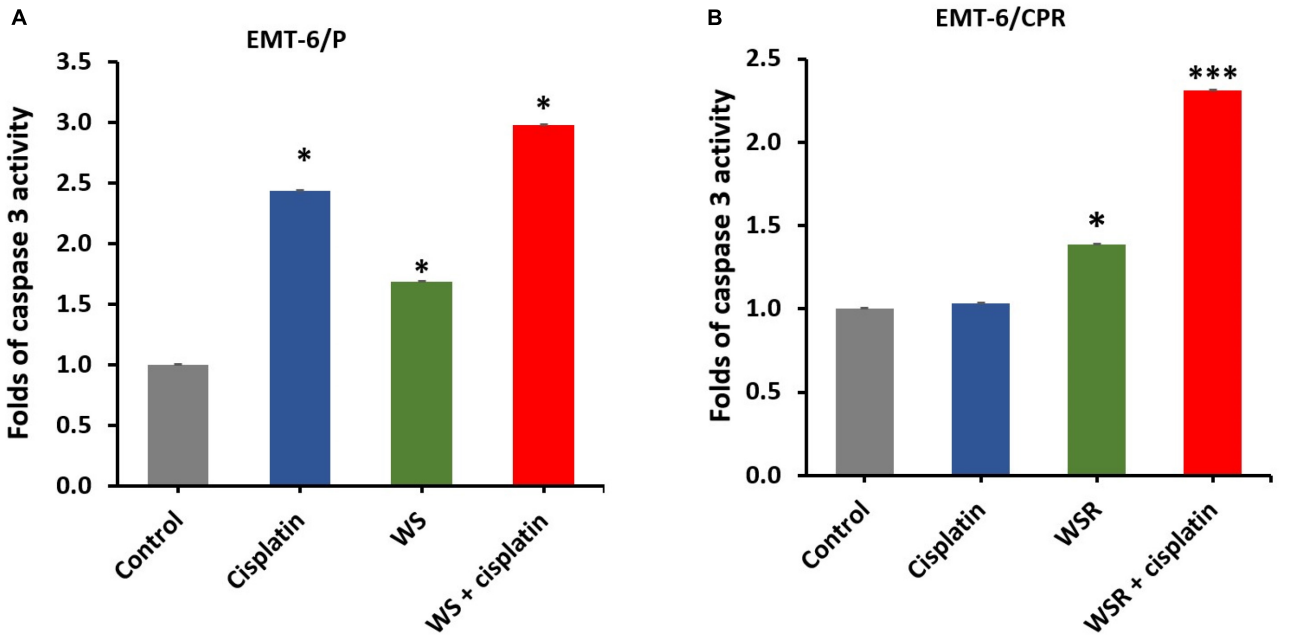

FIGURE 5 | Caspase-3 activity in breast cancer cells after treatment with WSR extract and cisplatin. **(A)** Treatment of EMT6/P cells with cisplatin (20 μM), WSR extract (2.9 mg/ml), and their combination (10 μM cisplatin with 0.54 mg/ml WSR extract). **(B)** Treatment of EMT6/C cells with cisplatin (54 μM), WSR extract (3.8 mg/ml), and their combination (27 μM cisplatin with 0.66 mg/ml WSR extract) in EMT6/C cell line. Results are expressed as means ± SEM (n = 3). *P < 0.05, ***P < 0.001. (*: treatments groups compared with the control group).

TABLE 3 | Results of WSR extract, intermittent fasting (IF), cisplatin and their combinations concerning tumor size changes, percentage of changes in tumor size, and average tumor weight in EMT6/P and EMT6/CPR cell line.

Treatment group	Av. initial tumor size (mm^3)	Av. final tumor size (mm^3)	(%) change in tumor size	(%) mice with no detectable tumor	Av. tumor weight (gm)
EMT6/P					
Control	358.05 ± 35.02	676.24 ± 170.41	88.87	0	0.54 ± 0.172
Cisplatin	291.80 ± 12.86	75.25 ± 4.77	–74.21	50	0.066 ± 0.004
WSR	367.13 ± 46.79	176.34 ± 33	–51.97	33.33	0.151 ± 0.021
IF	381.63 ± 24.84	180.33 ± 19.30	–52.75	33.33	0.127 ± 0.023
WSR + IF	330.25 ± 18.14	130.37 ± 12.87	–60.52	50	0.105 ± 0.007
WSR + cisplatin	389.23 ± 21.22	75.50 ± 7.72	–81.12	50	0.069 ± 0.001
WSR + IF + cisplatin	351.42 ± 37.10	0.0	–100	100	0.0
EMT6/CPR					
Control	215.38 ± 15.57	344.65 ± 64.71	60.02	33.33	0.275 ± 0.04
Cisplatin	200.54 ± 34.27	162.84 ± 17.19	–18.80	33.33	0.132 ± 0.026
WSR	248.48 ± 26.55	147.88 ± 4.96	–40.49	50	0.135 ± 0.008
IF	210.35 ± 23.37	132.06 ± 9.30	–37.22	33.33	0.085 ± 0.009
WSR + IF	246.99 ± 16.34	115.20 ± 3.30	–53.36	66.66	0.08 ± 0.001
WRS + cisplatin	247.23 ± 21.63	75.42 ± 3.21	–69.49	66.66	0.05 ± 0.001
WSR + cisplatin + IF	212.85 ± 22.57	0.0	–100	100	0.0

Av., average; mm‡, cubic millimeter; gm, gram. Data are expressed as means ± SEM (N = 6).

extract and for the triple combination with IF, respectively). Additionally, the effect of WSR extract, IF, and their combination on ALT levels were 36.66, 25.12, and 26.94 IU/L, respectively (**Figure 9A**). However, serum AST levels were normal for all treatment groups compared with the tumor nonbearing mice as they recorded an insignificant difference with $p > 0.05$. The cisplatin-treated group achieved a higher value than the tumor nonbearing mice (74.04 and 63.32 IU/L, respectively). However, this difference was insignificant ($p = 0.195$). Moreover, combination treatments, including cisplatin, registered lower

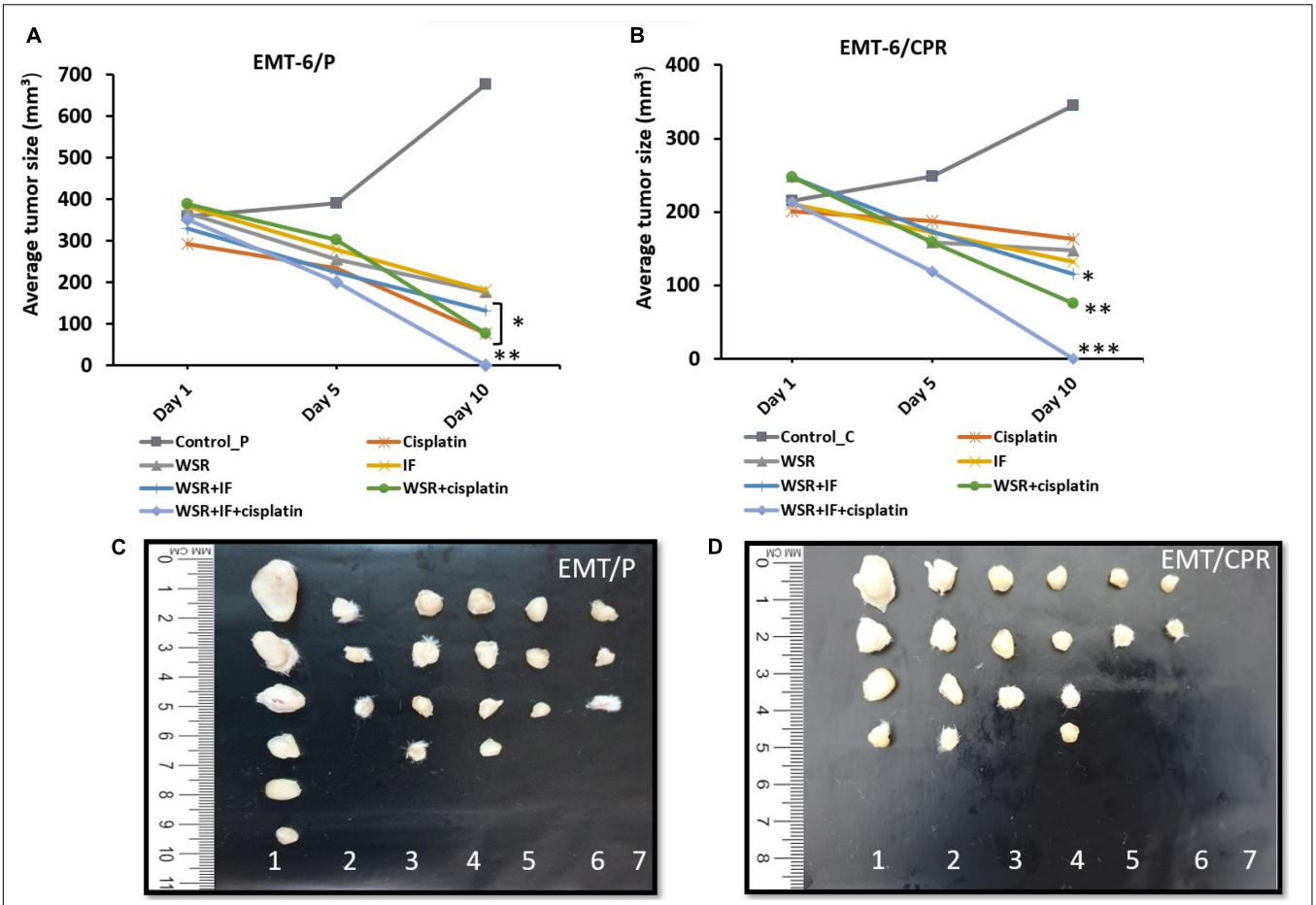

FIGURE 6 | Effect of treatments on tumor size in vivo. **(A)** A plot of change in average tumor size (mm‡) vs. time (days) of treatment in the EMT6/P cell line. **(B)** A plot of change in average tumor size (mm‡) vs. time (days) of treatment in EMT6/CPR cell line. **(C)** Tumor sizes in EMT6/P cells after dissection on day 10 in all groups compared to each other. **(D)** Tumor sizes of EMT6/CPR cells after dissection at day 10 in all groups compared to each other. (1 = negative control; 2 = cisplatin; 3 = WSR; 4 = IF; 5 = WSR + IF; 6 = WSR + cisplatin; 7 = WSR + IF + cisplatin). Results are expressed as means ± SEM (n = 6). *P < 0.05, **P = 0.001, ***P < 0.001. (*: treatment groups compared with the control group).

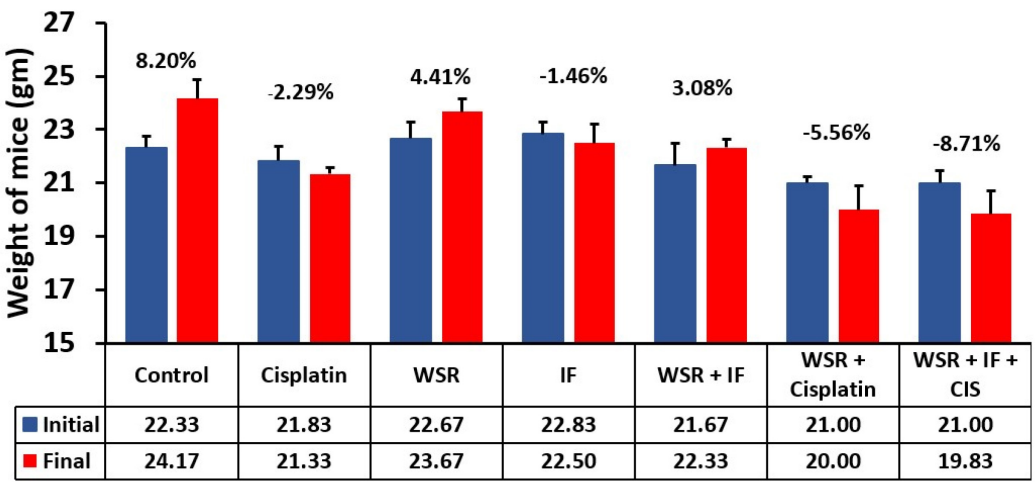

FIGURE 7 | Mice average weight (gm) on days 1 and 10 in all groups compared with each other. (%): percentage of change in body weight. Results are expressed as means ± SEM (n = 6).

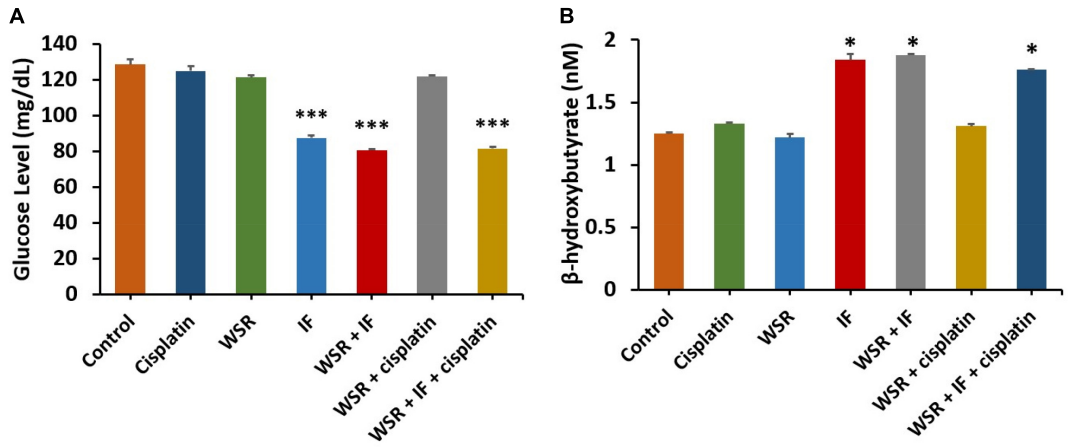

FIGURE 8 | Effect of treatments on glucose and β-hydroxybutyrate (BHB). **(A)** Serum level of glucose for different treatments. **(B)** Serum level of BHB for different treatments. Results are expressed as means ± SEM ($n = 3$). $^{*}P < 0.05$, $^{***}P < 0.001$. (*: treatment groups compared with the control group).

values of AST (57.77 and 50.83 IU/L for cisplatin with WSR extract and the triple combination with IF, respectively; **Figure 9B**).

In the case of serum creatinine, normal levels of creatinine were observed between tumor nonbearing mice and the other mice groups, which were treated with the above-mentioned treatments. However, the single treatment of cisplatin increased creatinine levels significantly (1.2 mg/dl with a p value of 0.001). Note that combined treatment of cisplatin with either WSR extract or WSR extract and IF showed lower creatinine levels than cisplatin alone, with values of 0.81 and 0.79 mg/dl, respectively (**Figure 9C**).

DISCUSSION

Diverse obstacles hinder the successful treatment of breast cancer due to toxicity to normal cells, the narrow therapeutic index of chemotherapy, and MDR. The latter is a major obstacle because MDR has accounted for the failure of treatments and subsequently death (26). The combination of chemotherapy with natural products is widely used these days to overcome MDR (27). As another option, IF can reduce tumor incidence, potentiate the effectiveness of chemotherapy, and improve the response to chemotherapy (28). In this research, water extract of WSR and IF was examined compared with cisplatin *in vitro* using EMT6/P and EMT6/CPR breast cancer cell lines and *in vivo* using female Balb/C mice inoculated with EMT6/P and EMT6/CPR breast cancer cell lines. *In vitro* and *in vivo* tests showed promising results. It is worth mentioning that the WSR extract, IF combination, and triple combination have not been tested before.

