



***In vitro* anti-proliferative activity of clove extract on human gastric carcinoma**

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Abstract

Background and objectives: Cancer cell resistance to common chemotherapy agents is on rise. Plants are considered valuable sources of herbal drugs for cancer therapy. The present study was conducted to investigate the *in vitro* antioxidant, anti-proliferative, and apoptosis-inducing properties of clove (*Syzygium aromaticum* L.) extract in human gastric carcinoma (AGS). **Methods:** Crude ethanol extract of *S. aromaticum* dried buds was prepared and *in vitro* anti-proliferative effects of the extract on AGS and normal Human dermal fibroblasts (HDF) cell lines were studied by MTT assay. To examine apoptosis induction, AGS cells were incubated with IC₅₀ concentrations of the extract, stained with propidium iodide (PI) and annexin V-fluorescein isothiocyanate (FITC), and analyzed by flow cytometry. Antioxidant activity and total phenolics and flavonoids contents were evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, Folin-Ciocalteu method, and aluminum chloride colorimetric method, respectively. **Results:** The IC₅₀ of DPPH and total phenolics and flavonoids contents of the extract were 10.05±1.93 µg/mL, 225.6±40 mg GAE/g, and 29.30±2.35 mgRUT/g, respectively. The IC₅₀ of the extract against HDFs was 649 µg/mL, higher than AGS cells, which was 118.7 µg/mL at 48 h after treatment. Flow cytometric analysis showed that the extract induced cell apoptosis. **Conclusions:** Crude ethanol *S. aromaticum* extract had high total phenolics content, and suppressed the proliferation of human gastric cancer cells, likely due to apoptosis induction. Further studies should be conducted to determine the mechanisms of its anticancer effects.

Keywords: antioxidant, apoptosis, human gastric carcinoma, proliferation, *Syzygium aromaticum*

Introduction

Cancer is a leading cause of death in the world and the number of cancer cases and deaths is expected to grow rapidly [1]. In 2012, the highest stomach cancer incidence and mortality rates among both males and females were found in Eastern and Western Asia, Latin America, and

some former Soviet European countries [2]. Most patients diagnosed with gastric cancer need chemotherapy, surgery or radiotherapy [3]. However, chemotherapy is widely known to lead to emesis, nephrotoxic toxicity and multi-drug resistance [4]. Therefore, it is necessary to find

new agents that can be used to enhance the anti-cancer effects of common chemotherapeutic drugs currently being used for gastric cancer treatment [5-7].

In recent years, due to the relatively inexpensive and nontoxic properties, phytochemical containing herbal medicine extracts have been evaluated for their anti-cancer effects [8,9] and also for treatment of some of human diseases [10-12]. Some of these extracts have been shown to have promising results [13-20].

Syzygium aromaticum L. commonly known as clove is an evergreen tropical plant which has been used for culinary and medicinal purposes, either alone or in combination with other species, in Europe, Asia and some other regions of the world [21]. The different species of *Syzygium* contain a wide spectrum of important chemical compounds such as sesquiterpenes [22], tannins[23], triterpenoids [24] and a phenolic compound called eugenol (4-allyl-2-methoxyphenol). Eugenol seems to act as an antioxidant, carminative, antispasmodic, antiseptic, and antimicrobial agent [25,26] and also has antimutagenic property [27]. Regarding that the prognosis of gastric cancer patient is very low, and the toxicity associated with therapy remains a problem, it is necessary to seek out new compounds and more optimal treatments for gastric cancer. Therefore, this research was aimed to evaluate the *in vitro* anti-proliferative, antioxidant, and apoptosis-inducing activities of the crude ethanol extract of dried clove buds (*S. aromaticum* L.) on human gastric carcinoma (AGS).

Experimental

Plant material and extraction

The flower buds of clove were purchased from a local market. Then, the species was identified at the Herbarium of Medical Plants Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran.

