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Basic nutritional investigation

Consumption of garlic and lemon aqueous extracts combination reduces tumor burden by angiogenesis inhibition, apoptosis induction, and immune system modulation

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ABSTRACT

Objectives: Dietary agents play an important role in cancer prevention and therapy because of their low toxicity and the perception that they are not a medicine. The aim of the present study was to investigate the anticancer effect of the administration of garlic and lemon aqueous extracts against breast cancer implanted in mice.
Methods: We used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to determine the antiproliferative effect of both extracts and their combinations. Isobolographic method was used to calculate the combination index. Balb/C mice were inoculated with EMT6/P breast cancer cells and received intragastric administration of one of three treatments (garlic alone, lemon alone, or a combination of both). Change in tumor size and survival rates were measured. TUNEL assay was used to measure apoptosis and enzyme-linked immunosorbent assay (ELISA) was used to measure vascular endothelial growth factor expression. Serum levels of interferon-γ, interleukin (IL)-2, IL-4, and IL-10 were measured using ELISA and levels of aspartate transaminase, alanine transaminase, and creatinine were determined.
Results: The combination of both extracts acts synergistically against breast cancer in mice. Of the treated mice, 80% were cured using this combination. This combination inhibited angiogenesis, induced apoptosis, and caused systemic activation in the immune system.
Conclusions: The combination of garlic and lemon aqueous extracts represents a promising option to develop an anticancer food for augmenting conventional anticancer therapies. However, further testing is essential to understand the exact molecular mechanisms of this combination and to test its therapeutic effect against other cancer models.

Introduction

Cancer is one of the main causes of death globally. It is estimated that by 2025, there will be an increased rate of 19.3 million new cases per year [1]. Such estimation is a reflection of the limited efficiency of conventional anticancer therapies [2].

Food plays an important role in cancer development and progression and recent studies showed a clear correlation between decreased cancer risk and the consumption of high-fiber, low-fat diets [3,4]. Additionally, many natural dietary products exhibit anticancer activity by different mechanisms, including metastasis inhibition, immune system activation, apoptosis induction, and augmenting therapeutic effects of anticancer agents [5–7]. Garlic (*Allium sativum* L.) is an edible crop with a wide range of traditional uses in treating different ailments including cancer, diabetes, and cardiovascular diseases [8]. Epidemiologic data suggest a correlation between reduced risk for gastric cancer and high consumption of garlic [9,10]. Similar results were reported for lung cancer, where protective association between raw garlic intake and lung cancer has been observed [11,12]. Experimental studies showed that raw garlic can induce growth arrest and redifferentiation of breast cancer cells in vitro [13]. Further testing revealed that consumption of a single raw garlic meal caused activation of genes related to immunity and apoptosis [14]. Garlic is rich in organosulphur

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compounds such as allicin, alliin, diallyl disulfide, *S*-allylcysteine, diallyl sulfide, and allyl mercaptan. These compounds were detected as major constituents responsible for the antitumor activity of garlic [15,16]. The anticancer activity of various garlic-derived organosulphur compounds is mediated by activation of apoptotic cell death, inhibition of metastasis, immune system modulation, and angiogenesis inhibition [3].

Despite well-documented anticancer effects of garlic extract, its strong smell and flavor reduce its daily consumption (as fresh plant) to very low levels. Additionally, most recipes depend on cooked garlic for food preparation. Recent studies showed that exposure of garlic to high temperatures abrogate its growth inhibition effect [17]. Exposure of garlic to 60 s of microwave heating or 45 min of oven heating destroyed its active allyl sulfur compound production and inhibited its anticancer activity [18]. Most garlic-containing meals are cooked in temperatures exceeding these values, which explains the limited therapeutic effects of cooked garlic. Additionally, heat treatment reduced garlic antioxidant capacity. This reduction is mainly due to the decomposition of some phenolic and sulfur-containing compounds [19].

Citrus fruits are rich in biologically active compounds that may inhibit cancer. Recent studies proved the anticancer activity of citrus peels with superior activity reported for lemon peels [20]. Also daily consumption of citrus fruits is associated with reduced risk for gastric cancer [21].

In this study, a combination consisting of garlic and lemon aqueous extracts was prepared and tested for its anticancer activity in vitro and in vivo. The hypothesis of this study is that lemon extract may reduce the strong smell and flavor of garlic extract and make it more suitable for consumption. Additionally, biologically active phytochemicals in lemon extract may act synergistically to enhance the anticancer activity of garlic phytochemicals.

Materials and methods

Animal care and use were conducted according to standard ethical guidelines, and all of the experimental protocols were approved by the Research and Ethical Committee at the Faculty of Pharmacy—Applied Science University. All experiments were carried out in accordance with the recommendations of the Research and Ethical Committee of the Faculty of Pharmacy at the Applied Science University.

Plant material and extracts preparation

Fresh garlic (*Allium sativum* L.) bulbs and lemon (*Citrus limon* L.) fruits were provided from local farms in Jordan. Plant materials were washed and dried in a shed. Peeled garlic bulbs and whole lemon fruits (peels and fleshy parts) were used to prepare extracts. To mimic the method for preparing lemon juice and other recipes, water was selected as a solvent to prepare extracts. We chopped 500 g of each plant material into small pieces and vigorously mixed them with 1 L of distilled water using electric mixer. The resulted solution was filtered to remove insoluble material and diluted to prepare different concentrations for each extract. The stock solution (500 mg/L) was diluted by tissue culture media to prepare increasing concentration (30–100 mg/mL) of each extract. Extracts were freshly prepared before each experiment to avoid deterioration due to storage.