In vitro, our results based on the viability assay indicated that WSR extract prevented EMT6/P and EMT6/CPR cell line viability in a concentration-dependent pattern. The present results agree with previous studies. Prasad et al. (29) observed a dose-dependent anti-breast cancer activity for the crude water extract of WSR on MCF-7 cell lines (29). This antiproliferative effect of the WSR extract is related to its content of anticancer components (**Table 1**). Based on the previous finding, succinic acid (68.52%) revealed an apoptotic effect on acute lymphoblastic leukemia (T-ALL cell line) and increased caspase-3 activity in human leukemic lymphoblasts (CCRF-CEM cell line) *in vitro* (30). Additionally, anthranilic acid (16.87%) has been widely used with its derivatives to fight cancer and it has an antiproliferative effect (31). Furthermore, the phenolic compounds, gallic acid (7.52%) can reduce viability and promote apoptosis (32) by upregulation of Fas and FasL and induction of p53 and caspase-3 (33). The latter is considered a key enzyme in the execution of apoptosis (5). Considering that WSR extract caused a dramatic increase in caspase-3 levels in both EMT6/P and EMT6/CPR cell lines, and that potentiates the apoptotic effect and reduce viability (**Figures 5A,B**). These outcomes were matched with a previous study where the caspase-3 activity was enhanced upon the use of WSR extract on MDA-MB231 (34).

Cisplatin was used as a positive control, along with the WSR extract. WSR extract with cisplatin showed a moderate synergism in both cell lines and reduced the used dose of cisplatin influentially, in addition to their ability to reduce the resistance fold of cisplatin (**Table 2**). Thus, it was concluded that WSR extract alongside cisplatin resulted in greater synergism than any other combination tested alone. Consistent with previous studies, withaferin A synergized the effect of paclitaxel on both drug-sensitive and drug-resistant NSCLC cells *in vitro* (35). Also, the antiapoptotic effect of this combination enhanced the level of caspase-3 effectively compared with the control, WSR extract-treated cells, and cisplatin-treated cells in both cell lines. As noted, WSR extracts remarkably potentiated the cisplatin response in the resistant cell line (**Figures 5A,B**), which means that WSR extract sensitized the resistant cell line to cisplatin at lower doses. Previously, Cohen et al. (36) showed that withaferin A along with sorafenib raised caspase-3 levels efficiently in papillary and anaplastic cancers (36).

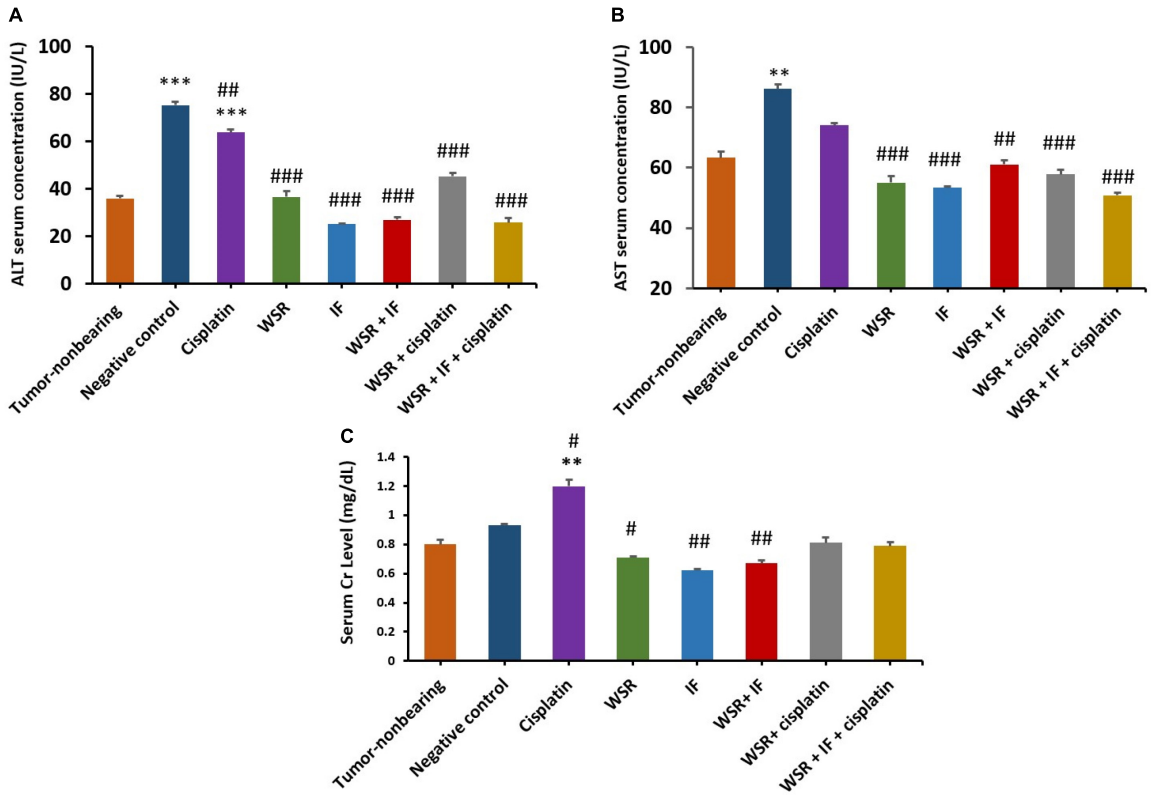

FIGURE 9 | Safety profile for the treatments [WSR extract (100 mg/kg/d), cisplatin (5 mg/kg/week), IF, their combinations]. **(A)** Serum alanine aminotransferase (ALT) level measured by (IU/L). **(B)** Serum aspartate aminotransferase (AST) level measured by (IU/L). **(C)** Serum creatinine level measured by (mg/dl). Results are expressed as means μ SEM ($n = 3$). $^{**}P = 0.001$, $^{***}P < 0.001$. $^{\#}P < 0.05$, $^{\#\#}P = 0.001$, $^{\#\#\#}P < 0.001$. (*: treatments group compared with the tumor-non-bearing group, $^{\#}$: treatments groups compared to the negative control).

Different treatments were tested *in vitro* in this research. In agreement with *in vitro* data, WSR extract reduced tumor size in EMT6/P and EMT6/CPR *in vivo* to 51.97 and 40.49%, respectively (**Table 3**). These findings follow previous studies where water extract of WS reduced tumor size in mice bearing cervical (HeLa) and colorectal (HT-29) cell-derived tumors (37). Another previous study observed that withaferin A, a component in WSR, showed tumor size reduction in mice injected with HeLa cells (38).

Another side of interest in this context is IF, which showed *in vivo* antitumor effect by reducing the tumor size in both EMT6/P and EMT6/CPR tumor-bearing mice by 52.75 and 37.22%, respectively (**Table 3**). It has been reported that IF reduced tumor size in a colon cancer xenograft mice model (CT26 cells) (39). Depending on that, the proposed antitumor effect of IF is due to reversing the Warburg effect and increasing BHB. Both have approved antitumor activity as mentioned before. Moreover, serum glucose level is an indicator for the Warburg effect (16), which was tested in this study. Based on the obtained results, cancer-induced mice that were treated with IF displayed a considerable serum glucose reduction compared with the control group (**Figure 8A**), which means that the Warburg effect was reversed and this was also proved before (40). The same groups had a higher level of BHB, which is considered an anticancer molecule (41) (**Figure 8B**).

Different combinations were explored *in vivo* in this research. First, combination treatment of WSR extract and cisplatin showed more size reduction of tumor (81.12 and 69.49% in mice bearing EMT6/P and EMT6/CPR cell lines, respectively; **Table 3**). These observations provided the basis that cisplatin and its combination with WSR extract resulted in greater efficacy and potency than the use of the drug alone in both cell lines. Interestingly, WSR extract sensitized the resistant cell line to cisplatin, remarkably.

Alternatively, a combination treatment of WSR extract and IF resulted in more tumor size reduction (60.52%) compared with a single treatment of WSR extract (51.97%) and IF (52.75%) in EMT6/P cells. Furthermore, combination treatment of WSR extract and IF resulted in more tumor size reduction (53.36%) compared with single treatment WSR extract (40.49%) and IF (37.22%) in EMT6/CPR cells. This combination has not been tested before; however, IF showed a synergistic effect with ascorbic acid vs. Kirsten rat sarcoma virus, a gene that makes a protein that is involved in cell signaling pathways that control cell growth, cell maturation, and cell death (KRAS) mutated cancers

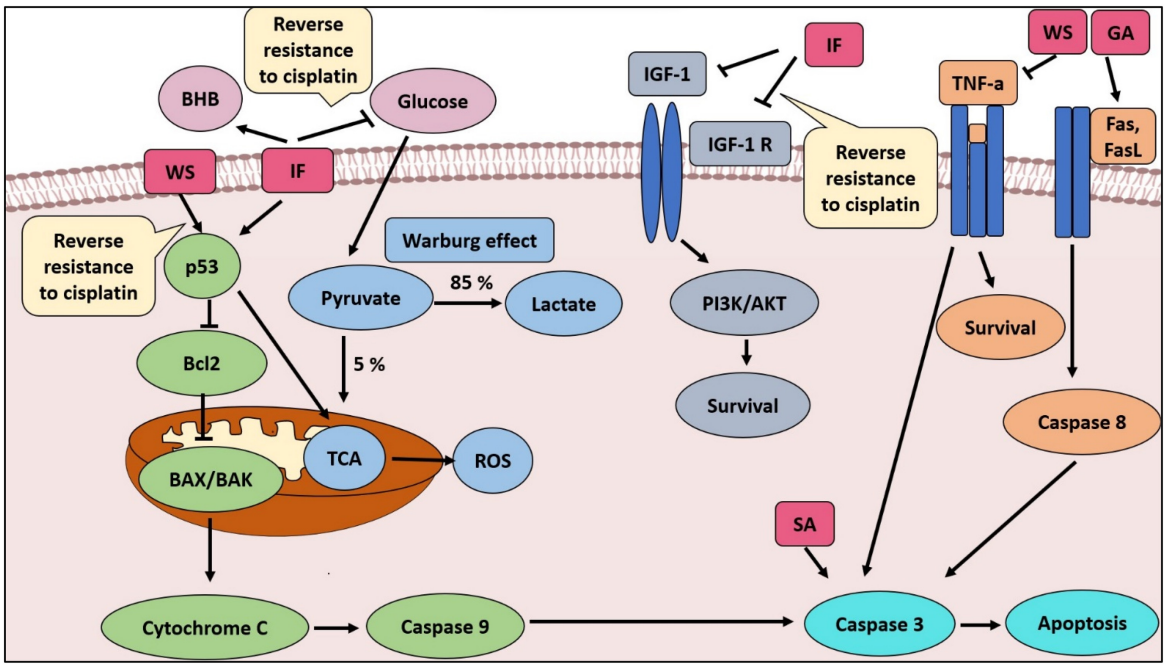

FIGURE 10 | The proposed mechanisms for WSR extract and IF combination and how they can evade cancer and sensitize resistant cells to cisplatin, when added, based on the results obtained from this study and former studies. SA, succinic acid; GA, gallic acid; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle.

(42). Others observed that the combination of WS and Maitake (*Grifola frondosa*) extract had a synergistic effect on immunity (43). Moreover, WSR extract induced oxidative stress, which could potentiate the effect of IF by reversing the Warburg effect (44). WSR extract countered the effect of IF on mice's weight significantly (**Figure 7**).

The mechanistic analysis provided in the literature demonstrates that WS can sensitize cancerous cells to chemotherapy through the apoptotic pathway, which is considered a predominant pathway for cisplatin-induced cell death. WS activates tumor suppressor p53, a fundamental element for inducting cisplatin-induced apoptosis and overcoming resistance (45). Primarily, A can block the autophagy flux in breast cancer cell lines MCF7 and MDA-MB-231, which is considered another method to reverse chemoresistance (46).

It was imperative to examine the last combination, which included IF, WSR extract, and cisplatin. Further reduction in tumor size was detected by adding IF to the extract and cisplatin leading to complete vanishment of the tumor in the sensitive and resistant cell lines. That supported the synergistic effect of IF in both cell lines. Moreover, IF succeeded to sensitize the resistant cell lines to cisplatin and that was confirmed by the complete reduction of the tumor. Interestingly, this combination has not been evaluated in the literature (**Figure 6**; **Table 3**). Nevertheless, IF can reverse MDR by several pathways as revealed in the literature. The first way is by increasing p53, which is substantial for sensitization (47). By the same token, IF suppresses IGF-1 and IGF-1 receptors and that increases sensitivity to chemotherapy (48). **Figure 10** illustrates the proposed mechanisms for WSR extract and IF combination and how they can evade cancer and can sensitize resistant cells to cisplatin, when added, based on the results obtained from this study and former studies.

The safety profile of anticancer agents is crucial to evaluating their toxicity. Liver enzymes (ALT and AST) and creatinine were used as indicators of liver and kidney functions, respectively. Results demonstrated that all treated groups had a normal level of ALT, except the cisplatin-treated group. As mentioned previously, cisplatin can induce hepatotoxicity. Combination treatments showed lower ALT levels than single treatment with cisplatin (**Figure 9A**). On top of that, AST levels were normal in the treated groups without exception. Nevertheless, combination treatments revealed lower AST levels than cisplatin alone (**Figure 9B**). In the literature, the extract of WSR showed hepatoprotective and antioxidant effects on radiation-induced hepatotoxicity (49). In contrast, creatine levels were normal in the treatment groups, excluding the cisplatin-treated group. The explanation for this finding is that cisplatin induces nephrotoxicity, which is one of the most serious obstacles that hinder cisplatin use (50). Despite that, combination treatment relieved the nephrotoxicity of cisplatin (**Figure 9C**). Formerly, water extract caused a remarkable elevation in the antioxidant activities of glutathione and superoxide dismutase to conserve renal tissue damage from gentamicin (51).

CONCLUSION

Based on the data presented here, we concluded that the combination of WSR extract and cisplatin has a synergistic anticancer effect on both the parent and the resistant cell lines *in vitro* and *in vivo,* better than cisplatin alone through apoptosis

induction and caspase-3 activation. On the other hand, the combination of IF with WSR extract has a superior ability to cause a reduction in tumor size. The activity of this combination was enhanced in the presence of cisplatin and caused complete tumor regression. These combinations are safer for the liver and kidney than the conventional therapy cisplatin. Such novel findings are worth the opportunity of expanding the range of research to establish better treatment for breast cancer in the future. Further studies are needed to evaluate the expression levels of antiapoptotic genes including Bcl2, BAX, and caspase 8 and to study morphological changes in cells after treatment.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by Research and Ethical Committee of Applied Science Private University.

AUTHOR CONTRIBUTIONS

SJ: experimental work, data collection, data analysis, and wrote the original draft. WT: conceptualization, the direction of the work, supervision, data analyses, wrote revision, and editing. Both authors contributed to the article and approved the submitted version.

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Chemoprevention effect of the Mediterranean diet on colorectal cancer: Current studies and future prospects

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Colorectal cancer (CRC) is the third most common cancer and the second most deadly cancer worldwide. Nevertheless, more than 70% of CRC cases are resulted from sporadic tumorigenesis and are not inherited. Since adenoma-carcinoma development is a slow process and may take up to 20 years, diet-based chemoprevention could be an effective approach in sporadic CRC. The Mediterranean diet is an example of a healthy diet pattern that consists of a combination of nutraceuticals that prevent several chronic diseases and cancer. Many epidemiological studies have shown the correlation between adherence to the Mediterranean diet and low incidence of CRC. The goal of this review is to shed the light on the anti-inflammatory and anti-colorectal cancer potentials of the natural bioactive compounds derived from the main foods in the Mediterranean diet.