The flower was powdered and extracted three times by maceration in 80% ethanol (Ghadir Industries, Iran) and kept at room temperature for

96 h. Then, the mixture was filtered and concentrated at 40 °C using rotary evaporator. The extract was kept in sterile bottles, in refrigerator, until further use when it was dissolved in dimethylsulphoxide (DMSO; Merck Germany) at 37°C to give a stock solution of 25 mg/mL, filtered (Millipore 0.22 mm) and stored at 4 °C. The small percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment.

Cells culture

AGS (human gastric carcinoma), cell line was purchased from Pasteure Institute, Iran and Human dermal fibroblasts (HDFs) cell line was kindly provided by Cellular and Molecular Research Center of Shahrekord University of Medical science, Iran. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 µg/mL of streptomycin (Gibco, USA), 100 UI/mL of penicillin (Gibco, USA) and 0.25 µg/mL amphotericin B (Gibco, USA), at 37 °C in a humidified air atmosphere containing 5% CO₂.

Determination of free-radical scavenging activity

The free-radical scavenging activity was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method described by Moon and Terao, with some modification [28]. Different amounts of the extract (10, 20, 30, 40, and 80 µg/mL) were added to a solution of 0.3 mg/mL methanol solution of DPPH to make up a total volume of 3.0 mL. After standing for 15 min at room temperature, the absorbance was measured at 517 nm using UV-Vis spectrophotometer (UNICO 2100, USA). High absorbance of the reaction mixture indicated low free radical scavenging activity. Butylated hydroxytoluene (BHT) was used as the positive control. Inhibition of free radicals by DPPH was calculated as follows:

$$\text{Antiradical activity (\%)} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$$

The IC₅₀ value, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% was calculated based on the linear regression of plots of the percentage antiradical activity against the concentration of the tested compounds [20]. The experiment was carried out in triplicate.

Determination of total phenolics content

The total phenolics content of the crude extract was determined using Folin-Ciocalteu method [29]. Briefly, 0.1 mL of each diluted samples was added to 0.5 mL of 10% (v/v) Folin-Ciocalteu reagent and kept at room temperature for 3-8 min. Subsequently, 0.4 mL of 7.5% (w/v) sodium carbonate solution was added to the mixture. After being kept in total darkness for 30 min, the absorbance of the reaction mixture was measured at 765 nm using a UV-Vis spectrophotometer (UNICO 2100, USA). Amounts of total phenolics were calculated using a gallic acid calibration curve. The results were expressed as gallic acid equivalents (GAE) g/g of dry plant matter.

Determination of total flavonoid content

The total flavonoid content of the extract was measured as according to a reported method [30]; in a way that, 0.5 mL of each diluted plant material was independently mixed with 1.5 mL of methanol, 0.1 mL of 10% (w/v) aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL distilled water. Following incubation at room temperature for 40 min, the absorbance of the reaction mixture was read at 415 nm for total flavonoid using a UV-Vis spectrophotometer. The results were expressed in mg of rutin equivalents of dry plant matter by comparison with the standard curve, which was made in the same condition.

MTT assay for proliferation assay

Cells were seeded into 96-well plates (SPL, Korea) at a concentration of 6000 cells per well (100 μ L per well) and incubated at 37 °C with 5% CO₂ for 24 h. Subsequently, the overlay medium was removed and the cells were incubated with 100 μ L/well of different

concentrations of crude ethanol extract (in triplicates) and incubated at 37°C with 5% CO₂ for further 48 h. The number of living cells in the culture medium was determined by the ability of the mitochondrial enzyme succinate dehydrogenase to cleave the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2yl) 2,5 diphenyltetrazolium bromide], and produce formazane with purple color as described previously [31]. Briefly, the supernatant was removed from the wells and 50 μ L MTT solution (1 mg/mL in PBS, Sigma, USA) was added to each well. The plates were incubated for 4 h at 37 °C, and 100 μ L of DMSO (Samchun, Korea) was added to the wells to dissolve the insoluble MTT crystals. The plates were placed on a shaker (IKA, Germany) for 15 min and the absorbance was read on an enzyme-linked immunosorbent assay (ELISA) reader (STATA FAX 2100, USA) at 570 nm. Each experiment was carried out in triplicate and the half maximal inhibitory concentration (IC₅₀) of each extract as the percentage survival of the treated cancer and normal cultured cells was calculated according to the formula provided below:

$$\text{Percentage of survival} = (\text{Absorbance of treated cells} / \text{Absorbance of control}) \times 100$$