Mice, cell line, and culture conditions

Forty Balb/C female mice (4–6 wk old, weight 21–25 g) were used in this study. Animals were kept in separate cages with bedding of wooden shavings. The temperature of the animal house was 25°C with alternating 12-h light/dark cycles and continuous air ventilation. The mouse mammary cell line (EMT6/*P*, ECACC 96042344) was purchased from the European Collection of Cell Cultures (Salisbury, UK). Minimum essential medium was used to culture EMT6/P cells and was supplemented with 10% fetal calf serum, 1% L-glutamine, 0.1% gentamycin, and 1% penicillin-streptomycin solution. Incubation conditions were 37°C, 5% carbon dioxide, and 95% humidity.

Antiproliferative assay

Actively growing EMT6/P cells were harvested by trypsinization and dispensed into 96-well tissue culture flat bottom plates at a concentration of 13 000 cells/well for overnight incubation. After incubation, cells were exposed (in triplicate) to increasing concentration of garlic aqueous extract (30–100 mg/mL), lemon aqueous extract (30–100 mg/mL), and different combinations of both extracts. All extracts were sterilized by filtration using 0.2-um syringe filters. Cells were incubated for 48 h; then cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. A microplate reader (Biotek, Winooski, VT, USA) was used to measure resulting color at 595 nm. Percentage cell survival was measured for all treatments and compared with untreated cells. Untreated cells were used as negative controls and cells treated with vincristine sulfate were used as positive controls.

Combination index calculation

The mode of interaction between garlic and lemon aqueous extracts was determined using isobolographic approach. The combination index (CI) was calculated for combinations of the two extracts against EMT6/P cells and results were interpreted as described here [22]:

$$CI = (D)\,1/(Dx)\,1 + (D)\,2/(Dx)\,2 + \alpha\,(D)\,1\,(D)\,2/(Dx)\,1\,(Dx)\,2$$

where (Dx) 1 = dose of garlic extract to produce 50% cell kill alone; (D) 1 = dose of garlic extract to produce 50% cell kill in combination with lemon extract; (Dx) 2 = dose of lemon extract to produce 50% cell kill alone; (D) 2 = dose of lemon to produce 50% cell kill in combination with garlic extract; $\alpha = 0$ for mutually exclusive or 1 for mutually nonexclusive modes of drug action. Interpreted as: CI >1.3 antagonism; CI 1.1 to 1.3 moderate antagonism; CI 0.9 to 1.1 additive effect; CI 0.8 to 0.9 slight synergism; CI 0.6 to 0.8 moderate synergism; CI 0.4 to 0.6 synergism; CI 0.2 to 0.4 strong synergism.

Antitumor activity on experimental animals

Actively growing EMT6/P cells were harvested by trypsinization and tested for their viability using trypan blue exclusion method. A tumor induction dose of 100 000 cells (in 0.1 mL) was injected subcutaneously in the abdominal area of each female Balb/C mouse. Injected cancer cells were allowed to grow for 14 d to form tumors and the dimensions of the new tumors were measured using digital caliper. The following formula: (A × B2 × 0.5) was used to measure tumor volumes. Where A was the length of the longest aspect of the tumor and B was the length of the aspect perpendicular to A [23]. Tumor-bearing mice were divided into four groups with 10 mice in each group. Group 1 was the control group and mice were injected intraperitoneally with vehicle (phosphate-buffered saline) 0.1 mL daily. Group 2 mice were gavaged daily with 50 mg/kg of garlic extract. Group 3 mice were gavaged daily with 50 mg/kg lemon extract. Group 4 mice were gavaged daily with 50 mg/kg garlic extract + 50 mg/kg lemon extract. All treatments continued for 14 d. Tumors were remeasured at the end of the treatment and mice were sacrificed, tumors removed and stored in 10% formalin.

Histologic evaluation of tumor sections

Paraffin sections were prepared from fixed tumors followed by staining using hematoxylin and eosin staining procedure. A light microscope (Zeiss, Munchen, Germany) equipped with a computer-controlled digital camera (Canon, Taipei, Taiwan) was used to examine stained slides.

Detection of apoptosis in tumor sections

The degree of apoptosis induction of different treatments was detected using the DeadEnd TUNEL colorimetric apoptosis detection system (Promega, Madison, WI, USA). Kit instructions were followed to stain paraffin-embedded tumor sections. Briefly, tumor sections were exposed to serial treatments for paraffin removal, gradual dehydration, and then fixation using 10% buffered formalin. Proteinase K solution (20 μg/mL) was added to each slide followed by refixation. Equilibration of sections was performed using equilibration buffer for 5 to 10 min at room temperature. Fragmented DNA was labeled by incubation with rTdT reaction mixture at 37°C in a humidified chamber. 2X SSC termination solvent was used to terminate the reaction and horseradish peroxidase (HRP)-labeled streptavidin was added followed by incubation with DAB for 20 min in the dark for color development. Finally, stained slides were mounted by glycerol and examined under the light microscope.

Measuring VEGF *expression in cultured EMT6/P cells*

Mouse vascular endothelial growth factor (VEGF) enzyme-linked immunosorbent assay (ELISA) kit (Sigma, Missouri, USA) was used to measure the effect of different treatments on the levels of expression of *VEGF*. Actively growing EMT6/P cells were cultured in small flasks at a concentration of 1.5 × 105 cell/mL and exposed to one of the following treatments for 48 h: 80 mg/mL garlic extract, 90 mg/mL lemon extract, 80 mg/mL garlic extract + 90 mg/mL lemon extract, and negative control, which only contained tissue culture media. Kit instructions were followed to process cells. Briefly, treated cells were collected and lysed using lysis buffer. After centrifugation, supernatants were collected and 100 μL were dispensed in each well of the 96-well microplates coated with *VEGF* capture antibody followed by incubation for 2.5 h then adding 100 μL of biotinylated detection antibody with 1 h incubation. Finally, 100 μL of HRP-conjugated streptavidin was added followed by adding 100 μL of 3,3′,5,5′-tetramethylbenzidine substrate solution with 30 min incubation in dark. Intensity of developed color was measured at 450 nm.