KEYWORDS

Mediterranean diet, inflammation, colorectal cancer, chemoprevention, natural compounds

Introduction

The Mediterranean diet (MD) are one of the many studied and well-known dietary pattern worldwide, and it has been associated with a broad range of benefits for health as well. Besides, the MD appears as the best diet pattern to reflect many characteristics of an ideal healthy diet. The roots of the traditional MD pattern are seen in civilizations encircling the Mediterranean Sea; historically, some of the 22 countries bordering the Mediterranean Sea. So that this pattern has been closely associated with the social behaviors and lifestyles of that region (1). The traditional MD is arranged from a high intake of plant foods (fruits, vegetables, pieces of bread and other cereals, potatoes,

beans, nuts, and seeds); or fresh fruits as a typical dessert, and olive oil as the vital source of fat, reaching to a low intake of foods like red meat and sweets containing sugars or honey. The health benefits of individual foods and components of the MD (e.g., extra-virgin olive oil and nuts) have been well-documented (**Figure 1**) (2, 3). The benefits of the MD are not due to exclusively one component, but it is the whole food pattern as well as the wide range of traditional cuisine and lifestyle (4). Many factors are associated with the positive outcomes of the MD, such as traditional cooking methods, fasting practices, unique recipes, and using home garden vegetables (5). As well, the MD has been subject to many changes that shaped the current form today, those changes including culture, religion, agriculture production, climatic conditions, poverty, and economy (5, 6). According to the United Nations Educational, Scientific and Cultural Organization (UNESCO), the MD is recognized as "a set of traditional practices, knowledge, and skills passed on from generation to generation and providing a sense of belonging and continuity to the concerned communities" (7). The most consistent and robust evidence for the health benefits of MD has been observed in cardiovascular conditions, type 2 diabetes, metabolic syndrome, obesity, cancer, cognitive decline, and many others (8, 9). In observational studies, higher adherence to the MD was inversely associated with different types of cancer, including breast, colorectal, head, neck, respiratory tract, bladder, and liver (10).

Colorectal cancer (CRC) is the third most familiar malignancy and the second most deadly cancer. It has been reported about 1.9 million incidence cases and 0.9 million deaths worldwide in the year 2020 (11). There is evidence of higher CRC risk in Westernized society whose behaviors are characterized by a more elevated consumption of red and processed meat than in people living along the Mediterranean coast, who have a decreased overall cancer mortality correlated to their eating habits such as MD (12). Chronic intestinal inflammation, such as Crohn's disease and ulcerative colitis, is predisposed to CRC (13). In addition, the upregulation of proinflammatory factors such as cyclooxygenase-2 is observed in inflammatory bowel disease-related CRC. The capacity for the MD to prevent CRC is likely due in part to the total anti-inflammatory effects exerted by the diverse food components that contribute to this dietary pattern, specifically those foods and beverages contributing a significant load of phenolic compounds (i.e., olive and fish oil, and plant-based foods) (14).

This review aims to summarize the most recent clinical and preclinical studies of the main micronutrients included in the components of the MD and to verify the correlation between their anti-inflammatory, gut microbiome modulation, and chemopreventive effects in CRC.

Factors associated with the development of colorectal cancer

Impact of genetic abnormalities on colorectal cancer initiation and progression

Colorectal cancer can be promoted when intestinal epithelial cells are exposed to different genetic and epigenetic modifications that make them hyperproliferative (15). There are several distinct molecular pathways that modulate the progression of adenoma-carcinoma sequences, including chromosomal instability, microsatellite instability, and CpG island methylation (15–18). Hence, chromosomal abnormalities may associate with mutations that occur in particular oncogenes or tumor suppressor genes like *APC, KRAS, PIK3CA, BRAF, SMAD4,* or *TP53* (19). Besides, when mutations happened in DNA mismatch repair genes this is known as microsatellite instability which is found in 10–15% of sporadic CRCs (20). Moreover, DNA CpG methylation is involved in the early stage of CRC development (21) and associated with *BRAF* and *KRAS* mutations as well as *MLH1* methylation (22). CRC development begins from normal cells changed to hyperplastic polyp and then converted to sessile serrated adenomas ending up with cancer (**Figure 2**) (23). As a result, CRC is classified into five stages: stage 0 (benign polyp), stage I (tumor invades the muscularis propria), stage II (tumor invades tissue in the serosa), stage III (involved of visceral peritoneum), and stage IV (metastasis) (**Figure 2**) (24).

Colorectal cancer and inflammation

In humans, up to 20% of all cancers result from chronic inflammation and persistent infections (25). CRC can be categorized as either sporadic, with inflammation following cancer onset, or colitis-associated CRCs induced by chronic inflammation. Both inflammatory bowel diseases, ulcerative colitis, and Crohn's disease have a clear correlation with a significantly increased CRC risk, indicating chronic inflammation's role in carcinogenesis (26). CRC that comes from inflammatory bowel disease (IBD) is responsible for about 2% of CRC mortality yearly. Despite optimal medical treatment, the chronic inflammatory condition associated with IBD raises the chance for high-grade dysplasia and CRC, along with the effect of the genetic and environmental risk factors and the microbiome (27).

Several studies have demonstrated the strong correlation between chronic inflammation and tumorigenesis. Chronic inflammation could be prompted by: infections (viruses and bacteria), environmental factors (smoking and pollution), dietary factors, stress, and obesity (28). Besides, inflammation

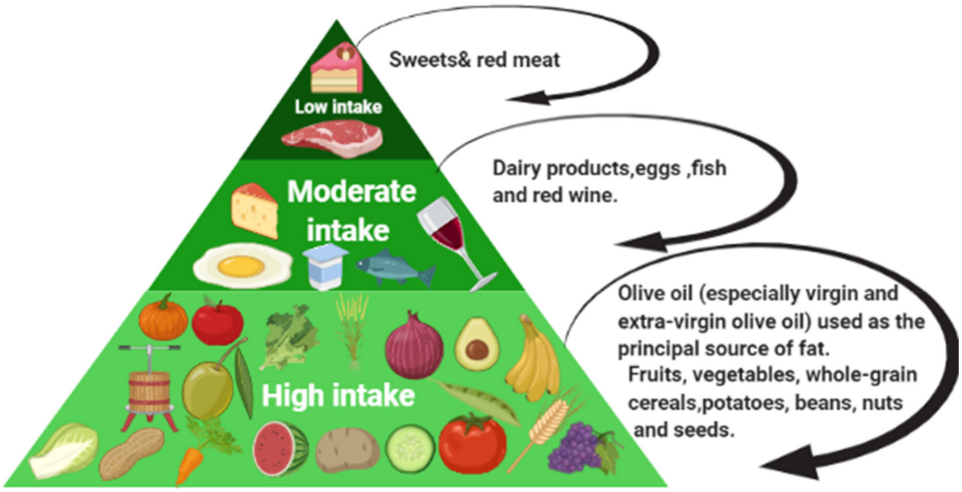

FIGURE 1
The Mediterranean diet intake level.

plays a role in tumor development, which mediates epigenetic alteration and modulation of oncogenes expression, DNA damage induced by oxidative stress and mutagens, as well as unrestricted tissue regeneration and proliferation (16, 29). In particular, chronic intestinal inflammation triggered different signaling pathways that augment tumor initiation and progression in CRC (**Figure 3**) (16). On the other hand, significant data have been associated between unbalanced gut microbiota and gastrointestinal tumorigenesis (30). The function of the intestinal epithelial barrier is affected by both chronic inflammation and microbial pathogens (31). They expanded gut permeability leading to ease in the translocation of microbial substances and stimulating an immune response (30, 31).

Colorectal cancer and microbiome

The gut microbiota is categorized into commensal and pathogenic bacteria. There are four distinct groups of bacteria found in the gut microbiota, including *Firmicutes, Bacteroidetes, Actinobacteria,* and *Proteobacteria* (32, 33). Healthy gut microbiota plays an essential role in keeping the immune system active and able to attack opportunistic bacteria through particular receptors (e.g., Toll-like receptors) or gut microbiota's metabolites (e.g., short-chain fatty acid) (34). Dysbiosis is the state of an imbalanced microbiome which can lead to a wide range of digestive problems such as irritable bowel syndrome (IBD) and CRC. An unhealthy gut microbiome can initiate inflammation and modify several signaling pathways resulting in the carcinogenesis of CRC (35). However, there are specific bacterial strains associated with the development of CRC, including *Fusobacterium nucleatum*, *Escherichia coli,* and *Bacteroides fragilis* (32). Tumorigenesis effects of dysbiosis can be summarized as the following: the genotoxicity (DNA damaging and mutations) effect of some bacteria and their metabolites, disrupting the gut surface permeability, which may promote inflammation, and modulation of the immune response (16, 32). Several studies have shown that modulation of the gut microbiome can improve the prognosis, treatment, and prevention of CRC (32).

Selected components of the Mediterranean diet and their effect on reducing colorectal cancer risk

Extra virgin olive oil: Phenolic compounds

Olive oil (OO) is an essential component of the MD with high nutritional values due to the presence of various bioactive compounds (36). Simple phenols, fatty acids, flavonoids, lignans, hydrocarbons, triterpenes, and phytosterols are the main chemical compositions of olive oil (37). Moreover, extraction methods play a critical role in determining the natural nutrients in (OO). Thus, applying cold extraction methods will produce extra virgin olive oil (EVOO), which is recognized with high phenolic content and low free fatty acids (38, 39). Extra virgin olive oil is known for its protective effect on CRC, conquers intestinal inflammation, and improves gut microbiota (40–42).

Hydroxytyrosol (3,4-DHPEA) and its secoiridoid derivatives are the main polyphenolic compounds in EVOO, and it originated from the hydrolysis of oleuropein during

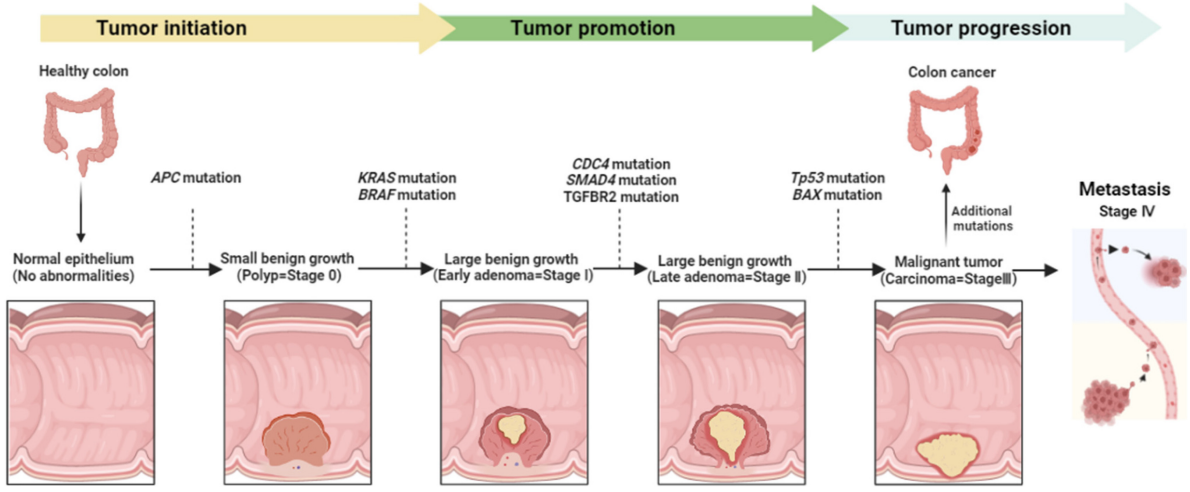

FIGURE 2
Colorectal cancer development, stages, and the main genetic modifications all along tumor progression.

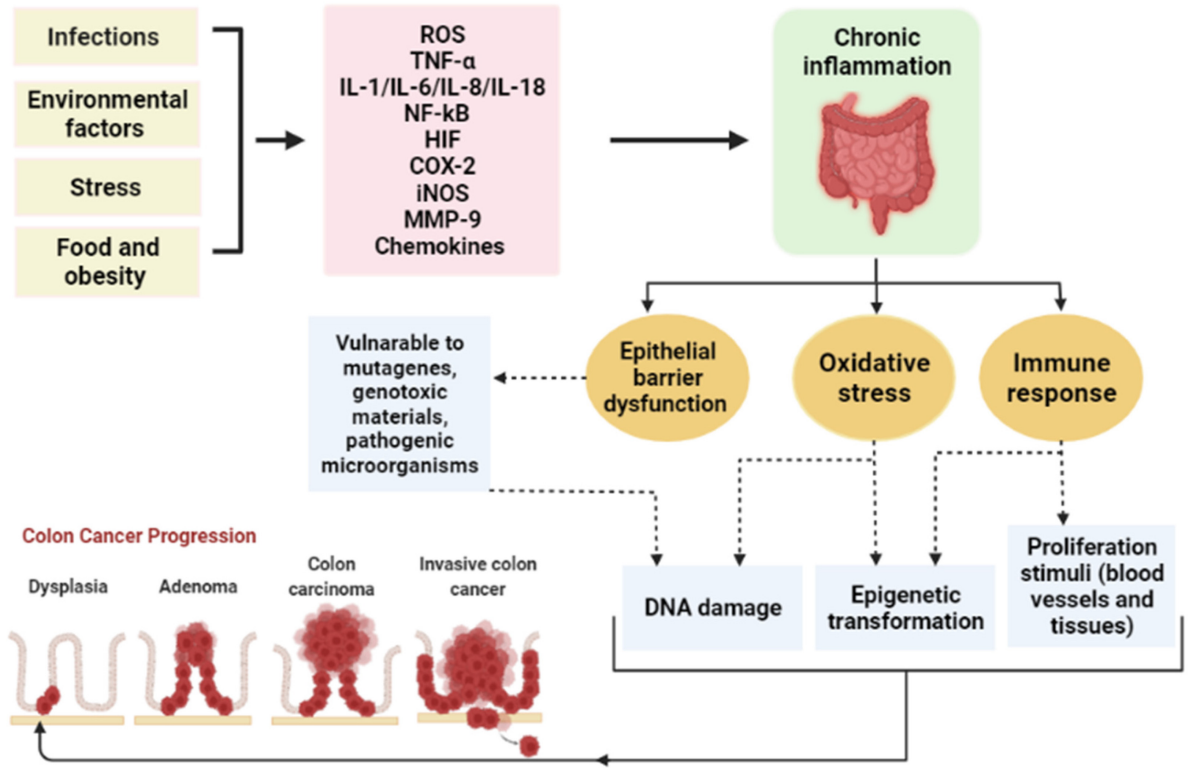

FIGURE 3
The correlation between chronic inflammation and the development of CRC. ROS, reactive oxygen species; TNF-α, tumor necrosis factor-alpha; IL, interleukin; NF-kB, nuclear factor kappa B; HIF, hypoxia-inducible factor; COX-2, cyclo-oxygenase-2; iNOS, inducible nitric oxide synthase; MMP-9, matrix metalloproteinases-9.

the ripening of olives (43). Hydroxytyrosol (HT) is known for its diverse pharmacological effects, including antitumor, anti-inflammatory, immunomodulatory, antimicrobial, and neuroprotective potential (44–48). Recently, Hydroxytyrosol has been tested in mice subjected to dextran sulfate sodium (DSS)-induced colitis (49). The study revealed a high potency

of (HT) in suppressing inflammation and alleviating colitis symptoms via downregulation of IL-6, IL-1β, TNF-α, and myeloperoxidase enzyme. Moreover, hydroxytyrosol was able to reduce NLRP3 inflammasome expression, and thus suppressed the expression of IL-18, IL-1β, and caspase-1 in DSS-induced ulcerative colitis (50). It also improved oxidative biomarkers and downregulated colon malondialdehyde, myeloperoxidase, and NO levels along with a significant reduction in mortality rate and disease activity index of albino rats with induced ulcerative colitis (51).