The IC₅₀ was defined by regression analysis and related models with regression probit model procedure using SPSS program.

Flow cytometric analysis of cell apoptosis

In order to assess the rate of cell death by apoptosis or necrosis, AGS cells were treated with one time IC₅₀ concentrations of the extract, stained with both Propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC) using an Annexin V-FITC apoptosis detection kit (BD Biosciences, USA) in accordance with the manufacturer's instructor, and analyzed by flow cytometry. AGS cells were seeded into 6-well plates with a concentration of 10⁵ cells/well (final volume of 2 mL per well). After incubation at 37°C with 5% CO₂ for 24 h, in order to let the cells attach to the bottom of each well, the cell

culture medium of cells was aspirated. The cells were incubated with 2 mL/well of one time IC_{50} concentrations of crude ethanol extract and incubated at 37°C with 5% CO₂ for further 48 hour. The cells were then collected, resuspended in 100 μ L of 1 \times binding buffer containing 2 μ L of FITC-conjugated annexin-V and 1 μ L (100 μ g/mL) of PI and incubated for another 20 min in the dark. The results were then analyzed using flow cytometry (Partec, Germany).

Results and Discussion

The antioxidant capacity, flavonoid, and phenolic content in *S. aromaticum* extract were measured. Total phenolics and flavonoid content of the extract were 225.6 \pm 4 mg GAE/g equivalent and 29.3 \pm 2.35 mg RUT/g, respectively. Our study indicated that the extract's scavenging effect on free radicals increased with increasing its concentration. The IC_{50} of the extract was obtained 10.05 \pm 1.93 μ g/mL, compared to BHT, a reference standard (IC_{50} =25.41 \pm 1.89 μ g/mL). The IC_{50} is the concentration of an antioxidant-containing substance required to scavenge 50% of the initial DPPH radicals. The lower the IC_{50} value, the more potent is the substance at scavenging DPPH and this implies a higher antioxidant activity. Based on our results, *S. aromaticum* extract was more potent than the control (BHT); more clearly, this extract acted as a potent antioxidant agent due to its capability to scavenge DPPH free radicals. Based on previous study, the methanol extract of *S. aromaticum*, contains a number of polyphenols, with numerous hydroxyl units attached to one or more rings, which may be divided into flavonoids, phenolic acids, stilbenes and lignans. These polyphenols have been credited with chemoprevention of several illnesses resulting, at least in part, from oxidative damage, because of their potent antioxidant and radical scavenging properties [32-34].

To evaluate the anti-proliferative activity of the extract on AGS and HDFs (noncancerous) cell lines, the cells were treated with different concentrations of this extract for 24, 48 and 72h

and cell viability was determined using MTT assay.

The results showed that cell viability significantly reduced in concentration and time dependent manner following treatment with the extract. Based on probit regression model, anti-proliferative activity of the crude ethyl alcohol extract on the two cell lines was significantly different ($p<0.05$, figure 1). The IC_{50} of the extract against HDFs was 649 μ g/mL, higher than that against AGS cells, which was 118.7 μ g/mL suggesting that the dried flower bud of clove extract was more toxic against cancer cells than normal cells. The low toxicity towards normal cells and high toxicity towards cancer cells indicated that the plant extract possessed anti-cancer compounds with cytotoxic effect on cancer cells without causing toxicity in normal cells [35]. The IC_{50} and CI 95% on AGS cell line were 381.1(288.1-589), 118.7 (108.6-129.4) and 116.2 μ g/mL (106.9-126.1) after 24, 48 and 72 h of incubation, respectively (figure 2).