Detection of IFN-γ, IL-2, IL-4 and IL-10 serum levels

Mouse T helper (Th)1/Th2 ELISA kit (Affymetrix eBioscience, California, USA) was used to measure the serum levels of interferon (IFN)-γ, interleukin (IL)-2, IL-4, and IL-10. Serum samples were prepared from blood collected from different treated groups. Cytokine levels were measured following kit instructions.

Measurement of aspartate transaminase, alanine transaminase, and creatinine serum levels

Aspartate transaminase (AST), alanine transaminase (ALT), and creatinine in serum samples were measured using commercially available kits (BioSystems, Barcelona, Spain).

Thiosulfonate determination and qualitative phytochemical screening

Thiosulfonate concentration in the extracts was measured using a previously described method [24]. The principle of this method depends on using hexane to extract thiosulfonate and measuring the extinction of the solution at 254 nm. Briefly, 0.5 g of dry extract was dissolved in 25 mL distilled water. Five mL of the solution were measured into a 100-mL Erlenmayer flask followed by adding 10 mL hexane with gentle swirling. After separation of the hexane layer, the aqueous layer was returned to the flask and 5 mL hexane was used to extract the remaining thiosulfonate. Both hexane extracts were combined and the absorbance was taken at 254 nm. The following equation was used to calculate the thiosulfonate concentration:

$$C = A/\varepsilon \times b$$

Where A is absorbance; b is path length (cm), C is the concentration of the solution (μmol/g), and ε is the molar absorptivity of thiosulfonate solution at 254 nm = 0.014 g/μmol cm.

Qualitative phytochemical screening of flavonoids, alkaloids, terpenoids, and phenols was conducted using standard procedures described by Evans [25].

Gas chromatography coupled to tandem mass spectrometry analysis

The Gas Chromatograph 2010 (ultra; Shimadzu, Tokyo, Japan) interfaced to an 8030 mass detector and controlled by GC MS LabSolution software was used to identify compounds in the combination extract. The glass apparatus was washed with hot water and detergent, rinsed three times with tap water, dried in an oven at 105°C for 1 h, and then cooled to room temperature. Sample bottles and equipment were rinsed with acetone followed by dimethylsulphoxide before use. Test samples were shaken and mixed using the ultrasound path for 6 min, then filtered using glass wool. The sample was drawn into small vials and then 1 μL was injected into the gas chromatography (GC)-mass

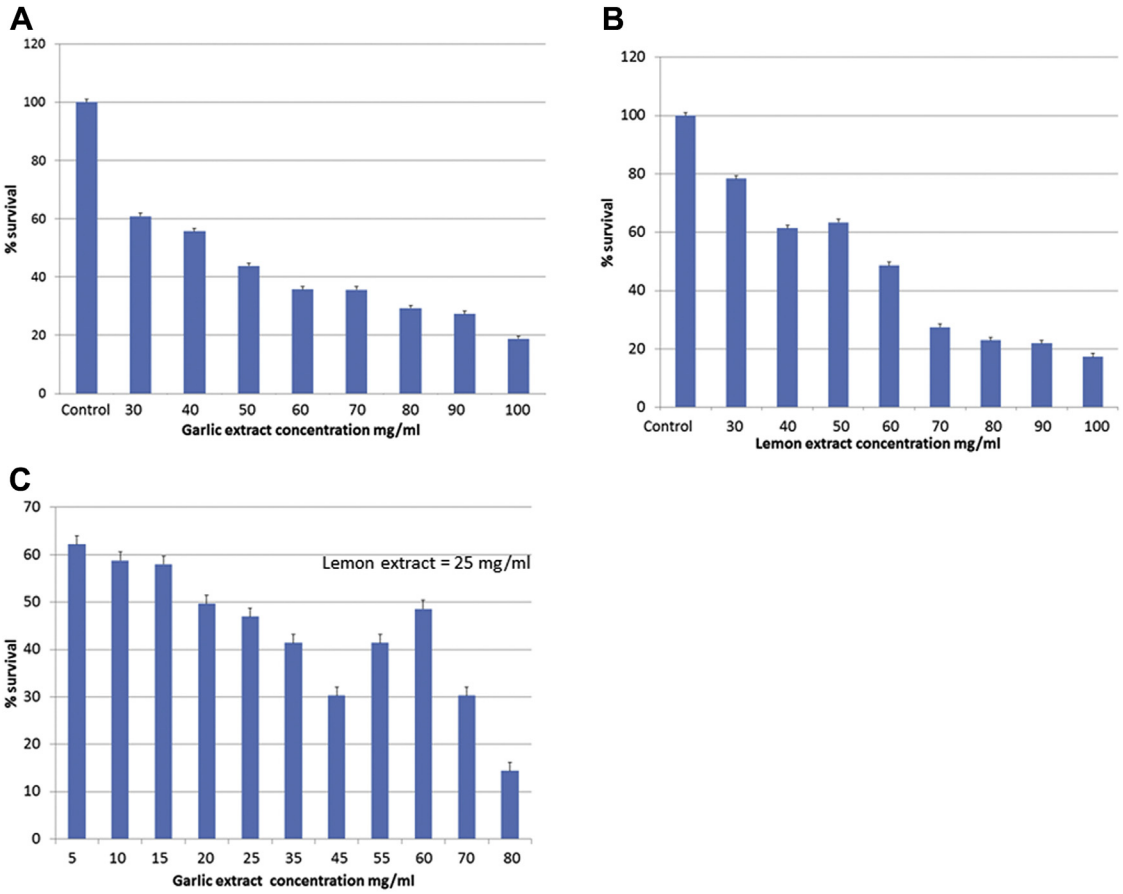

Fig. 1. Antiproliferative effect of aqueous extracts of garlic, lemon, and their combination on the viability of EMT6/P. (A) Treatment of EMT6/P cells with increasing concentration of garlic aqueous extract. (B) Treatment of EMT6/P cells with increasing concentration of lemon aqueous extract. (C) Treatment of EMT6/P cells with increasing concentrations of garlic extract combined with fixed lemon extract concentration (25 mg/mL).