Besides the anti-inflammatory effects of (HT), it is also known to have anticancer properties and is involved in cancer hallmarks modification and tumor regression effect (52, 53). Hormozi et al. reported that (HT) induced apoptosis via upregulation of the caspase-3 gene and increased BAX/Bcl2 ratio in a human CRC cell line (LS180 cells) (54). It enhanced the expression of the antioxidant enzymes, which emphasized the antiproliferation effect (54, 55). As well, another anticancer mechanism of hydroxytyrosol was the inhibition of thioredoxin reductase 1(TrxR1) enzyme and promoted G1/S cell cycle arrest (48). It is noteworthy that a high level of (TrxR1) enzyme has been detected in CRC cells, which correlated with poor prognosis and chemotherapy response (56, 57). Moreover, hydroxytyrosol repressed the growth of human colorectal adenocarcinoma cells (HT-29) in both models *in vitro* and *in vivo* through downregulation of epidermal growth factor receptors (58). As well, it increased the expression of the CRC -associated-1 gene (COLACA1) in the same cell line leading to reduce tumorigenesis and an upraised survival rate (59).

Oleuropein is the ester form of hydroxytyrosol with β-glucosylated elenolic acid. It is found in olive leaves and EVOO with different content, and it gives olives a bitter and pungent taste (39, 60). Oleuropein has been involved in many pharmacological applications due to its properties, such as antioxidant, anti-inflammatory, and antineoplastic properties (61, 62). Oleuropein exhibited an anti-inflammatory effect via the suppression of inflammatory mediators, including NF-Kβ, COX-2, caspase-3, TNF-α, and Inos (61, 63, 64). Besides, it conquers inflammation by inhibiting of MAPK/NF-κB signaling pathway (65) and reduces the expression of IL-6, TNFR60, TNFR80, and ICAM-1 (66). In a recent study, Motawea et al. suggested that oleuropein was effective in reducing the following pro-inflammatory cytokines: IL-1β, TNF-α, IL-10, COX-2, iNOS, TGF-β1, MCP-1, and NF-κB in an induced colitis rat model (67). Besides, nanostructured lipid carrier-oleuropein was tested in the DSS-induced colitis experimental model and it exhibited a modulation of the inflammatory biomarkers via decreasing the level of TNF-α, IL-6, and hindering neutrophil infiltration (68). As well, oral intake of a diet supplemented with olive cream and probiotics revealed a synergistic anti-inflammatory effect in DSS-induced chronic colitis (69).

Previous studies also demonstrated the anticancer effect of oleuropein, including cell proliferation and migration inhibition, apoptosis induction, and growth signals modulation (39, 62, 70). Cárdeno et al. suggested that oleuropein significantly improved apoptotic mediators and decreased HIF-α expression in human CRC (HT-29 cells) (71). Besides, oleuropein repressed the activity of the main transcription proteins, including NF-κB, STAT3, PI3K/Akt, and β-catenin in AOM/DSS-induced CRC mice (72).

Tomato: Lycopene

Vegetables are consumed in the MD abundantly, and tomatoes are one of the universal MD components in the countries of the Mediterranean basin (73). Tomatoes are the edible fruits of the tomato plant (*Solanum lycopersicum*) that belongs to the *Solanaceae* family (74). The consumption of tomatoes has been related to a low incidence of chronic degenerative diseases, and various types of cancer (74, 75). These health benefits are expected to be associated with the presence of a wide range of phytochemicals, including carotenoids (lycopene and β-carotene), and polyphenolic compounds (flavonoids, flavanones, and flavones). Besides, high concentrations of other nutrients such as vitamin A, ascorbic acid, potassium, and folate have been reported in the chemical composition of tomatoes (73, 76). According to the chemical structure of the carotenoids, they are classified into carotenes (purely hydrocarbons such as lycopene and β-carotene) and xanthophylls (having oxygen in their structure such as lutein, zeaxanthin, and β-cryptoxanthin) (77, 78). Lycopene (LC) is a lipid-soluble pigment of natural carotenoids, which could be found in fresh tomatoes and processed tomato products (79, 80). Moreover, it is responsible for the red color of many fruits and vegetables like tomatoes, pink grapefruit, red guava, and watermelons (77). Many epidemiological studies have shown the biological activities of lycopene, including antioxidant, anti-inflammatory, cardioprotective, and anticancer effects (73, 81, 82).

From an anti-inflammatory perspective, lycopene was able to reduce the following inflammatory biomarkers: TNF-α, IL-1β, and IL-6 in the acetic acid-induced ulcerative colitis rat model (83). As well, it has significantly suppressed the level of NF-kB, TGF-β1, and caspase-3 along with upregulation of GSH expression and catalase activity in the same experimental model (84). A recent study has shown the effect of lycopene on colitis progression by lowering the disease activity index score, improving the colon length, and rising the expression of catalase, GSH-Px, and SOD (85).

On the other hand, several studies have described the antiproliferation activity of lycopene in CRC cells. Lin et al. have shown the chemo-preventive effect of lycopene in human colon cancer cells (HT-29). It downregulated the MMP-7 expression and hindered tumor development and tumor cell invasion (86). In a mouse xenograft model, LC decreased the expression

of PCNA and β-catenin proteins, which are associated with tumor growth and progression. As well, lycopene attenuated the level of MMP-9 in tumor-bearing mice (87). MMPs are known to enhance the tumor microenvironment and promote cell invasion resulting in poor prognosis and low survival rate in CRC patients (88).

Herbs and spices

Medicinal plants have remained the primary source of medications; many of the pharmaceuticals that are now accessible were derived from them, either directly or indirectly. Many plants have been shown to have vital roles in the treatment and prevention of various illnesses in various regions of the globe (89, 90). The bioactive phytochemical elements of many plants have traditionally been employed in Asian medicine (91, 92). Health care services are provided by the traditional healer plants, which are founded on religious and cultural backgrounds, knowledge, attitudes, and beliefs (90, 91, 93). Recent years have seen a surge in interest in assessing plant foods and discovering phytochemicals with the potential to inhibit carcinogenesis.

Onion: Quercetin

Onions (*Allium cepa*) are members of the Liliaceae (94). The Liliaceae family has around 250 genera and 3700 species (95–97). *Allium cepa* is one of the world's oldest and most frequently grown vegetables, growing in practically every climate zone, from tropical to cold temperate (98). Although *A. cepa* is referred to as the "Queen of the Kitchen," it is distinguishable by the color of its outer scales (yellow, red, or white), its taste (sweet or bitter), and whether it is consumed fresh or powdered (99, 100).

Onion is roughly 90% water, with a significant concentration of nutritional fiber and carbohydrates. In terms of vitamins and minerals, onion has a low salt level while being rich in vitamin B6, vitamin C, folic acid, and minerals (Ca, Fe, S, Se, Mg, Ph, and k) (101–103). On the other hand, has a low lipid content and a pool of free amino acids (104, 105). Onions are high in a range of phytochemicals with beneficial properties, including organosulfur compounds, phenolic compounds, polysaccharides, and saponins (106–113). Sulfur compounds such as DATS, diallyl disulfide (DADS), ajoene, and sallylmercaptocysteine (SAMC), onionin A (114). Two flavonoid subgroups are abundant in onion: anthocyanins, which give certain kinds of their Reddish-purple color, and the primary pigments are flavonols, which include quercetin, which is found conjugated as quercetin 4′–*O*-glycopyranoside, quercetin 3,4′–O-diglycopyranoside, and quercetin 3,7,4′–O-triglycopyranoside (115–117). Along with quercetin, additional flavonols found in onions include kaempferol, luteolin, and isorhamnetin (118, 119).

Onion bulbs are used not only for their taste and aroma but also for the nutritional value they provide to the human diet. Several studies have demonstrated that onion and its bioactive components have a variety of pharmacological effects (120), including anti-inflammatory (121), anti-obesity (121), anti-spasmodic agent (100, 122), anticancer (123–125), and wound healer (126). Additionally, it has long been recognized as a helpful therapy for a variety of medical conditions including diabetes (95, 127), cardiovascular disease (128), hypertension (106), anxiety (129), and asthma (130). Furthermore, *A. cepa* demonstrated that it suppressed gram-positive bacteria more efficiently than gram-negative bacteria (131) and that it reduces DNA damage and breaking owing to the presence of quercetin, a potent antioxidant (109, 132).

The onion bulb extract has been shown to both prevent and reverse colitis by modulating several pro-inflammatory signaling pathways, including the mechanistic target of rapamycin (mTOR), the mitogen-activated protein kinase family (MAPK), cyclooxygenase-2 (COX-2), and tissue inhibitors of metalloproteinases (TIMP), as well as several molecules involved in the apoptotic pathway (121, 133, 134). They also found several phytochemicals such as saponins, tannins, and anthocyanin can help fight inflammation (104, 105). In the MC3T3-E1 preosteoblastic cell line, quercetin in a dose-dependent manner strongly inhibited the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-jB) (135). Furthermore, in animal and human investigations, quercetin lowered TNFa/IL-10 and IL-8/IL-10 ratios (136–138). Umoh et al. showed that red onion may reduce inflammation by inhibiting NF-jB, MARK, and STAT-1, perhaps via the action of its active component quercetin (139). Besides, quercetin was able to reduce inflammation in DDS-induced colitis mice via upregulation of GSH levels (140).

In recent years, scientists have concentrated their efforts *in vitro*, *in vivo,* and human investigations on the prevention of cancer through diets with a high percentage of onion. Inhibiting cell cycle, triggering apoptosis, inhibiting tyrosine kinase, regulating p53 protein, and inhibiting antioxidant activity that interferes with several phases of cancer cell creation, development, differentiation, and metastasis (117, 139, 141–145). Onion extracts or their key bioactive constituents have shown strong anticancer activity against prostate, stomach, breast, lung, colorectal, laryngeal, and esophageal cancers, pancreatic, adenocarcinoma, and glioblastoma (146, 147). Quercetin, a novel onion component, might cause G (2) phase arrest, reduce colon cancer cell growth, and trigger autophagic cell death (148). In particular, it has been detected the endocannabinoid receptor (CB1-R) expression, PI3K/Akt/mTOR pathways, and the pro-apoptotic JNK/JUN pathways in Caco2 and DLD-1 cells. It was found a considerable increase in the expression of the endocannabinoid receptor (CB1-R), as well as suppression of important survival signals including PI3K/Akt/mTOR (145). The administration

of quercetin was obsessively monitored for 48 h. In both the Colo-320 and Colo-741 cell lines, there was an increase in BAX immunoreactivity after quercetin treatment, but only in the Colo-320 main cell line was there existing a substantial reduction in Bcl-2 immunoreactivity (149). By a remarkable mechanism, quercetin (5 M) was able to significantly reduce the migratory and invasive potential of Caco-2 cells, resulting in decreased MMP-2 and MMP-9 expressions, whereas *E*-cadherin was downregulated. furthermore, quercetin has been shown to inhibit the production of inflammatory mediators such as TNF-α, COX-2, and IL-6 (150). Male Wistar rats were given (200 mg/kg, 28 days) or (0.5 g, 27 weeks) of onion-rich of quercetin had a big impact on ACF formation, mucin depletion, mitosis, and increasing the apoptosis percent in the treatment group. Although significant influence on cell proliferation and the expressions of p53 and BAX (151). (152) demonstrated the quercetin-loaded MPEG–PCL nanomicelles (Qu-M) dispersed entirely in water and released quercetin for a long time *in vitro* and *in vivo*. *In vitro*, Qu-M enhanced apoptosis induction and inhibited cell proliferation in CT26 cells. Furthermore, the mice (BALB/c) subcutaneous CT26 colon cancer model was constructed to assess the therapeutic effectiveness of Qu-M in greater detail. Qu-M investigates a high impact on cell death, preventing tumor angiogenesis, and limiting cell proliferation (152). Supplying quercetin (30 mg/kg, 4 weeks) to AOM/DSS-induced colon cancer mice (Wild-type C57BL/6J mice) decreased the number and size of tumors by a significant margin including, reduce the inflammation produced by AOM/DSS, recovered leukocyte numbers, also reduces oxidative stress indicators such as lipid peroxide (LPO), nitric oxide (NO), superoxide dismutase (SOD), glucose-6-phosphate (G6PD), and glutathione (GSH) (153).

Garlic: Allicin

Garlic or *Allium sativum* L. is a bulbous plant of the Alliaceae family that grows in the Mediterranean region (154, 155). In addition to its medicinal properties, garlic is also widely used as a food and spice (156, 157). Garlic is distinguishable from other members of the allium family by its clove-shaped bulbs and flat leaves (158, 159). Garlic includes at least 33 sulfur compounds, various enzymes such as peroxidase, allinase, and myrosinase, 17 amino acids, and minerals such as selenium, calcium, copper, iron, potassium, magnesium, and zinc, as well as vitamins A, B1, and C, fiber, and water (156, 160–163). It has the highest concentration of sulfur compounds of all Allium species (158, 163). Sulfur compounds are responsible for garlic's strong odor as well as many of its therapeutic properties (163). Saponins (proto-eruboside B, eruboside B, sativoside), lectins, and flavonoids are some of the other elements found in this plant (159, 160). A compound called allicin is one of the most important biologically active compounds. Garlic does not have allicin until it is crushed or cut. The enzyme allinase, which breaks down alliin into allicin, is activated when the garlic bulb is damaged. Once it's made, it quickly breaks down, but the speed of this reaction changes depending on the temperature. Allicin can still be found in garlic that has been refrigerated for a few days. At room temperature, it breaks down into smelly sulfur compounds like diallyl di- and tri-sulfides, ajoene, and vinyldithiins in just a few hours (164–168).

Garlic was highly prized in ancient Egyptian, Greek, and Chinese cultures as a food and medicinal (169, 170). It has been studied clinically for a variety of illnesses, including hypertension, hypercholesterolemia, diabetes, common cold, and cancer prevention (171–178). This plant has antibacterial, antifungal, antioxidant, immune system stimulant, and anti-parasitic properties (159, 162, 176). Garlic's medicinal potential has also been investigated in a variety of inflammatory illnesses, including allergic rhinitis, allergic asthma, IBD, rheumatoid arthritis, and atherosclerosis (158, 177). Breath odor is a common adverse effect of using garlic, both orally and intravenously (162, 179).

A person's risk of developing malignant tumors may be increased or decreased depending on the amount of some foods consumed or the number of others omitted from their diet. On this premise, dietary treatments are regarded to have the capacity to prevent or modify malignancies. One of these natural compounds, garlic (*Allium sativum*), has been studied for medicinal purposes. Allicin, a compound found in garlic, has been shown to inhibit CRC metastasis by strengthening the immune system and limiting the growth of tumor arteries (180).

Using allicin, an active ingredient derived from the popular seasoning agent or condiment *Allium sativum* L., was able to reduce the secretion of pro-inflammatory factors such as interleukin-6 (IL-6), prostaglandins (PG), nitric oxide (NO), interleukin-1 (IL-1), interleukin-6 (IL-6), and TNF-a, while simultaneously increasing anti-inflammatory cytokines such as IL-10 (181–184). Rats with Acrylamide (AA)-induced intestinal damage were used to examine the possible therapeutic benefits of allicin food supplementation. Allicin significantly reduced the expression of Toll-like receptor 4 (TLR4), NF-kB signaling pathway proteins, and proinflammatory cytokines in AA-treated rats by boosting the synthesis of SCFAs (185). In Caco-2 cells, allicin (25 mg/ml) can significantly inhibit p-38 and the JNK pathway activation. Allicin also suppressed the production of TNF-α and IL-6 generated by IL-1 at the mRNA and protein level in a dosage-dependent manner (186). According to Li et al. (186), an oral dose of allicin enhanced the colonic histopathology score and investigated the synergistic effects of allicin (30 mg/kg)-mesalazine (30 mg/kg) and allicin (30 mg/kg)-sulfasalazine (100 mg/kg) on TNBS (50 mg/kg) induced Wistar rats. Therapy with allicin-mesalazine decreased the colonic histopathology score from 5.83 to 2.10, whereas treatment with allicin sulfasalazine decreased it to 3.38. TNF-a levels were lowered by

allicin-mesalazine therapy to 2.65 (pg/ml) from 6 (pg/ml), while allicin or mesalazine treatment alone reduced them to roughly 3.8 (pg/ml). TNBS therapy decreased IL-4 concentration to less than 4 (pg/ml), while mesalazine-allicin treatment increased their concentration to 5.76 (pg/ml), but neither allicin nor mesalazine alone could boost their expression to synergistic levels.