In recent years, remarkable attempts have been made to explore novel natural compounds and related synthetic agents that can prevent the progression and recurrence of cancer. Several natural compounds, such as phenols, flavonoids, indoles, aromatic isothiocyanates, and dithiolethiones, have been shown to induce apoptosis in several types of tumor cells.

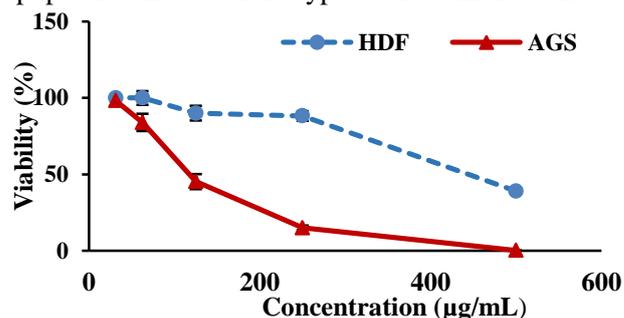


Figure 1. Anti-proliferative activity of various concentrations of *Syzygium aromaticum* extract on human gastric carcinoma (AGS) and normal (HDFs) cell lines for 48 h incubations. AGS and HDFs cell lines were treated with different concentrations of the extract for 48 h and cell viability was determined using MTT assay. The data indicate means \pm SEM of three independent experiments.

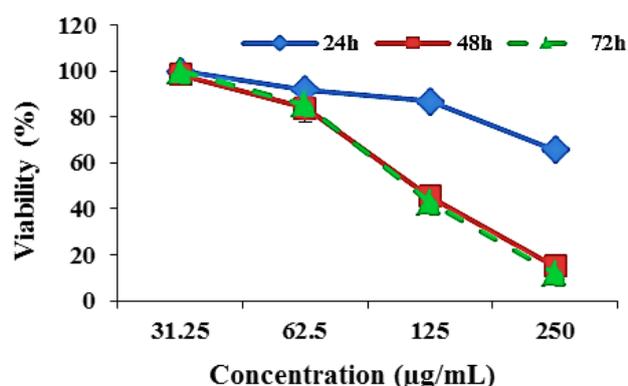


Figure 2. Antiproliferative activity of different concentrations of *Syzygium aromaticum* extract on human gastric carcinoma (AGS) cell lines for 24, 48, and 72h incubations. AGS cell lines were treated with different concentrations of the extract for 24, 48, 72 h and cell viability was determined using MTT assay. The data indicate means \pm SEM of three independent experiments.

These evidences may indicate the role of some of the natural compounds in cell cycle inhibition of cancerous cell lines [36]. Therefore, it is important to evaluate anti proliferative activity of natural products for potential development of anticancer compounds.

The results of previous researches on cloves support the ability of this spice as an excellent cytotoxic agent. Clove is being admired in cancer studies because of its ability to induce apoptosis in various cancer cells [37]. *Syzygium aromaticum* is also a source of betulinic acid and other triterpenes, which can act as chemopreventive agents against breast cancer. [38]. Different researches show that this extract is ideal for cancer studies because it raises apoptosis and inhibit cell proliferation [39-41]. The significantly high presence of phenolic and flavonoids in the extract might be responsible for the antiproliferation effects on AGS cell line. This is in agreement with the earlier work of Lee *et al.* [42] and Saleem *et al.* [43] who have shown that *Terminalia* species containing phenolic compounds that are rich in the hydroxy group and are believed to be responsible for the antiproliferation activity. Most of the phenolic

compounds in plants such as gallic acid, caffeic acid, flavonoids, and its derivatives are known to display different pharmacological actions like antioxidant, free radical scavenging, pro-oxidant toxicity, and apoptosis [44,45].