Table 1
The IC_{50} values (mg/mL) and CI for garlic and lemon aqueous extracts against EMT6/P cell line

IC_{50} of garlic extract	IC_{50} of lemon extract	IC_{50} of garlic extract in combination	IC_{50} of lemon extract in combination	IC_{50} of vincristine sulfate (μM)	CI	Interpretation
78.91	89.32	25	24.59	54	0.673	Synergism

CI, combination index

spectrometry (MS). The chromatographic separation was achieved using a HP5-MS capillary column (30.0 m × 250 m × 0.25 m). The column stationary phase comprised of 5:95% diphenyl:dimethylpolysiloxane blend. The operating GC condition was an initial oven temperature of 35°C for 3 min, then programmed to 280°C at the rate of 100°C/min, and then kept constant at 280°C (23 min). The injector and detector temperatures were set at 270°C and the carrier gas was nitrogen flowing at a rate of 1.2 mL/min. The MS was operated in the electron impact mode at 70 eV. Ion source and transfer-line temperature was kept at 280°C. The MS were obtained by centroid scan of the mass range from 40 to 800 amu. Identification of the constituents was done on the basis of retention index, library mass search database (NIST and WILEY) and by comparing with the mass spectral data.

Statistical analysis

Data are presented using mean ± SE from three independent experiments. The statistical significance among the groups was determined by using one-way analysis of variance. $P < 0.05$ was considered significant. The IC_{50} values obtained with the different concentrations of garlic or lemon extracts or a combination of the two were calculated using nonlinear regression in Statistical Package for the Social Sciences version 18 (SPSS Inc. Chicago, IL, USA).

Results

Garlic and lemon extracts act synergistically to inhibit breast cancer cell line

A dose-dependent inhibition of cell growth and proliferation was observed after treatment of EMT6/P cells with serial dilutions of garlic aqueous extract (30–100 mg/mL) with IC_{50} value of 78.90 mg/mL (Fig. 1A). Similar inhibition was observed after treating cells with serial dilutions of lemon extract with IC_{50} value of 89.32 mg/mL (Fig. 1B). A synergistic interaction was reported for combinations of garlic and lemon aqueous extracts with a reduction in IC_{50} values for both extracts and CI value of 0.673 (Table 1 and Fig. 1C). Significant linear inhibition was observed with increasing concentration of all treatments.

Combination therapy enhanced angiogenesis inhibition

Treatment of EMT6/P cells with garlic aqueous extract (80 mg/mL) caused significant ($P < 0.05$) inhibition in the level of *VEGF* expression compared with the negative control. The levels of *VEGF* were 324.69 for garlic extract and 848.7 pg/mL for the negative control. Inhibition of *VEGF* was significant in cells treated with lemon aqueous extract (90 mg/mL), which caused a reduction in *VEGF* levels to 491.5 pg/mL. However, treatment of cells with a combination of both extracts resulted in the most important inhibition compared with other treatments with *VEGF* levels of 144.90 pg/mL (Fig. 2).

Combination therapy reduced tumor size and increased survival percentage

Daily treatment of tumor-bearing mice with garlic extract (50 mg/kg) for 14 d caused significant ($P < 0.05$) decrease in tumor size with a percentage change in tumor size of (−87.43%) compared with an increase of 566.45% in the control group. A slightly lower percentage change (−83.04%) was observed in the group treated with lemon extract (50 mg/kg). Combining both treatments resulted in the highest reduction in tumor size (−91.69%; Table 2). Tumors disappeared in 60% of the mice treated with either extract. However, the highest percentage (80%) of undetectable tumors was reported for mice treated with the combination therapy (Fig. 3). Slight differences in average tumor weight were recorded for garlic and lemon extract groups with average tumor weight of 0.403 and 0.0384 g, respectively. However, these values were significantly ($P < 0.05$) lower than the value of the negative control (1.958 g). On the other hand, the lowest average tumor weight was reported in the combination group with average tumor weight of 0.16 g. All treated groups exhibited reduction in body weight with percentage change in body weight around (−6%) compared with the negative control group, which showed an increase of 0.94% in body weight (Table 2).

Garlic and lemon extracts enhanced apoptosis and induced geographic necrosis

Apoptosis was detected in tumor sections obtained from mice treated with either garlic or lemon extracts. The degree of apoptotic cells was highest in tumor sections obtained from mice that received a combination of garlic and lemon extracts (Fig. 4).

Small necrotic regions were observed in tumor sections treated with lemon extract. The necrotic regions were larger in tumor sections of mice treated with garlic extract. However, the most potent effect was reported for tumor bearing mice treated with combination therapy, which induced geographic necrosis in tumor sections (Fig. 5).

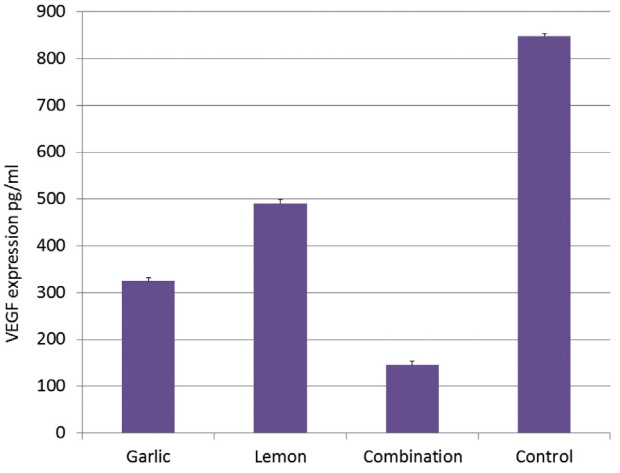

Fig. 2. The effect of different treatments on the expression of vascular endothelial growth factor (VEGF). Concentration of VEGF (pg/mL) in cells treated with 80 mg/mL garlic extract, 90 mg/mL lemon aqueous extract, and a combination of both, as well as in untreated control cells. Each treatment was performed in duplicate. Results are expressed as means (*bars*) ± SEM (*lines*). The lowest VEGF levels were detected in the combination group. *Significant values.