It was investigated the impact of allicin on cell proliferation in colon cancer cell lines HCT-116, LS174T, HT-29, and Caco-2 *in vitro*, as well as the underlying processes. Allicin has been demonstrated to have chemo-preventive effects on critical cellular processes such as mitochondrial membrane potential maintenance, intracellular redox control, and cell division. Allicin triggers G2/M arrest alters intracellular glutathione (GSH) levels (187) and causes a transitory decrease in intracellular GSH levels (187). Allicin treatment caused apoptotic cell death in HCT-116, as shown by increased hypodiploid DNA content, reduced levels of B-cell non-Hodgkin lymphoma-2 (Bcl-2), increased levels of BAX, and increased capacity to release cytochrome c from mitochondria to the cytosol. Allicin also caused NF-E2-related factor-2 (Nrf2) to be translocated to the nucleus of HCT-116 cells. Although Allicin's cytotoxic effects were considerable when evaluated in four distinct human colon cancer cell lines (188). Experiments on animal models of carcinogenesis showed that components of garlic (e.g., allyl sulfides) suppress both the start and promotion phases of tumorigenesis in a wide range of malignancies including colorectal, lung, and skin (189). Perez-Ortiz et al. (190) Test the efficacy of a thiosulfinate-enriched garlic extract in combination with 5-fluorouracil (5-FU) or oxaliplatin chemotherapy in colon cancer cells as a new chemotherapy regimen that may also lower the cost of clinical treatment. The cytotoxic effects of an *Allium sativum* extract enriched in thiosulfate were investigated in two distinct human colon cancer cell lines, Caco-2 and HT-29, respectively. The doses of allicin (43–60 g/mL) were discovered to substantially decrease colon cancer cell growth and induce apoptosis. The impact of Allicin on the azoxymethane/dextran sodium sulfate (AOM/DSS) CRC mice model on STAT3 is being studied. Through various ligand-mediated phosphorylation, STAT3 plays key roles in cytokine signaling pathways, as well as cell proliferation and death. STAT3 activation causes the transcription of target genes such as Bcl-2, Bcl-xL, Mcl-1, and p21, all of which are important in cell survival and proliferation (180, 191). STAT3 activation may increase cell proliferation, angiogenesis, and inhibit apoptosis in human cancer cells. According to the western blot results, Allicin reduced the levels of phosphorylated STAT3. Allicin also inhibited the expression of Mcl-1, Bcl-2, and Bcl-xL. Therefore, allicin may be able to prevent colonic carcinogenesis in AOM/DSS mice *in vivo* (180). As an example, Diallyl trisulfide (DATS), an organosulfur compound isolated from garlic, has shown anticancer activity both *in vitro* and *in vivo* by reducing tumor mitosis and enhancing histone acetylation of H3 and H4 in both tumors and healthy cells (168).

Oregano: Carvacrol

Oregano is one of the most highly prized spices in the world, both commercially and culinary (192). oregano is formed from the terms "Oros" and "Ganos," which both refer to the beauty of the mountains in ancient Greek (193). Oregano is the common name for at least 61 different species spread over 17 different genera and six different families (194). The family Lamiaceae contains the genus *Origanum*, which is the primary source of well-known oregano spice (194). All of the other plant families (Rubiaceae, Scrophulariaceae, Apiaceae, and Asteraceae) play a minor role (194–196). Monoterpenes and sesquiterpenes make up the majority of the essential oils in the Lamiaceae family. Their action is linked to the presence of carvacrol and thymol, which are combined with the primary elements of oregano, *p*-cymene, and terpinenes (196–198). Also, Oregano has a high nutritional value since it includes considerable quantities of vitamins and minerals while having a low salt level (195, 199, 200). Numerous studies have demonstrated the beneficial effects of oregano on human health, including its use in the treatment of a wide range of ailments, including respiratory tract disorders, gastrointestinal disorders (anti-stomachic and tonic agent), as an oral antiseptic, analgesic, urinary tract disorders (as a diuretic and antiseptic agent), anti-inflammatory, and even anticarcinogenic properties (201–205).

Oregano plant species have been extensively utilized in traditional medicine to treat inflammation-related disorders via a variety of mechanisms, including Reduced synthesis of proinflammatory cytokines such as TNF-α, IL-1, and IL-6, and increased production of anti-inflammatory cytokines such as IL-10 (206), as well as inhibition of aldose reductase and lipoxygenase (206, 207). Oregano, which is high in essential oils, has been shown to inhibit the COX-2 enzyme, which is linked to tissue inflammation (208). Carvacrol may also play a role in reducing the side effects of chemotherapy, in addition to its anticancer properties. The anticancer medication irinotecan hydrochloride causes a condition in which inflammation and cell damage are triggered by the transient receptor potential cation channel subfamily A, member 1 receptor. Carvacrol is an agonist of this receptor leading to an effective reduction of inflammation biomarkers, such as nuclear factor b and cyclooxygenase 2, as well as oxidative stress, as measured by glutathione, malondialdehyde, and NOx levels in a mouse model of inflammatory arthritis (209).

There are a lot of different Flavonoids and phenolic compounds in species of oregano, and some of them have been shown to fight colon cancer (210). As a result, research has looked into whether oregano flavonoids and phenolic compounds could be used as anti-colon cancer.

The "shutting down" of many cancer survival pathways, including the ERK/MAPK and PI3K/Akt pathways, may be

responsible for the overall suppression of colon cancer cell growth following treatment with *Origanum syriacum* ethanol extract (211). The whole extract of *Origanum vulgare* is responsible for the apoptosis-inducing action (212). Carvacrol significantly slowed the growth, migration, and invasion of colon cancer cells by stopping cells at the G2/M phase and causing them to die, reduced Bcl-2 expression, phosphorylated extracellular regulated protein kinase (p-ERK) and p-Akt, and increased BAX and c-Jun N-terminal kinase (p-JNK) expression (LoVo and SW620 metastatic cells line) (213). Showcase of Mexican oregano in colon cancer cells, there was an increase in the expression of BAX (apoptotic protein) and a decrease in the expression of Bcl-2, PARP, and Survivin (anti-apoptotic proteins), as well as an increase in the expression of caspase-3 in various oregano cultured plants (wild type, *in vitro* and *ex vitro* plant tissue culture) (202). Thymol, another phenolic component typically found in *O. syriacum*, was discovered to suppress the growth of bladder and colon cancer cells (HCT116, LoVo, and Caco2 cells line) *in vitro*, which was promising (214, 215). The key mechanistic activity of thymol's action was identified to be the inhibition of JNK and p38 as the main mediators (214, 215). In another investigation, oregano aqueous infusion had the strongest radical scavenging efficacy in HT29 cells. Oregano's strong antioxidant activity has been linked to a variety of substances including carvacrol and other phenolic compounds (202).

Saffron: Crocin

Saffron, the world's most expensive botanical spice, commonly known as "red gold," is made from the dried stigmas of *Crocus sativus* L., a member of the broad Iridaceae family (216, 217). It is an autumn-flowering, high-value, low-volume spice crop plant that originated in the Middle East and is now farmed in China, India, Iran, Azerbaijan, Turkey, Egypt, Morocco, Greece, Spain, Italy, France, and Mexico (218–221). In addition to the elements that already contained in saffron, such as protein, fiber, lipids, vital minerals, and vitamins B1 and B2 (222–224). There are various key metabolite components, including carotenoids (crocetin, crocin, zeaxanthin), monoterpene aldehydes (picrocrocin and safranal), monoterpenoids, and phenolic compounds (anthocyanins and flavonoids) that contribute to the diverse pharmacological effects of this substance (216, 225–227). Three major bioactive chemicals (crocin, picrocrocin, and safranal) are found in significant amounts among these metabolites, and they are responsible for the Safran's distinctive red color (crocin), bitter taste (picrocrocin), odor, and aroma (safranal), and other characteristics (146, 228, 229).

Saffron consumption correlates with a lower risk of many types of cancer (227, 230–232), improvement of depression and memory loss (226, 227), regulation of menstruation (233), accelerated wound healing in burn injuries, and relief of cough and asthmatic breathing (229, 230). It has also been shown to be an antihypertensive (225, 234), antianxiety (233), insulin resistance lowering agent (233, 235), cardioprotective (236), and gastroprotective properties (233).

Various research has shown that the anti-inflammatory and antioxidant effects of saffron constituents are due to their significant inhibitory effects against cyclooxygenase 1 and 2 enzymes and prostaglandin E2 production (237), attenuating endoplasmic reticulum stress signaling, blocking pro-inflammatory cytokine production such as TNF-α, inhibiting transcription factors such as nuclear factor kappa B (NF-kB), which exacerbates chronic inflammation, and suppressing inflammatory gene expression (223, 238). Additionally, the anti-inflammatory properties of saffron derivatives affect neuroinflammation (237). Following the study (239), the researcher concluded that crocin protects rat gastric mucosa ethanol-induced injury through the expression of anti-inflammatory, antioxidant, antiapoptotic, and mucin secretagogue mechanisms, which are most likely mediated through increased PGE2 release. After only 4 weeks, Kawabata et al. found that crocin feeding could prevent Dextran Sulfate Sodium (DSS)-induced colitis and decrease TNF-α expression, IL-1β, IL-6, IFN-γ, NF-kB, COX-2, and iNOS in the colorectal mucosa and increased nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression (240). Concerning the effectiveness of saffron in the treatment of ulcerative colitis (US), studies have shown that oral administration of crocetin to mice (25–100 mg/kg per day) for 8 days significantly ameliorated TNBS-induced UC, as evidenced by a reduction in NO, neutrophil infiltration, and lipid peroxidation in the inflamed colon, favorable expression of TH1 and TH2 cytokines, and down-regulation of the NF-kB (241).

According to the literature, saffron and its components have chemopreventive activity via the inhibitory effect of saffron on cellular DNA and RNA synthesis, modulation of lipid peroxidation, antioxidants, immune modulation, enhancement of cell differentiation, inhibition of cell proliferation, modulation of carcinogen metabolism, cell cycle arrest through p53 dependent and independent mechanisms causing apoptosis, the interaction of carotenoids with topoisomerase II (227, 242–247). While the majority of *in vivo* and *in vitro* studies focused on isolated bioactive compounds from saffron, Aung et al. (248) revealed that *C. sativus* and its primary ingredient, crocin, effectively reduced the proliferation of CRC cell lines (HCT-116, HT-29, SW-480) and non-small cell lung cancer cell lines (NSCLC) by MTS test while not affect normal cells. Aung et al. (248) demonstrated that saffron crudes and its main compound crocin can be used to supplement current CRC treatments by limiting cancer cell proliferation and motility progression by targeting the Metastasis-Associated in Colon Cancer 1 (MACC1) as a major causal metastasis-inducing gene. Crocetin (0.8 mmol/L) significantly triggered cell cycle arrest and P21 induction and caused cytotoxicity in SW480 cells by increasing apoptosis and lowering DNA repair capability in a

time-dependent manner (249). Another research revealed that long-term intraperitoneal injection of crocin (400 mg/kg body weight) improves survival and inhibits tumor development in female rats with colon cancer generated by subcutaneous injection of rat adenocarcinoma DHD/K12 PROb cells (227). Crocin exhibited antiproliferation activity against the HCT116 cell line via induction of apoptosis and attenuation of the ratio of p-STAT3/STAT3 (250). As well, it suppressed tumor growth in colitis-associated CRC mouse model via modulation of the Wnt/PI3K pathway. Crocin was also able to lower disease-activity index and mucosal ulcer inflammation by regulating antioxidant markers, including catalase (CAT) activity and malondialdehyde (MDA) (251).

Rosemary: Rosmarinic acid

Rosmarinus officinalis (Rosemary) is a typical houseplant growing around the globe that belongs to the Lamiaceae family (252, 253). The chemical composition of rosemary extract was examined to determine its active principles, which indicated the existence of many compounds, including rosmarinic acid (RA), caffeic acid (CA), chlorogenic acid, carnosic acid, rosmanol, and carnosol (252, 254–256). Therefore, three types of chemicals have been linked to the biological activity of *R. officinalis* L.: a volatile fraction, phenolic compounds, and di and triterpenes (254, 255).

Rosemary has a long history of usage in food to change and improve tastes. On the other hand, rosemary extracts have anti-inflammatory, antioxidant, antimicrobial, antitumor, antispasmodic, and anti-diabetic bioactivities. The low toxicity and strong cardioprotective, hepatoprotective, neuroprotective, diuretic effect, estrogenic effect, as well as memory enhancement and pain relief have been investigated in the reviewed literature (253, 257–262). On the other hand, rosmarinic acid (RA) was able to reduce inflammation in AOM/DSS-induced colon cancer mouse model by inhibiting NF-kB and STAT3 pathways (263). (264) suggested that RA alone or in combination with black rice extract can suppress colitis disease in DSS-induced colitis mice. The results of the study have shown a reduction in the inflammatory mediators expression, including IL-6, IL-1β, TNF-α, iNOS, and COX-2 (264).

Rosemary has been shown anticancer properties in both models: *in vitro* and *in vivo*. Several of these properties have been ascribed to its principal constituents, including carnosic acid, carnosol, ursolic acid, and rosmarinic acid (252, 265). Rosemary has been shown to have significant antiproliferative activity against several human cancer cell lines (266–269), induce apoptosis via nitric oxide production (270, 271), antioxidant activity (265, 266, 272), decreased TNF-α, IL-6, and COX2 levels (273, 274), suppress lipid peroxidation (261, 275, 276), prevent carcinogen-DNA formation (265), stimulation of p53 and BAX (277), reduction of Bcl-2, Mdm2, and Bcl-xL expression, and stimulation of caspase-3 and -9 expression (272, 274, 277, 278). Another probable method is via inhibiting Akt phosphorylation, which is required for cancer cell proliferation, growth, and survival (279). A study has tested the antiproliferation activity of rosemary extract on HT-29 and SW480 cells. Rosemary extract suppressed tumor cell growth via increasing intracellular ROS, reducing in G0/G1 phase, and improving cells accumulation in the G2/M phase (280). Moreover, Valdés et al. reported that carnosol-enriched extract showed significant inhibition of cell growth by inducing G2/M cell cycle arrest in colon cancer cells (SW480 and HT-29 cell lines) (281). A recent study suggested that rosmarinic acid (RA) can reduce the rate of adenocarcinoma formation in azoxymethane-induced CRC in rats. It upregulated the total antioxidant status (TAS) and decreased the expression of IL-6 and MCP-1 (282). Using male Wistar rats with DMH-induced colon cancer, RA exhibited a chemoprotective effect via suppressing tumor formation and decreasing lipid peroxidation (276). **Figure 4** summarized the anticancer effect of herbs and spices in CRC.

Whole grains and cereals: β-D-glucan

Whole grains are cereals that have the complete grain kernel (bran, germ, and endosperm) in contrast with refined grains that contain the endosperm only. There is considerable evidence that chronic diseases could be avoided by the consumption of whole-grain cereal products, which can reduce the risk of obesity, metabolic syndrome, type 2 diabetes, cardiovascular disease, cancer, and mortality from these chronic diseases (283, 284). Whole-grain cereals are an abundant source of fiber and bioactive compounds. For example, whole-grain wheat consists of 13% dietary fiber and at least 2% bioactive compounds excluding fiber. In the bran and germ fractions, still greater proportions are attained: about 45 and 18% of dietary fiber, and about 7% and at least 6% of bioactive compounds, respectively (285). The total dietary fiber of wheat ranges from 9 –to 20% and it consists of both soluble and insoluble portions. The two major components of dietary fiber in wheat are arabinoxylan (AX) and β-D-glucan (283). While barley and oats contain β-glucan as the primary fiber in the whole kernel, AX is present in much less content (286). As well, cellulose and hemicellulose are the major fiber components of corn bran and brown rice (287).

