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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.