Table 2
Effect of different treatments on tumor size and cure percentage

Treatment	Initial tumor size (mm) ± SEM	Final tumor size (mm) ± SEM	% Change in tumor size	% Of mice with no detectable tumor	Average tumor weight (g)	% Change in body weight
Garlic extract	207.491 ± 12.146	26.077 ± 16.159	−87.432*	60%	0.403*	−6.23*
Lemon extract	199.511 ± 8.676	33.83 ± 14.383	−83.043*	60%	0.384*	−5.96*
Combination	212.994 ± 11.695	17.685 ± 9.539	−91.696*	80%	0.160*	−6.07*
Negative control	202.117 ± 19.292	1347.017 ± 21.303	566.454	30%	1.958	+0.94

* One-way analysis of variance was performed to evaluate the effect of each treatment on change in tumor size; $P < 0.05$ was considered statistically significant.

Combination therapy increased serum levels of IFN-γ, IL-2, and IL-4

Treatment of tumor-bearing mice with garlic extract resulted in an increase in serum levels of IL-2 and IL-4 with values of 267.30 and 278.5 pg/mL, respectively. The same treatment caused no change in serum levels of IFN-γ (391.5 pg/mL) and a decrease in serum levels of IL-10 (197.3 pg/mL) compared with untreated tumor-bearing mice that exhibited values of 390, 246.7, 190.1, and 347.8 pg/mL for IFN-γ, IL-2, IL-4, and IL-10, respectively. On the other hand, different results were obtained for mice treated with lemon extract with a decrease in IFN-γ and IL-2 serum levels to 80.7 and 128.4, respectively. This treatment also increased the level of IL-4 (210.08 pg/mL) and decreased the level of IL-10 (269.1 pg/mL). Tumor-bearing mice treated with a combination of both extracts showed the highest levels of IFN-γ, IL-2, and IL-4 with serum levels of 438, 423.8, and 349.2 pg/mL, respectively (Fig. 6).

Combination therapy exhibit no liver or kidney toxicity

Serum levels of liver enzymes (AST and ALT) and creatinine were lower than normal control for all treatments. AST and ALT serum levels in the untreated group were lower than normal control group. However, creatinine levels in this group were significantly ($P < 0.05$) higher than the reported levels in the normal group with creatinine levels of 223.85 and 78.69 μmol/L, respectively (Fig. 7).

Phytochemical screening

Phytochemical screening of the three extracts revealed the presence of high concentrations of thiosulfonate in garlic (112.14 μmol/g) and combination (132.14 μmol/g) aqueous extracts. On the other hand, low levels of thiosulfonate (7.29 μmol/g) were detected in the lemon aqueous extract (Table 3). High levels of flavonoids and terpenoids were observed in garlic extract and were low or absent in lemon extract. Low levels of alkaloids were detected in garlic extract and medium levels were measured in lemon extract. Phenolic compounds were absent in garlic extract and detected in medium concentrations in lemon extracts. All tested phytochemicals were detected in the combination aqueous extract with the highest levels of alkaloids followed by terpenoids and phenolic compounds. Flavonoids were detected in low levels in the combination of two extracts (Table 3).

Fig. 3. Effect of different treatments on tumor size and cure percentage. Combination therapy resulted in the highest cure percentage and smallest tumors size. There were 10 mice in each group.

Allicin and limonene are the major components in the combination extract

Further analysis of the combination aqueous extract using GC-MS/MS revealed the presence of high concentration of allicin (thiosulfonate) and limonene with percentages in the extract of 24.398% and 23.271%, respectively (Table 4). Most detected compounds are originated from garlic extract. These compounds include 3-Vinyl-1,2-dithiacyclohex-4-ene (11.009%), α-Zingiberene (6.859%), 3-Chlorothiophene (3.894%), and Diallyl disulphide (3.725%). The remaining compounds were detected in lower percentages (Table 4).

Discussion

In recent years, there has been a growing preference for using natural products in different fields, including agriculture and medicine. This trend is based on the assumption that natural products are available and less toxic compared with their chemically synthesized counterparts [26]. In the present study, a combination of garlic and lemon aqueous extracts was tested to inhibit breast cancer in vitro and in vivo. The combination of two extracts exhibited superior anticancer activity compared with single aqueous extracts.

The in vitro results showed potent antiproliferative activity of garlic aqueous extracts against mouse breast cancer cell line. The antiproliferative effect of this extract was further confirmed by its ability to reduce tumor size, inhibit *VEGF* expression, and apoptosis induction. These results are consistent with the previous studies that reported the ability of garlic extracts to inhibit proliferation and induce apoptosis in breast, prostate, hepatic, colon, and mouse macrophageal cell lines [27]. Inhibition of angiogenesis was reported as an anticancer mechanism of garlic extract [28]. The anticancer activity of garlic extracts is a result of the presence of organosulphur compounds such as allicin, diallyl disulfide, and diallyl sulfide [29]. Allicin is well known for its anticancer activity against various cancer cell lines by inducing apoptosis both in caspase-dependent and caspase-independent manner [30,31]. The biosynthetic pathway of this compound involves the activity of alliinase, which hydrolyze alliin to