Dietary fibers provide numerous benefits, including a lower risk of cancer and enhanced colon health, Where low dietary fiber consumption has been linked to both local and systemic chronic inflammation (288). Recently, a study using a synbiotic composed of arabinoxylan (AX) and *Lactobacillus fermentum* HFY06 was tested to determine its ability to relieve DSS-induced colitis. AX and *L. fermentum* HFY06 inhibited the activation of the NF-κB signaling pathway, downregulated the mRNA expression levels of NF-kBp65 and inhibited the TNF-α, and exerted anti-colitis effects (289). As well, the short-chain fatty acids (SCFA), particularly butyrate, are byproducts of dietary fiber fermentation by certain microorganisms in the

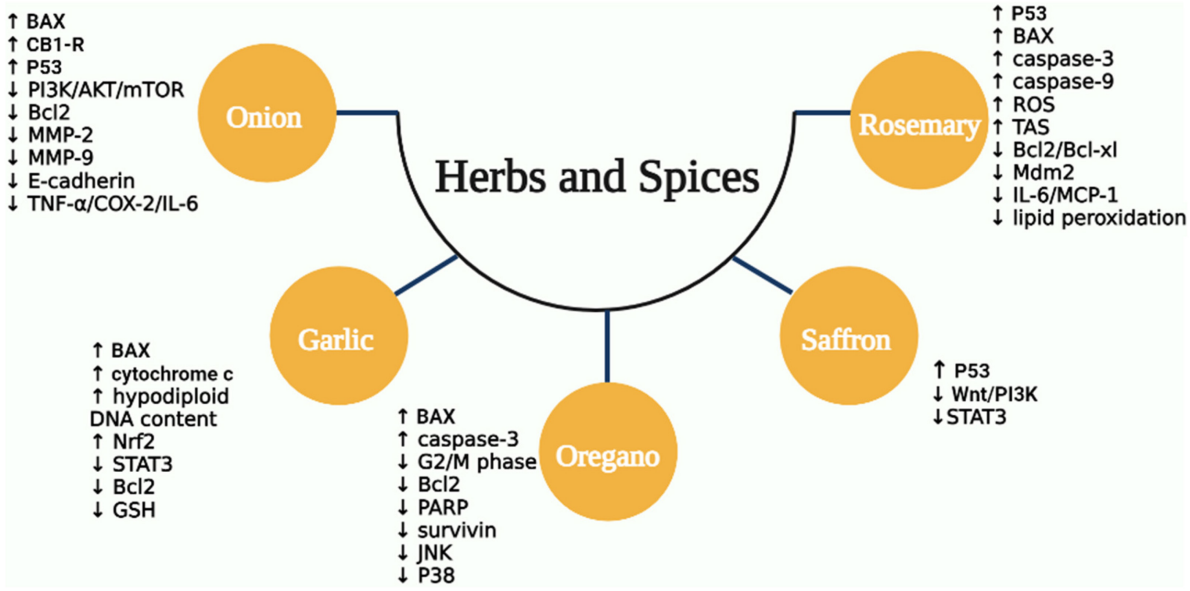

FIGURE 4
The mechanisms of anticancer activity for some types of MD herbs and spices. ROS, reactive oxygen species; TNF-α, tumor necrosis factor-alpha; IL, interleukin; COX-2, cyclo-oxygenase-2; MMP-9, matrix metalloproteinases-9; JNK, Jun N-terminal kinase; BAX, Bcl-2-associated X protein; PARP, poly adenosine diphosphate ribose polymerase; Nrf2, nuclear factor-erythroid factor 2; STAT3, signal transducer and activator of transcription 3; GSH, glutathione; CB1-R, cannabinoid receptor type 1; Mdm2, mouse double minute 2 homolog; MCP-1, monocyte chemoattractant protein-1; Wnt, wingless-related integration; PI3K, phosphoinositide 3-kinase.

intestinal colon, and they exhibited anti-inflammatory actions on both gut epithelial and immune cells. Hence, inflammation signaling pathways involving nuclear factor kappa-B (NF-kB) and deacetylase are inhibited by SCFA (288, 290). Several studies have been established to investigate the anti-inflammatory effect of the major types of dietary fibers. Such as the beneficial effect of oat β-D-glucan has been tested on Sprague–Dawley rats with TNBS-induced colitis. The results showed a significant reduction in IL-6, IL-10, C reactive protein (CRP), and IL-12. As well, β-D-glucan reduces some selected inflammatory markers, including COX, PGE2, and thromboxane A2 (TXA2). The results indicate the therapeutic effect of dietary oat beta-glucan supplementation in colitis (291). In another study, oat beta-glucan has been tested also on male Sprague Dawley rats with TNBS-induced colitis. The results proved the in-direct antioxidant effect of beta-glucans by agonistic binding of immune cells to membrane receptors, which results in increased antioxidant response and removing systemic effects of colon inflammation (292).

Another well-known function of dietary fiber is to lower the CRC incidence, as it reduces the concentrations of carcinogens and procarcinogens in the feces. Furthermore, it shortens the residence time of carcinogens in the lower gastrointestinal tract, reducing their absorbance and contact time with colon epithelium cells (293). As previously stated, dietary fibers digested by intestinal bacteria produce SCFA which has a protective effect against the growth of cancer cells (294, 295). (296) examined the role of dietary fiber in polyposis by using TS4Cre × cAPCl◦x 468 mice. The results showed that a high fiber diet significantly increased SCFA-producing bacterium as well as SCFA levels. This was associated with an increase in SCFA butyrate receptor and a significant decrease in polyposis. The prebiotic activity of fiber could be the key mechanism for the protective effects of fiber on colon carcinogenesis. Overall, the findings revealed that insoluble fermentable fiber may protect against CRC (296). As well, a dietary fiber (β-glucan) with quercetin anti-colonic cancer effect has been tested. The findings demonstrated that alternating β-glucan and quercetin consumption alleviated colon damage and reduced mortality in CRC mice, with a 12.5% reduction in mortality. Consumption of β-glucan and quercetin alternated dramatically reduced TNF-α, increased the relative frequency of Parabacteroides, and downregulated three genes linked to inflammation and cancer (Hmgcs2, Fabp2, and Gpt) (297). Moreover, β-glucan was tested in human colon cancer cells (SNU-C4) and it exhibited antiproliferation activity by reducing Bcl2 expression and upregulation of BAX and caspase-3 levels (298).

Fish: PUFA/n-3 fats

Fish is another important component of the traditional MD diet. In recent years, there has been a lot of emphasis on the positive effects of fish eating, which has been reinforced by the

idea that the ocean is a fantastic source of new compounds. Fish meat is abundant in anti-inflammatory n-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have been proven to reduce the risk of cardiac mortality by 30–45%, and lessen ischemic stroke incidence (299, 300). As well, n-3 PUFA appears to be protective against dementia and Alzheimer's in the elderly (301). It has been proven to affect various types of cancer, including breast, ovarian, prostate, lung, skin, colon, colorectal, pancreatic, and stomach cancers. These benefits of n-3 PUFA are a result of their antioxidant, anti-inflammatory, anti-apoptotic, and neurotrophic properties (302). As recently reviewed, n-3 PUFAs have anti-inflammatory effects against inflammatory diseases, including IBD, psoriasis, and rheumatoid arthritis by lowering arachidonic acid (AA) proinflammatory activities, increasing the production of endocannabinoids with EPA or DHA in their structure and thus anti-inflammatory properties, lowering the production of inflammatory cytokines like IL-1, IL-6, and TNF-α, lessening in T-cell proliferation and the formation of IL-2 (303). Moreover, they increase the production of anti-inflammatory markers; e.g., soluble IL-6r, IL-10, transforming growth factor-β (TGF-β) and lowering the expression of adhesion molecules on immune cells and endothelium. As well, the homeostasis of tissues has been restored after inflammation as a result of n-3 PUFA metabolites production such as resolvins, protectins, and maresins, which act as specialized pro-resolving mediators. These pro-resolving bioactive lipids act as "stop-signaling" of the inflammatory response and have important anti-inflammatory and anti-carcinogenic properties, by increasing macrophage phagocytosis, efferocytosis, and leukocytes egress (304, 305).

Recently, the impact of n-3 PUFA and probiotics have been investigated in BALB/c mice subjected to 2,4-Dinitrobenzenesulfonic acid (DNBS) induced-chronic colitis. Administration of combination reduced the concentrations of inflammatory mediators such as IFN-γ, TNF-α, and IL-17A. The findings showed that the combined effect of probiotics and n-3 PUFA might have a protective effect against colon injury and inflammation by creating synergistic effects (306). Moreover, the anti-inflammatory effect of n-3 PUFA was investigated in a rat model of acetic acid-induced UC. The results showed that after administration of n-3PUFAs, the expression levels of IL-1 and Caspase-3 were downregulated, whereas Bcl-2 was upregulated. These findings imply that n-3 PUFAs protect the colonic mucosa of rats against acetic acid-induced UC, and may aid in the repair of UC via anti-inflammatory and anti-apoptotic properties, as well as a regenerative endogenous antioxidant mechanism (307).

Interestingly, n-3 PUFA is also known to have anticancer potentials and is associated with the alteration of cancer hallmarks and tumor regression activity. It can modulate cyclooxygenase (COX) metabolism and reduce the production of several prostanoids including prostaglandin (PG) E2 in tumors whilst possibly increasing the production of lipid mediators involved in the resolution of inflammation such as lipoxins and resolvins, which may have anti-cancer properties (308). As well, n-3 PUFA has been found to bind to the plasma membrane of cancer cells, changing the content and fluidity of the lipid membranes. This can cause signal transduction to be inhibited, reducing cancer cell viability and encouraging apoptosis (309). Other CRC-promoting signaling pathways, including the Wnt/ß-catenin pathway, the MAPK/ERK pathway, and the PI3K-PTEN system have been reported to be downregulated by n-3 PUFA (310). In a recent study on CRC cells, SW620 and HCT-116 parental and HCT-116 mutant cells isogenic for constitutively active PI3K were treated with free or ethyl esterified n-3 PUFA. The results showed the ability of n-3 PUFA ethyl esters to inhibit PI3K activity confers their potency to reduce CRC cell invasion, but not proliferation. (311) Furthermore, n-3 PUFAs can activate pro-apoptotic signaling by interacting with G protein-coupled receptors (GPCRs), resulting in an anti-CRC activity. Non-epithelial cells including adipocytes and macrophages have been found to express these GPCRs. Activation has the potential to change macrophage polarization and reduce inflammation, both of which are critical for n-3 PUFA anti-cancer action (312). Additionally, in the LS174T human CRC cell line, the effect of EPA derived from n-3 PUFAs on cell number, cell proliferation rate, and caspase-3 enzyme activity was studied. When EPA concentrations were increased, caspase-3 activity rose by 3.4 times relative to untreated control cells at 200 mol EPA and reduces the number of CRC cells and their growth rate (313). A further study examined the effect of DHA on migration in CRC cell lines and found that 100 mM DHA inhibited Granzyme B expression in three human CRC cell lines (HCT116, CSC4, and HT-8), limiting their capacity to undergo epithelial-mesenchymal transition (EMT) (314).

Grapes: Resveratrol

Grapes have been associated with health benefits for many years, despite a lack of scientific evidence, and have been closely linked with diet since ancient times, particularly in Mediterranean countries. Several studies conducted around the world over the last two decades have shown that consuming grapes have beneficial impacts on antioxidant capacity, lipid profile, and the coagulation system (315).

Grape juice is made mostly from European *(Vitis vinifera)* and American grape species (*Vitis labrusca*). Both species have high levels of polyphenolic compounds, such as caffeic acid, gallic acid, *p*-coumaric acid, and stilbenes (*trans*-resveratrol). Resveratrol (RV) is one of the most important polyphenols found in grapes. As well as, Flavonoids such as quercetin, rutin, myricetin, catechin, and epicatechin (315, 316). The amount of RV in grapes varies by grape genotype, cultivar, growing

season, and climatic factors. It can be found in the leaf, skin, bud, stem, seed, bud, and root. Regardless, the majority of it is found in the grape skin, with much less in the juice and wine. Grape skin and juice contain more glycosylated RV than free RV (317). The benefits of RV are numerous. The most well-known benefits include anti-inflammatory, anti-proliferative, and chemopreventive. Multiple lines of evidence from *in vivo* and *in vitro* laboratory research suggest that the anti-inflammatory properties of RV can be explained by preventing the synthesis of anti-inflammatory factors (318). Chronic inflammation is one of the main mechanisms involved in colon cancer. Therefore, the anti-inflammatory effect may be beneficial in the treatment of CRC.

Resveratrol has been shown *in vitro* to reduce the production of pro-inflammatory mediators such as IL-1 and IL-6, as well as to down-regulate both mRNA expression and protein secretion of IL-17 in a dose-dependent manner. Resveratrol is also associated with the inhibition of 5-lipoxygenase, cyclo-oxygenase-2 (COX-2), and Nuclear Factor-kB (NF-kB). As well, suppressing the expression of tumor necrosis factor α (TNF-α), interleukin 8 (IL-8), and interferon-γ (IFN-γ) will lead to a decrease in the ulcerative colitis process (319–321). Ren et al. investigated the anti-inflammatory effect of RV on HEK-293T cells and HeLa cells the results have shown that RV suppressed endogenous NF-kB activity and TNF-α induced NF-kB activation (322). Another study has demonstrated the anti-inflammatory activity of RV in the DSS-induced colitis mouse model. The results exhibited a reduction in IL-2, IFN-γ, GM-CSF, IL-1β, IL-6, KC/GRO, and TNF-α, along with a significant effect on gut microbiota by increasing the abundance of *Bifidobacterium* (323).

Besides the anti-inflammatory effect of resveratrol in grapes, it also has a high potential for inhibiting tumor initiation, development, and promotion. The majority of research on grapes' cancer-preventive properties focuses on resveratrol (324). The anticancer activity of resveratrol is mediated by a variety of molecular mechanisms and signaling pathways. Such as activation of the mitochondrial and caspase cascade enzymatic systems, as well as death-induced cytokines and their receptors are upregulated, as are cyclin-dependent kinase inhibitors, and tumor suppressor genes. Additionally, resveratrol will lead to downregulation of survivin, cFLIP, cIAPs, and antiapoptotic proteins (Bcl-2 and Bcl-xL), all of which are linked to the development of chemoresistance. Although resveratrol suppresses tumor cell proliferation by activation of proapoptotic proteins (P21 and P53) and suppression of hippo–YAP, inhibition of MAPK, phosphoinositide 3-kinase (PI3K)/Akt, nuclear factor β (NF-β), activating protein-1 (AP-1) HIF-1α and signal transducer and activator of transcription 3 (STAT3) (325–327).

Several studies were conducted to investigate the effect of resveratrol on different cancer cell lines. Such as *in vitro* study on human CRC cells HCT116 and SW620, the results indicated that RV dose-dependently upregulated the expression of several proapoptotic proteins such as BAX, cytochrome *c*, cleaved caspase-9, and caspase-3, while anti-apoptotic protein Bcl-2 expression levels was reduced in RSV-treated CRC cells. Overall, resulted in the suppression of CRC cell viability, increased cell apoptosis, and ROS levels compared with the control group, as well as activated the mitochondrial apoptotic pathway (328). In another *in vitro* study of resveratrol on HCT116, a human CRC cell line, the treated cells showed cell growth inhibition and apoptosis induction, as well as downregulation of intracellular AKT1 and IL-6 expression (329). Moreover, an *in vitro* cellular model of aggressive and resistant colon cancer enriched in CSCs was chosen as doxorubicin-resistant LoVo/Dx cells (a subline of the LoVo cell line), which were treated with RV and celastrol. The results have shown that celastrol and resveratrol produce an antitumor activity against metastatic LoVo cells and cancer stem-like by inducing apoptosis and cell cycle arrest, by increasing *SIRT*1 gene expression, resulting in overcoming apoptosis resistance in LoVo colon cancer cells (330). Furthermore, a study using CRC-derived cell lines, LoVo and HCT116, found that resveratrol inhibited CRC invasion and metastasis by suppressing Wnt/-catenin signaling and, as a result, the expression of its target genes such as c-Myc, MMP-7, and MALAT1, which leads to the inhibition of CRC invasion and metastasis (331).