To determine whether the antiproliferation of the crud extract involved in the induction of apoptosis, AGS cells were treated with the extract, stained with both propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC), and analyzed by flow cytometry.

The analysis of flow cytometric results showed that, the percentages of apoptotic cells treated with *S. aromaticum* extract was 21.61% (figure 3). This showed that the extract induced cell death through early apoptosis. Apoptosis is characterized by distinct morphological features including the chromatic condensation, cell and nuclear shrinkage, membrane blabbing, and oligonucleosomal DNA fragmentation. Therefore, the anti-proliferative effect was due to the induction of apoptosis as shown by the annexin-V flow cytometric approach. Further studies are needed to fully recognize the mechanism involved in cell death.

A number of points should be considered in future studies. First, this study was performed *in vitro* with only one gastric cancer cell line. In order to consolidate our findings, other validation studies should be conducted by using various gastric cancer cell lines. Secondly, the apoptosis assay was performed only at IC₅₀ concentration after 48 h treatment. The time and dose dependency of apoptosis induction should also be evaluated. Thirdly, Apoptotic cell death was measured using Annexin V/PI assay by flow cytometry. The molecular mechanisms of apoptosis should be studied as well.

The crude ethyl alcohol extract of clove had great antioxidant activity and the high total phenolic content. It suppressed the proliferation of human gastric cancer cells probably due to induction of apoptosis. Further investigations are needed to elucidate the mechanism of anticancer actions.

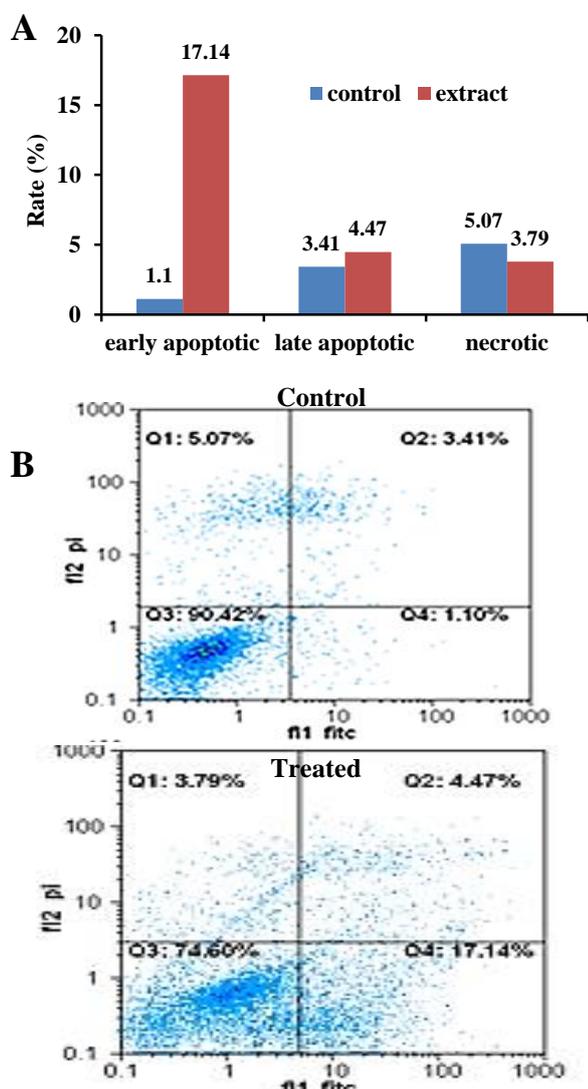


Figure 3. Flow cytometric analysis of apoptosis in AGS cells: cells were treated with the onetime IC₅₀ concentrations of crude ethyl alcohol extract of *S. aromaticum*, stained with propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC), and analyzed by flow cytometry; A: the apoptosis ratio; B: Flow cytometry analysis of apoptosis induced activity of AGS cells, control and treated

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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