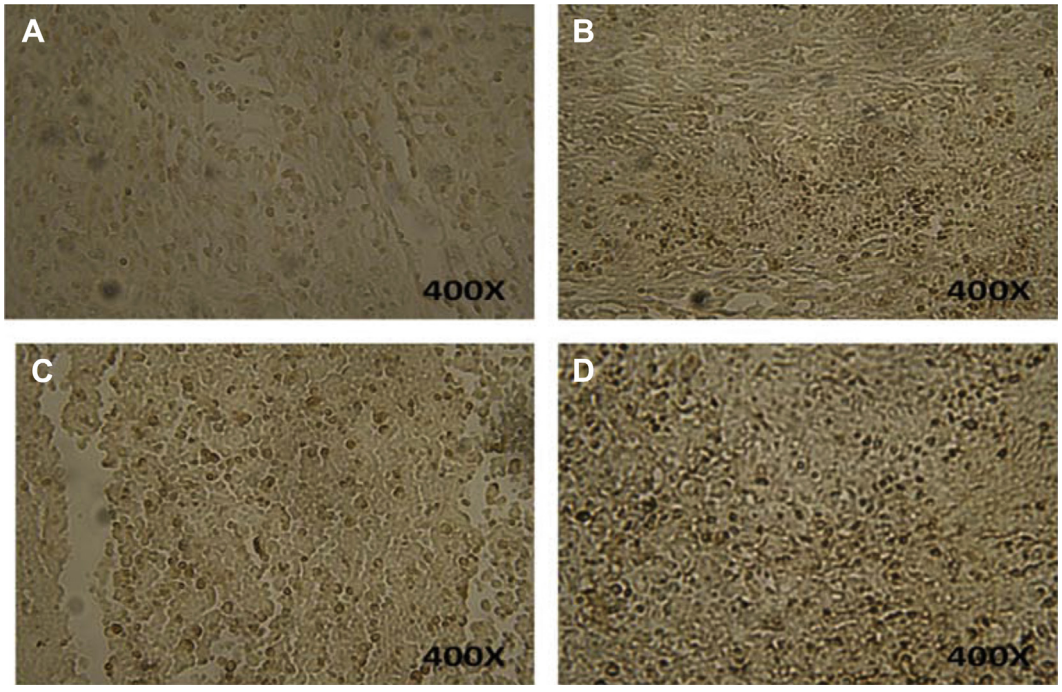

Fig. 4. Tumor sections assayed by DeadEnd colorimetric TUNEL system to indicate cell apoptosis: (A) negative control; (B) tumors treated with 50 mg/kg garlic extract; (C) tumors treated with 50 mg/kg lemon extract; (D) tumors treated with a combination of garlic and lemon extracts. Brown stained nuclei indicate DNA fragmentation and nuclear condensation. Tumors of five mice for each treatment were examined to detect apoptosis.

produce allyl sulfenic acid, which condenses spontaneously to produce allicin [32]. The yield of allicin and other organosulphur compounds is the highest at 35°C and under acidic environment [33]. Phytochemical screening of garlic aqueous extract showed the presence of high concentrations of thiosulfonate, flavonoids, and terpenoids. Although thiosulfonate are the most active anticancer agents in garlic extract, the observed anticancer effect may be explained by the combined effect of these phytochemicals.

In our attempts to enhance the anticancer activity of garlic extract, we prepared a combination consisting of garlic and lemon aqueous extracts. This combination increased garlic anticancer activity by providing the acidic environment needed to enhance organosulphur compounds production and by adding more phytochemicals with possible anticancer activity.

In the present study, lemon aqueous extract exhibited high ability to inhibit breast cancer cells in vitro. Its activity was obvious in vivo by reducing tumor size, inhibiting *VEGF*

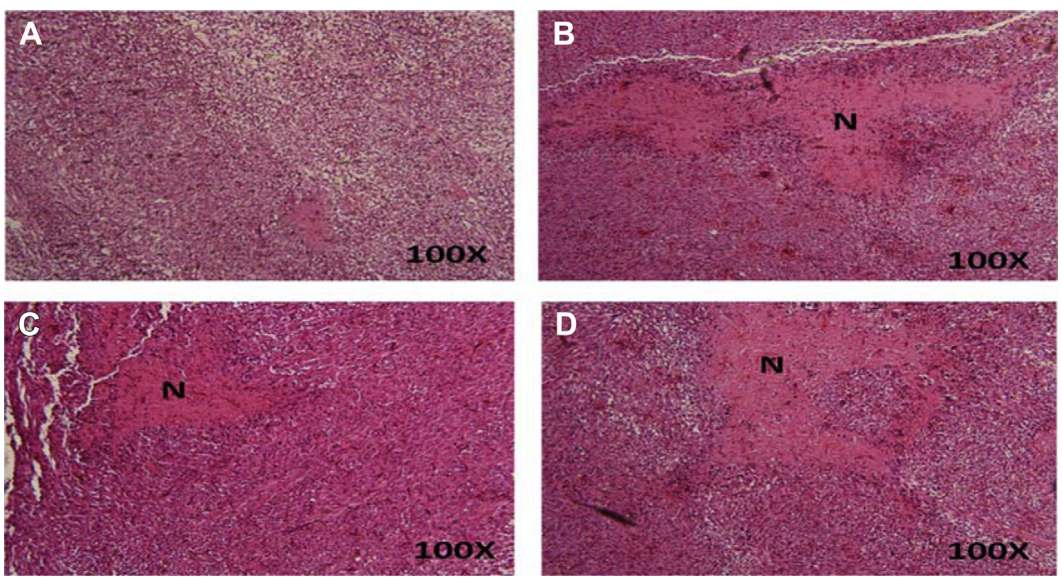

Fig. 5. Hematoxylin and eosin staining of tumors treated with vehicle: (A), 50 mg/kg garlic extract (B), 50 mg/kg lemon extract (C), and a combination of garlic and lemon extracts (D). Extensive necrosis was evident in tumors treated with a garlic extract and combination therapy. Five mice were examined for each treatment. N, necrotic area.