Nuts: Hazelnuts/β-sitosterol

Hazelnuts (HN) produced by *Corylus avellana* L., a member of the genus Corylus of the Betulaceae family, are widely consumed around the world, and the common hazel is widely dispersed along the southern European coast and the Black Sea region (332). HN has several health benefits. Many studies showed the effect of HN on the reduction of LDL-C levels, and a tendency to lower total cholesterol accumulation. Besides, it reduced the incidence of certain chronic diseases such as cancers (333). The main chemical compositions of HN are simple phenols, lipids, and monounsaturated fatty acids, as well as, a source of minerals, tocopherols, tocotrienols, squalene, triterpenes, and phytosterols. The total phytosterol content of HN varies between 133.8 mg/100 g and 263 mg/100 g of oil. The most common is β-sitosterol (BS), which accounts for 83.6% of the total (334, 335).

β-sitosterol has immunomodulatory and anti-inflammatory activity, several studies suggested that BS can suppress inflammation through the NF-κB pathway (336, 337). Furthermore, BS stimulates the activity of T helper (Th) cells, as well as T cells and natural killer cells (338). A study investigated the effects of both stigmasterol and BS on DSS-induced colitis in C57BL/6J male mice. The results showed that both BS and stigmasterol significantly inhibited colon shortening, minimized fecal hemoglobin contents, and lowered

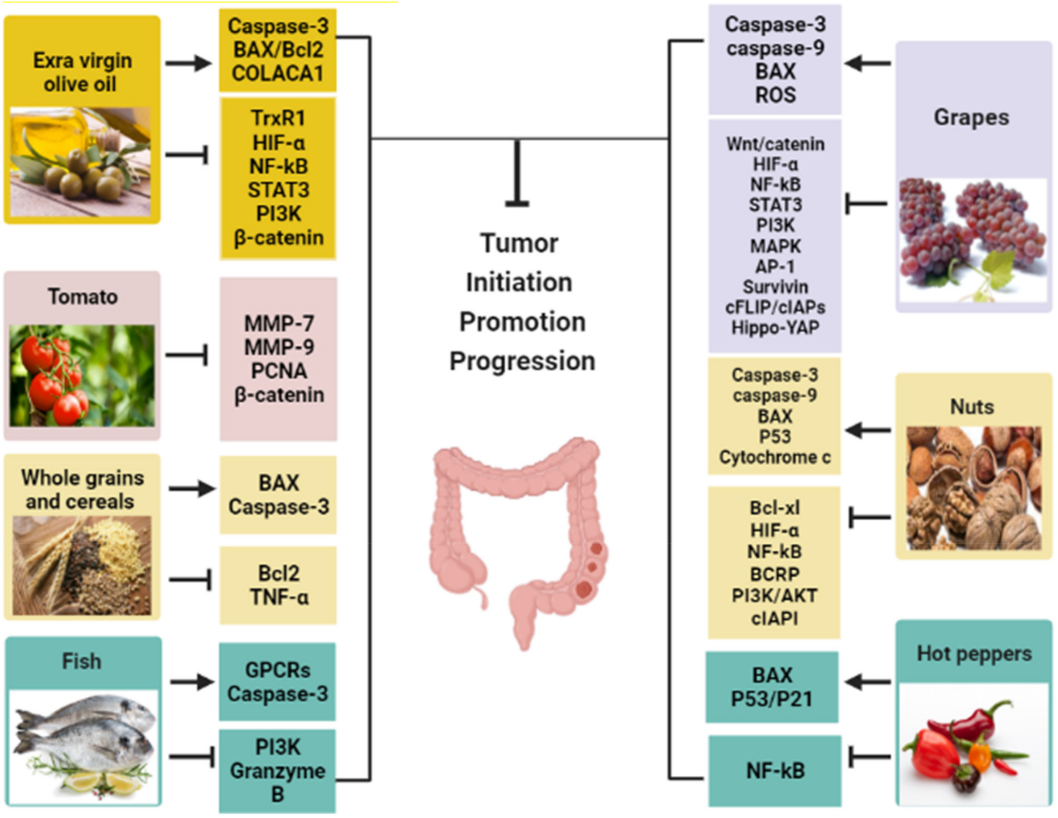

FIGURE 5
The Mediterranean diet components with their effects on cancer biomarkers. (→, activation; T, inhibition; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-alpha; IL, interleukin; MMP-9, matrix metalloproteinases-9; BAX, Bcl-2-associated X protein; Wnt, wingless-related integration; PI3K, phosphoinositide 3-kinase; PCNA, proliferating-cell nuclear antigen; TrxR1, thioredoxin reductase 1; COLACA1, colorectal cancer associated-1 gene; Bcl2, B-cell lymphoma 2; GPCRs, G protein-coupled receptors; MAPK, mitogen-activated protein kinase; AP-1, activator protein-1; c FLIP, cellular (FAAD-like IL-1β-converting enzyme)-inhibitory protein; cIAPs, cellular inhibitory of apoptosis proteins; BCRP, breast cancer resistance protein.

the severity of colitis in the middle and distal colon. As well, they significantly suppressed the activation of the inflammatory master regulator NF-kB (339). Moreover, the effect of BS on 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice was also examined to see if it also exhibits anti-colitis properties. The study showed that BS inhibited colon shortening and resulted in lower macroscopic scores and myeloperoxidase activity. In the colons of TNBS-induced colitis mice, BS reduced the expression of proinflammatory cytokines TNF-α, IL-1, and IL-6, as well as COX-2 and activation of NF-kB. These data suggest that BS may help with colitis by suppressing the NF-kB pathway (340). Furthermore, in DDS-induced colitis in male C57BL/6 mice, BS was able to reduce the levels of TNF, IL-6, and IL-1 in intestinal tissue, indicating that β-sitosterol administration significantly reduced inflammatory damage to colonic tissues, including colon edema, crypt distortion, goblet cell loss, and mononuclear cell infiltration. These findings suggest that it could be useful in treating chronic colitis (341).

Besides the anti-inflammatory effects of BS found in hazelnuts, it has also been shown to protect against cancers such as breast, colon, colorectal, and prostate cancer. BS can halt tumor development and promote programmed cell death in cancer cells (342). A recent study tested the anticancer effect of BS-mediated silver nanoparticles (AgNP) on human colon cancer (HT-29) cells. The results suggested that BS improved apoptosis via inducing p53 expression in HT-29 cells (343). Furthermore, it inhibited the expression of breast cancer resistance protein (BCRP) and restored oxaliplatin (OXA) sensitivity in drug-resistant CRC cells. The study also found that BS could activate p53 by disrupting the p53–MDM2 interaction, resulting in increased p53 translocation to the nucleus and silencing the NF-κB pathway, which is required for BCRP expression. These findings showed that β-sitosterol can regulate CRC response to chemotherapy by mediating the p53/NF-B/BCRP signaling axis (344). Shen et al. (345) have reported that liposomal β-sitosterol can prevent tumor migration of colon carcinoma via downregulation of MMP-9 expression

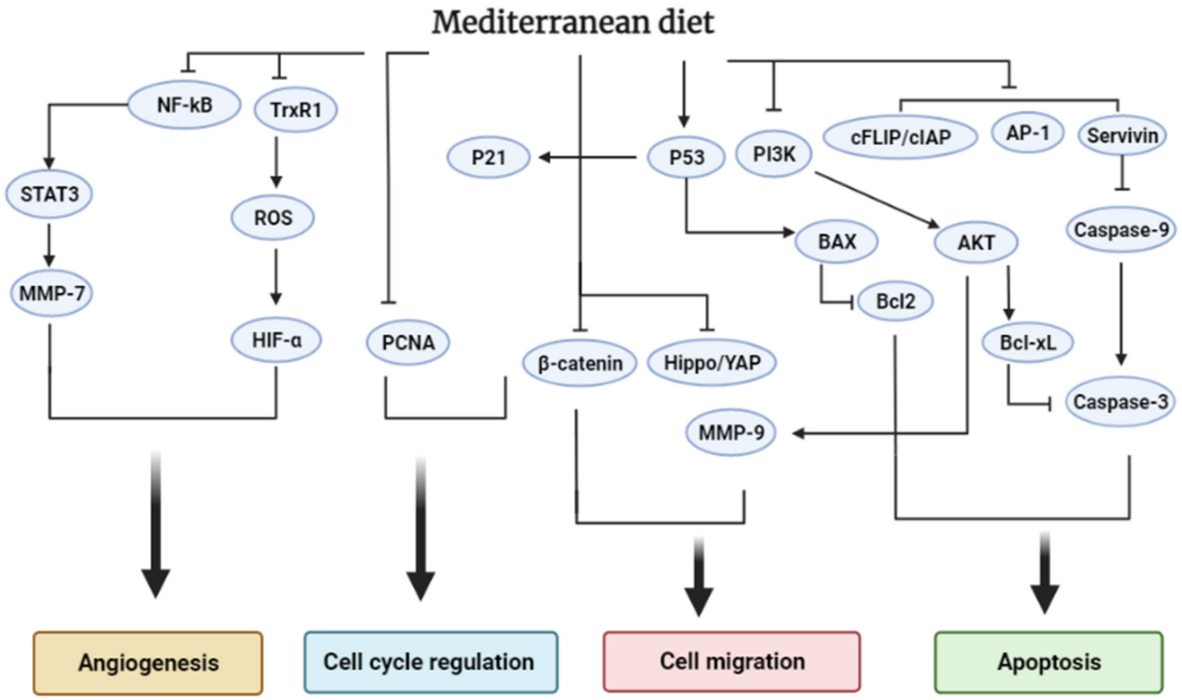

FIGURE 6
The effect of the Mediterranean diet on the cellular signaling pathways that regulate colorectal cancer progression.

and modulation of Th1 immune markers. Additionally, BS, campesterol, and stigmasterol have been tested on colon cancer cells (Caco-2). The results revealed BS and other polyphenols induced reversible arrest in phase G_0/G_1 of the cell cycle (346). In HCT116 cells, BS induced apoptosis was accompanied by a decrease in anti-apoptotic Bcl-2 protein and mRNA and a concurrent rise in proapoptotic BAX protein and mRNA, as well as cytochrome c release from the mitochondria into the cytoplasm. The expression of cellular inhibitor of apoptosis protein-1 (cIAP1) was also suppressed by BS treatment (347). As well, the anti-CRC effects of BS were investigated in BALB/c nude mice. The study has proved that the treatment of mice with β-sitosterol decreased tumor growth by lowering PI3K/Akt expression, promoting Bad activation, decreasing Bcl-xl, and increasing cytochrome-c release, resulting in caspase-9 and caspase-3 activation, PARP cleavage, and apoptosis.

Hot pepper: Capsaicin

Hot pepper (*Capsicum annuum* L.), usually called chili, is a diploid, facultative, self-pollinating crop that belongs to the Solanaceae family (closely related to the potato, tomato, eggplant, tobacco, and petunia). Hot pepper contains many essential vitamins, minerals, and nutrients that have a significant role in human health (348–351). Peppers are a rich origin of both vitamins C and E (352, 353). The major components of most capsicum species are capsaicin (69%), dihydrocapsaicin (22%), nordihydrocapsaicin (7%), homocapsaicin (1%), and homodihydrocapsaicin (1%), respectively (354). Capsaicin (8-methyl-*N*-vanillyl-*trans*-6-non-enamide) is a naturally occurring alkaloid derived from chilies. It is responsible for its hot pungent taste, characterized by its odorless fat-soluble compound that is rapidly absorbed via the skin.

Lycium barbarum polysaccharides and capsaicin have a protective effect in rats with dextran sulfate sodium-induced ulcerative colitis through anti-inflammation and antioxidation actions (355). Oral capsaicin has downregulated IL-6 and protein expression of TRPV1 and TRPA1 as well as increased SOD level and catalase activities (356). Besides, dietary capsaicin exhibited an anti-colitis effect in DSS-induced colitis wide-type (WT) mice by improving Na^+ absorption, and reducing Cl^- level. It ameliorated intestinal inflammation by suppressing the hyperactivity of TRPV4 channels (357).

Capsaicin has an anticancer impact on HCT116 and LoVo cells (human colon cancer cells) through influencing cell cycle G0/G1 phase arrest and apoptosis, which was associated with an elevate of p21, BAX, and cleaved PARP. The capsaicin anticancer mechanism was attributed to the stabilization and activation of p53. It lengthened the half-life and boosted the transcriptional activity of p53 (358). Synergistic induction of both apoptosis and inhibition of cell proliferation was shown in HCT116, SW480, LoVo, Caco-2, and HT-29 (human CRC cells) that were treated with the combination of capsaicin and 3,3'-diindolylmethane.

Also, these two compounds activated the transcriptional activity of NF-kB and p53 synergistically. The combination treatment stabilized nuclear p53 and up-or downregulated expression of several target genes that are downstream of NF-κB and p53 (359).

Figure 5 demonstrated the seven MD components and their effects on cancer biomarkers and CRC development. **Figure 6** displays the molecular mechanism of the MD in CRC.

Table 1 summarized the anti-inflammatory effects of all the discussed MD components. **Table 2** summarized the anticancer effects of the MD components.

Role of the Mediterranean diet and its components in modulation of the gut microbiome

Several studies have shed the light on the role of the diet in the modulation and improvement of the gut microbiome (363). In particular, the MD which is recognized for its high plant-based food has shown a remarkable impact on gut microbiota profile (364, 365). It was found that adherence to MD has increased the presence of SCFA and fiber-degrading bacteria as well as reduced the presence of *E. coli* bacteria (365, 366). Besides, the positive impact of the MD on the gut microbiota content has been recognized by Garcia-Mantrana et al. (367), it was observed an improvement in Bifidobacteria abundance and higher production of SCFAs. Concurrent literature has described the beneficial modulation of the gut microbiome by the different components of the MD. Interestingly, the intervention treatment of hydroxytyrosol, a main phytochemical in EVOO, has modulated the gut microbiota leading to a lower density of inflammation-related microbes and enhancing the presence of probiotics (49, 50). Recently, Rocchetti et al. reported that oleuropein derived from olive leaf extract and EVOO has improved gut microbiota and potentiated the growth of bacteria associated with healthy metabolic markers (368). (360) reported the anti-inflammatory effect of lycopene in DSS-induced colitis mice. The study suggested that lycopene reduced the expression of TNF-α, IL-1β, TLR-4, and iNOS as well as modulated the gut microbiome by decreasing the density of proteobacteria and improving the presence of *Bifidobacterium* and *Lactobacillus* (360). Furthermore, allicin altered the structure of the gut microbiota and raised the number of beneficial bacteria. Koch and Lawson found that allicin suppresses the development of *Escherichia coli* and *Staphylococcus aureus* (369). Allicin (100 mg/kg/d) dramatically increased the relative abundance of *Ruminococcaceae*, *Clostridiales*, *Bacteroidales*, and *Facklamiaets* while decreasing the relative abundance of *Firmicutes*, *Corynebacteriales*, and *Lactobacillales* (370). Dietary supplementation with 100 mg/kg of carvacrol–thymol (CV–TH) (1:1) blend as animal feed for 14 days reduced weaning-induced intestinal oxidative stress and inflammation in piglets by decreasing tumor necrosis factor mRNA levels. It is worth noting that the CV–TH blend increased the population of *Lactobacillus* species and decreased the populations of *Enterococcus* and *E. coli* (361). Moreover, dietary fiber consumption enhances the creation and maintenance of a healthy, viable, and diversified colonic microbiota, acting as prebiotics. Prebiotics are ingredients that are resistant to gastric acidity and hydrolysis by enzymes (295). On the other hand, β-sitosterol maintained gut microbiota compositions leading to the production of beneficial metabolites including SCFAs that promote tumor apoptosis (362). As well, pungent food, particularly Capsaicin, has a positive action on gut flora, by decreasing the disease-causing enteric pathogens and encouraging the growth of useful bacteria (371).