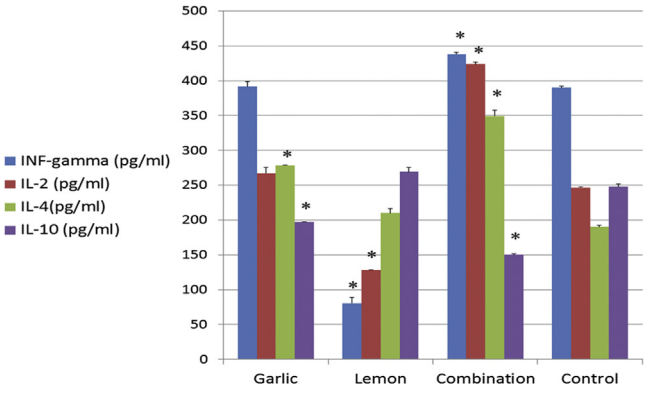

Fig. 6. Effect of different treatments on serum levels of IFN-γ, IL-2, IL-4, and IL-10. Concentration of serum cytokines (pg/mL) in mice treated with 50 mg/kg garlic extract, 50 mg/kg lemon aqueous extract, and a combination of both, as well as in untreated control cells. Each treatment was performed in duplicate. Results are expressed as means (*bars*) ± SEM (*lines*). The highest level of IFN-γ and IL-2 were detected in the combination therapy. IFN, interferon; IL, interleukin. *Significant values.

expression, and apoptosis induction. These results agree with the previous studies that showed high anticancer activity of lemon extract against breast cancer [20]. Lemon extract contains many biologically active phytochemicals including D-limonene. This compound is a monocyclic monoterpene and represents a major component of citrus oils [34]. Inductions of apoptosis and angiogenesis inhibition are the main mechanisms of action of limonene against cancer [35]. In the present study, high concentrations of limonene were detected in the combination as well as lemon extract. The observed anticancer effect of lemon extract could be explained by the presence of high concentrations of limonene in lemon extract. Phytochemical analysis revealed the presence of phenolic compounds, flavonoids, and alkaloids in lemon aqueous extract [36]. These results are consistent with the previous study that showed the presence of phenols, alkaloids, flavonoids, and terpenoids in lemon juice. Additionally, the results obtained in the present study are in agreement with previous studies that reported the presence of high flavonoid concentrations in lemon juice. High concentrations of hesperidin, eriocitrin, and diosmin were detected in lemon juice [37]. Apoptosis induction was determined as a method of action of citrus hesperidin to inhibit human hepatocellular carcinoma [38]. Apoptosis induction also was observed

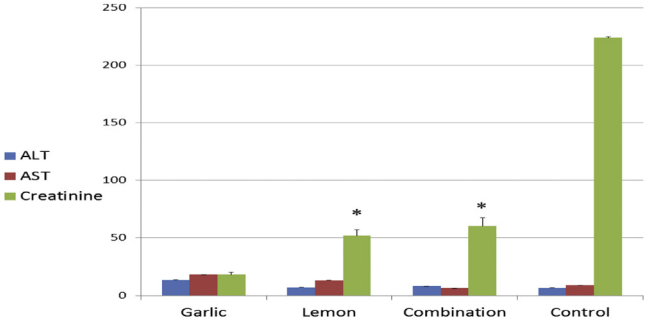

Fig. 7. Effect of different treatments on serum levels of AST, ALT, and creatinine. Concentrations of AST and ALT are expressed by IU/L and serum creatinine levels are expressed by μmol/L. Mice were treated with 50 mg/kg garlic extract, 50 mg/kg lemon aqueous extract, and a combination of both. Each treatment was performed in duplicate. Results are expressed as means (*bars*) ± SEM (*lines*). ALT, alanine transaminase; AST, aspartate transaminase. *Significant values.

Table 3
Phytochemical screening of garlic, lemon, and combination aqueous extracts

Ingredient	Garlic	Lemon	Combination
Thiosulfonate, μmol/g	112.14	7.29	132.14
Flavonoids	+++	+	+
Phenolic compounds	–	++	++
Alkaloids	+	++	+++
Terpenoids	+++	–	++

–, absent; +, low concentration; ++, medium concentration; +++, high concentration

after treatment of hepatocellular carcinoma with eriocitrin from lemon [39]. Additionally, diosmin-induced genotoxicity and apoptosis in prostate cancer cells [40].

Testing the anticancer effect of a combination consisting of garlic and lemon aqueous extracts revealed more potent anticancer effects in vivo and in vitro. The combination worked synergistically to inhibit breast cancer cells proliferation in vitro and reduced tumor size >90% in vivo. Additionally, extensive apoptosis, geographic necrosis, potent *VEGF* inhibition, and enhanced production of IFN-γ, IL-2, and IL-4 cytokines were observed as anticancer mechanisms of this combination. The combined effect of these mechanisms resulted in 80% tumor disappearance of treated mice, which was higher than the percent observed in single extract therapy. On the other hand, 30% of mice in the negative control group had no detectable tumors at the end of the study. This spontaneous tumor regression reduces the observed antitumor effect to lower values and different results may be obtained if different cell line was used. This result is consistent with the previous study that reported the ability of tomato and garlic combination to induce apoptosis and inhibit carcinoma development [41]. However, the results obtained in the combination of garlic and lemon aqueous extracts showed a potent anticancer response mediated by diverse anticancer mechanisms. The presence of different groups of phytochemicals in the combination aqueous extract may explain the activation of diverse anticancer mechanism including immune modulation. The observed angiogenesis inhibition in the group treated with combination extract is mainly due to the presence of high concentrations of antiangiogenic agents. Allicin has the highest concentration in the combination extract. This compound exhibits high ability to inhibit angiogenesis by targeting *VEGF* as well as other basic steps in the angiogenesis process [42]. The second highly concentrated compound in the combination extract is limonene, which was detected in levels close to the levels of allicin. Limonene antiangiogenic activity was explained by its ability to block *VEGF* binding with its receptor [43]. IFN-γ and IL-2 are the signature cytokines for Th1 anticancer immune response and IL-4 is the cytokine dominating Th2 immune response. Balanced ratio of Th1/Th2 cytokines was observed in healthy individuals. High levels of Th2 cytokines were observed in patients with different types of cancer [44]. In the present study, garlic induced high levels of IFN-γ, whereas lemon extract caused an increase in IL-4 levels. The combination of garlic and lemon extracts caused systemic activation in the immune system resulting from an increase in IFN-γ, IL-2n, and IL-4 levels. This enhanced immune activation augments other anticancer mechanisms to get the high cure percentage obtained in this study. In the present study, high levels of sulphur-containing compounds were detected in the combination extracts. Immunomodulatory effect of these compounds was reported previously [45] and enhanced natural killer activity was reported due to increased IFN-γ expression after allicin treatment [46]. An increase in IL-2 and IL-4 production was observed

Table 4
Major compounds identified in the combination aqueous extract using GC-MS/MS method

No	Compound	Molecular formula	MW	% In combination aqueous extract	Source
1.	Allicin	$C_6 H_{10} OS_2$	162.26	24.398	G
2.	Limonene	$C_{10} H_{16}$	136	23.271	L
3.	3-Vinyl-1,2-dithiacyclohex-4-ene	$C_6 H_9 S_2$	144	11.009	G
4.	α-Zingiberene	$C_{15} H_{24}$	204	6.859	G
5.	3-Chlorothiophene	$C_4 H_3 ClS$	118	3.894	G
6.	2-Butanone,4-(-hydroxy-3-methoxyphenyl) azafluorenone, phenylimine	$C_{11} H_{14} O_3$	194	3.867	G
7.	Diallyl disulphide	$C_6 H_{10} S_2$	146	3.725	G
8.	3-Vinyl-[1,2-dithiacyclohex-5-ene thiadiazol-5-yl]-,ethylester	$C_6 H_9 S_2$	144	2.467	G
9.	Methanehydrazonic acid, *N*-[3-(methylthio)-1,-2,4-thiadiazol-5-yl]-,ethylester	$C_6 H_9 N_4 OS_2$	218	2.426	G
10.	1,4-Diathiane	$C_4 H_8 S_2$	120	1.449	G
11.	1-propene-3, 3-thiobis	$C_6 H_{10} S$	114	1.148	G
12.	Disulphide, methyl-2-propenyl	$C_4 H_8 S_2$	120	1.113	G
13.	Methyl-4-deoxy-2-0-methyl.beta.1-threo-hex-4-	$C_8 H_{12} O_4$	204	1.031	G
14.	Disulphide, methyl-2-propenyl	$C_4 H_8 S_2$	120	0.924	G
15.	Acetamide, n-tetrahydrofurfuryl-2-methoxy methylbutyramide	$C_8 H_{15} NO_3$	173	0.875	G
16.	Acetic acid, chloro-2-butoxyethyl ester	$C_6 H_{15} ClO_3$	196	0.746	G
17.	1,2,3-Thiadiazole,5-methyl-	$C_3 H_4 N_2 S$	100	0.708	G
18.	Amidinothiourea	$C_2 H_6 N_4 S$	118	0.683	G
19.	Propanoic acid, 2-chloro	$C_6 H_5 ClO_2$	108	0.508	G
20.	Ethyl trifluoromethyl trisulphide	$C_3 H_5 F_3 S_3$	194	0.456	G

G, garlic extract; GC-MS/MS, gas chromatography coupled to tandem mass spectrometry; L, lemon extract; MW, molecular weight

after treatment of rat lymphocytes with garlic extract [47]. Our results are consistent with these findings and garlic extract is the main stimulator of the immune system in our combination as low levels of these cytokines were detected in lemon extract single treatment.

Further analysis of the combination extract revealed the presence of allicin, limonene, α-Zingiberene, and diallyl disulphide as major components in the extract. These compounds occupy more than 50% of total compounds identified in extract. Allicin was identified as a major anticancer component in garlic aqueous extract [48] and its apoptosis inducing ability is well documented [49]. Apoptosis induction was reported for limonene as its main anticancer mechanism [50]. On the other hand, cytotoxic activity of α-Zingiberene was observed against various cell lines [51], whereas induction of apoptosis and differentiation, histone modification, and inhibition of angiogenesis and invasion are the main anticancer mechanisms activated by diallyl disulphide [52]. In the combination used in the present study, the combined anticancer effects of these four compounds in addition to other active minor ingredients are expected to cause tumor regression by activating different anticancer mechanisms. Garlic extract was more active in immunomodulatory and anti-angiogenic activities, whereas a similar apoptosis-inducing effect was observed for both extracts.

Conclusions

The present study demonstrated that using a combination of garlic and lemon aqueous extracts inhibited breast cancer in mice. The anticancer activity of this combination is mediated by apoptosis induction, angiogenesis inhibition, and immune system modulation. The combination is safe and showed no liver or kidney toxicity. Combination of garlic and lemon extract can be used for future development of anticancer functional foods. However, careful evaluation using larger sample size and more molecular techniques is needed to fully understand the anticancer effect of this combination. Although this combination showed high therapeutic activity against Balb/C breast cancer, this effect may be tissue specific, and further testing is necessary to explore the therapeutic spectrum of this combination against other cancer models.

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