Chemopreventive effect of the Mediterranean diet and its components: Clinical studies

Despite the effectiveness of colonoscopy screening and recent improvements in cancer therapy, CRC remains one of the most prevalent and deadly types of cancer (372). Many studies have shown that a diet rich in fruits, vegetables, and tea is associated with lower rates of cancer, particularly colon cancer (373–375). In this context, a double-blind randomized clinical trial, case-control study, and meta-analysis revealed a substantial impact on colon disease and cancer patients. Fruit and deep-yellow vegetables, dark-green vegetables, onions, and garlic are moderately associated with a lower risk of colorectal adenoma, a precursor to CRC (376–378). Case-control studies generally revealed a lower risk of CRC with onion eating. In Argentina, the effect was more pronounced for consumption of a combination of garlic, onions, and pepper (379). In other case-control studies, the data points to a positive outcome. A food frequency questionnaire was used to analyze the intake of onions and garlic in a network of Italian and Swiss research that comprised 1037 cases and 2020 controls (380). The researchers discovered that onions and garlic were both protective against large bowel cancer. All levels of onion consumption were related to a lower risk of CRC. Also, Garlic usage at intermediate and high levels was linked to a lower risk of CRC. Several studies have indicated that flavonols, such as quercetin, can reduce the incidence of colon cancer with or without additional supplement therapy such as aspirin or NSAIDs (381–383). A dose-response meta-analysis revealed that an increase in dietary flavonols (such as quercetin) intake of 10 mg per day was significantly related to a reduced risk of CRC (383). Furthermore, taking 3.65 kg of garlic supplements per year for 2 years was connected with a

TABLE 1 Anti-inflammatory effects of the main Mediterranean diet components.

MD components	Active phytochemicals	Model of the experiment	Result of the study	References
Extra virgin olive oil	Hydroxytyrosol	DDS-induced colitis mice	↓ IL-6, IL-1β ↓ TNF-α ↓ Myeloperoxidase enzyme ↑ Probiotics	(49, 50)
		DDS-induced ulcerative colitis mice	↓ NLRP3 inflammasome ↓ IL-18, IL-1β ↓ Caspase-1	(50)
		Induced ulcerative colitis albino rats	↓Malondialdehyde, myeloperoxidase ↓ NO ↓ Mortality rate ↓ Disease activity index	(51)
	Oleuropein	Induced colitis rats	↓ IL-1β, IL-10 ↓ TNF-α ↓ COX-2 ↓ iNOS ↓TGF-β1 ↓ MCP-1 ↓ NF-κB	(67)
		DDS-induced colitis mice	↓ TNF-α ↓ IL-6 ↓ Neutrophil infiltration	(68)
Tomato	Lycopene	Ulcerative colitis rat	↓ IL-6, IL-1β ↓ TNF-α	(83)
		Ulcerative colitis rat	↓ NF-κB ↓TGF-β1 ↓ Caspase-3 ↓ GSH ↓ Catalase activity	(84)
		DSS-induced colitis mice	↓ TNF-α ↓ IL-1β ↓ iNOS ↓ TLR-4 ↓ *Bifidobacterium* and *Lactobacillus* ↓ Proteobacteria	(360)
		DSS-induced colitis mice	↓ Disease activity index score ↓ Colon length ↓ Catalase ↓ GSH-Px ↓ SOD	(85)
Onion	Quercetin	MC3T3-E1 preosteoblastic cell line	↓ NF-?B	(135)
		DSS-induced colitis mice	↓ GSH	(140)
		AOM/DSS-induced colon cancer mice	↓ LPO ↓ NO ↓ SOD ↓ G6PD ↓ GSH	(153)
Garlic	Allicin	Caco-2 cells	↓ p38 ↓ JNK	(185)
		AA-induced intestinal damage rats	↓ TLR4 ↓ NF-κB ↓ SCFAs	(185)
		TNBS-induced colitis Wistar rats	↓ TNF-α ↓ IL-6	(186)
Oregano	Carvacrol–thymol	Weaning-induced intestinal oxidative stress and inflammation piglets	↓ TNF ↑ *Lactobacillus*	(361)

(Continued)

TABLE 1 (Continued)

MD components	Active phytochemicals	Model of the experiment	Result of the study	References
	Carvacrol	Irinotecan-induced intestinal mucositis mice	↓ NF-κB ↓ COX-2 ↓ Oxidative stress ↓ NOx ↓ Malondialdehyde	(209)
Saffron	Crocin	colitis-associated CRC mice	↑ Catalase ↓ Malondialdehyde	(251)
		DSS-induced colitis mice	↓ COX-2 ↓ TNF-α ↓ NF-?B ↓ iNOS ↓ IL-6, IL-1β ↓ Nrf2	(240)
	Crocetin	TNBS-induced ulcerative colitis mice	↓ NO ↓ Neutrophil infiltration ↓ Lipid peroxidation ↓ NF-?B	(241)
Rosemary	Rosmarinic acid	AOM/DSS-induced colon cancer mice	↓ NF-?B ↓ STAT3	(263)
		DSS-induced colitis mice	↓ IL-6, IL-1β ↓ TNF-α ↓ iNOS ↓ COX-2	(264)
Whole grains and cereals	Arabinoxylan	DSS-induced colitis mice	↓ NF-?B ↓ NF-κBp65 ↓ TNF-α	(289)
	β-D-glucan	TNBS-induced colitis Sprague-Dawley rats	↓ IL-6, IL-10, IL-12 ↓ CRP ↓ COX ↓ PGE2 ↓ TXA2	(291, 292)
Fish	n-3 PUFA	DNBS-induced chronic colitis BALB/c mice	↓ TNF-α ↓ IFN-γ ↓ IL-17A	(306)
		Induced ulcerative colitis rats	↓ IL-1 ↓ Caspase-3 ↑ Bcl-2	(307)
Grapes	Resveratrol	HEK-293T cells and HeLa cells	↓ NF-κB ↓ TNF-α	(322)
		DSS-induced colitis mice	↓ IL-2, IL-1β, IL-6 ↓ IFN-γ ↓ TNF-α ↓ KC/GRO ↓ GM-CSF	(323)
Hazelnuts	β-sitosterol/stigmasterol	DSS-induced colitis C57BL/6J male mice	↓ Colon shortening ↓ Hemoglobin in feces ↓ NF-κB	(339)
	β-sitosterol	TNBS-induced colitis mice	↓ Colon shortening ↓ Myeloperoxidase ↓ TNF-α ↓ IL-1, IL-6 ↓ COX-2 ↓ NF-κB	(340)
		DDS-induced colitis male C57BL/6 mice	↓ IL-1, IL-6 ↓ TNF-α	(341)
Hot pepper	Capsaicin	DSS-induced colitis rats	↓ IL-6 ↓ TNF-α	(355)
		DSS-induced colitis rats	↓ IL-6 ↓ TRPV1, TRPA1 ↑ SOD ↑ Catalase	(356)
		DSS-induced colitis wide-type mice	↓ TRPV4	(357)

TABLE 2 Anticancer effects of the main Mediterranean diet components.

MD components	Active phytochemicals	*In vitro*/ *In vivo*	Result of the study	References
Extra virgin olive oil	Hydroxytyrosol	*In vitro*	- Upregulation of the caspase-3 - Increase BAX/Bcl2	(54)
		In vitro	- Inhibition of TrxR1 - G1/S cell cycle arrest	(48)
		In vitro/In vivo	- Downregulation of epidermal growth factor receptors - Increased the expression of (COLACA1)	(77, 78)
	Oleuropein	*In vitro*	- Decreased HIF-α expression	(71)
		In vivo	- Repressed the activity NF-κB, STAT3, PI3K/Akt, and β-catenin	(72)
Tomato	Lycopene	*In vitro*	- Downregulated the MMP-7 expression - Hindered tumor development and tumor cell invasion	(86)
		In vivo	- Decrease the expression of PCNA and β-catenin proteins	(87)
Onion	Quercetin	*In vitro*	- G 2 cell cycle arrest	(148)
		In vitro	- Increased in the expression of CB1-R - Suppression of PI3K/Akt/mTOR signals	(145)
		In vitro	- Increased in Bax immunoreactivity	(153)
		In vivo	- Increased the apoptosis, cell proliferation and the expressions of p53 and Bax	(151)
		In vivo	- Reduced the LPO, NO, SOD, G6PD, GSH	(153)
Garlic	Allicin	*In vitro*	- Decreased in intracellular GSH levels	(187)
		In vitro	- Increased hypodiploid DNA content - Reduced levels of Bcl-2 - Increased levels of BAX, and cytochrome c	(188)
		In vivo	- Reduced the levels of phosphorylated STAT3 - Inhibited of Mcl-1, Bcl-2, and Bcl-xL	(180)
Oregano	Carvacrol	*In vitro*	- Increased in BAX - Decreased in Bcl-2, PARP, Survivin increased in the expression of caspase-3	(202)
		In vitro	- The G2/M cell cycle arrest - Reduced Bcl-2 - Phosphorylated p-ERK and p-Akt - Increased BAX and p-JNK expression	(213)
	Thymol	*In vitro*	Inhibition of JNK and p38	(214, 215)
Saffron	Crocin	*In vitro*	- Targeting MACC1 as a major causal metastasis-inducing gene	(248)
		In vitro	- Modulation of the Wnt/PI3K pathway - Regulating (CAT)and (MDA)	(251)
		In vitro	- Induction of apoptosis - Attenuation of the ratio of p-STAT3/STAT3	(250)
		In vivo	- Improved survival rate - Inhibited tumor development	(227).
	Crocetin	*In vitro*	- Cell cycle arrest - P21 induction - Increasing apoptosis - Lowering DNA repair capability	(249)
Rosemary	Rosmarinic acid	*In vivo*	- Reduced the rate of adenocarcinoma formation. - Upregulated TAS - Decreased the expression of IL-6 and MCP-1	(282)
		In vivo	- Suppressing tumor formation - Decreasing lipid peroxidation	(276)
Whole grains and cereals	β-glucan	*In vitro*	- Reducing Bcl2 expression - Upregulation of BAX and caspase-3 level	(298)
	β-glucan and quercetin	*In vivo*	- Reduced TNF-α - Increased the Parabacteroides - Downregulated Hmgcs2, Fabp2, Gpt	(297)

(Continued)

TABLE 2 (Continued)

MD components	Active phytochemicals	*In vitro/ In vivo*	Result of the study	References
Fish	n-3 PUFA	*In vitro*	- Modulated COX metabolism - Reduced the PGE2 in tumors -increasing lipoxins and resolvins	(308)
		In vitro	- Downregulated:Wnt/ß-catenin pathway, the MAPK/ERK pathway, and the PI3K-PTEN system	(310)
		In vitro	- Activated pro-apoptotic signaling by interacting with GPCRs	(312)
		In vitro	- Inhibited signal transduction - Reducing cancer cell viability -encouraging apoptosis	(309)
Grapes	Resveratrol	*In vitro*	- Activation of P21 and P53 - Suppression of hippo–YAP - Inhibition of MAPK, (PI3K)/Akt, (NF-β) - Activating protein-1 (AP-1) HIF-1α - Signal transducer and activator of transcription 3 (STAT3)	(325–327)
		In vitro	- Upregulated BAX, cytochrome c, cleaved caspase-9, and caspase-3 - Reduced Bcl-2 expression levels	(328)
		In vitro	- Suppressing Wnt/-catenin signaling	(331)
		In vitro	- Downregulation of intracellular AKT1 and IL6 expression	(329)
Hazelnuts	β-sitosterol	*In vitro*	- Improved apoptosis via inducing p53 expression	(343)
		In vitro	- Decreased in Bcl-2 and mRNA -raised BAX protein and mRNA -suppressed cIAP1	(347)
		In vitro	- Restored oxaliplatin (OXA) - Disrupting the p53–MDM2 interaction - Silencing the NF-κB pathway	(344)
		In vivo	- Lowering PI3K/Akt expression, -promoting Bad activation - Decreasing Bcl-xl - Increasing cyto-c release	(362)
Hot pepper	Capsaicin	*In vitro*	- The G0/G1 cell cycle arrest - Elevated p21, BAX, and cleaved PARP.	(358)

lower incidence of colorectal adenoma, a precursor of CRC (375, 376). Epidemiological investigations of randomized controlled trials revealed that treatment of aged garlic extract reduced colon adenomas and CRC in patients with CRC via increasing NK cell activity (384). In a Japanese study, patients with both colorectal aberrant crypt foci (ACF) and colorectal polyps who were planning polypectomy had a double-blind, randomized controlled experiment to investigate the effectiveness of omega-3 FAs in humans. After a month of supplementation, EPA (2.7 g per day) was found to be more effective at inhibiting colorectal aberrant crypt foci than the placebo control group (385). Another clinical trial looked at how co-supplementing with vitamin D and omega-3 fatty acids affected inflammatory markers and the tumor marker CEA in chemotherapy-treated CRC patients. Eighty-one patients with stage I or stage II CRC were given two omega-3 fatty acid capsules and a 50,000 IU vitamin D soft gel once a week for 8 weeks. The findings demonstrated that, when compared to baseline, omega-3, vitamin D, and co-supplementation significantly reduced serum levels of TNF-, IL-1, IL-6, IL-8, and tumor marker CEA. In comparison to baseline, NF-kB activity was significantly reduced in the vitamin D and co-supplementation groups (386). Numerous clinical pilot investigations have demonstrated that resveratrol in high doses is generally safe. Twenty CRC patients received resveratrol before surgery, at doses of 0.5 g or 1.0 g taken orally for 8 days. According to the findings, resveratrol was well tolerated. In CRC resection tissue, resveratrol and its metabolites were discovered. Resveratrol (0.5 or 1.0 g) was sufficient to provide anticarcinogenic effects in colon cancers by reducing tumor cell proliferation by 5% ($P < 0.005$) (387) Furthermore, in nine patients with colon cancer and liver metastases, a daily injection of 5 g micronized resveratrol resulted in a 39% rise in cleaved caspase-3, a marker of apoptosis (388). In a double-blind, randomized, placebo-controlled study, tomato lycopene extract exhibited a chemopreventive effect in colon cancer patients ($n = 56$) via downregulation of insulin-like growth factor-1 levels (389). As well, an Italian case-control study confirmed that high adherence to MD can reduce CRC risk (390). However, more research involving human clinical studies is needed to prove the therapeutic effects of these phytochemical substances in the treatment of CRC.

Future prospects

The future of the MD is rather unclear, and the MD's health-protective qualities might be lost even before we completely realize the activity of the chemicals and the processes by which health results are attained. To maximize the potential health benefits, it is also essential to pay closer attention to

the preservation of traditional foods and a faithful reflection on cultural traditions and the MD diet. Many studies have shown that high adherence to the Mediterranean pattern could significantly reduce the incidence of CRC. Hence, the recommendation of these diet patterns is usually as chemopreventive and in particular, cases can be applied as a complementary treatment to reduce tumor recurrence or protect from second tumors in recovered patients. However, more clinical research is required to determine the suitable and effective food patterns that can be administrated in CRC cases either for prevention or as a therapy. Besides, focusing on investigating the molecular mechanisms of MD components and their phytochemicals will be essential to upgrading the complementary therapies to the rank of established anticancer agents.

Conclusion

The MD components are rich in phytochemicals with spectacular medicinal properties. It is believed that these components exert a nutritional synergy when consumed in combination. Many preclinical and clinical studies have demonstrated the cancer-preventive effects of the natural compounds involved in the MD patterns. Based on the collected facts in this review, these nutraceuticals could prevent CRC by either reducing inflammation or preserving a healthy microbiota in the intestine.

Author contributions

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

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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