Antiproliferative and Apoptotic Effect of Dried Flower Buds of Syzygium Aromaticum L. Extract on Human Cervical Cancer (Hela) Cells

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ABSTRACT

Cancer leads to over seven million deaths each year. The current therapeutic approaches have failed to completely treat cancer, and it is essential to seek out new effective anticancer drugs. Because of nontoxic properties, relatively low cost, and phytochemical compounds, medicinal plant extracts have been evaluated for anticancer effects. The present study investigated antiproliferative, apoptosis-inducing, and total phenolic content of the flower bud extract of dried Syzygium aromaticum L. on human cervical cancer (HeLa) cells. Ethanolic extract of dried flower buds was prepared and total phenolic and flavonoid content determined. In vitro antiproliferative activity of the extract in Hela and normal human dermal fibroblasts (HDFs) was evaluated using MTT assay. To determine apoptosis induction, HeLa cells were incubated with one time IC50 concentrations of extract, stained with both annexin V-fluorescein isothiocyanate and propidium iodide, and flowcytometrically analyzed. Total phenolic and flavonoid content was 225.6±4 mg GAE/g and 29.3±2.35 mg RUT/g, respectively. Antiproliferative activity results showed that cell viability significantly decreased in dose- and time-dependent manner after extract treatment (p<0.05). The extract IC50 against HeLa cell was less than that against HDFs. Flow cytometry results showed that the extract induced Hela cell apoptosis (apoptosis ratio: 66.77%). The ethanolic extract of dried S. aromaticum flower bud had the greatest phenolic content, and suppressed the proliferation of HeLa cells probably by inducing apoptosis. Further studies may identify the main anticancer ingredients of this extract.

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Introduction

As one of the leading causes of death across the world, cancer leads to death of over seven million people each year [1]. Cervical cancer is the second leading clinically diagnosed and one of the leading causes of death particularly among women in developing countries [2, 3]. The current therapeutic measures including surgery, radiotherapy, and chemotherapy cannot completely treat cancer, and it is therefore essential to seek out new effective anticancer drugs [4-6]. Because of having nontoxic properties, being relatively inexpensive, and containing phytochemical compounds, medicinal plants extracts have been evaluated for their potential anti-cancer [7, 8] and therapeutic effects on certain diseases in human [9-11]. Some of these extracts have been reported to exert useful effects [12-15].

The species of Syzygium are popular spices with pharmaceutical applications. They contain highly bioactive compounds so that they exert therapeutic effects. Syzygium aromaticum L. (commonly known as clove), from the family Myrtaceae, is a herbal medicine that has been used for culinary and medicinal purposes across the world [16]. The species of Syzygium contain a wide spectrum of important chemical compounds such as tannins [17], sesquiterpenes [18], triterpenoids [19] and eugenol (a phenolic compound). S. aromaticum and its main component, eugenol, seem to act as an antioxidant, antispasmodic, antiviral, carminative, antiseptic, antimicrobial and antimutagenic agent [20-25].

Given that treatment-associated toxicity remains a problem, it is necessary to seek out new compounds and more efficient treatments for cancer. Therefore, this study is to investigate the total phenolic and flavonoid content, antiproliferative activities, and apoptosis induction of S. aromaticum extract in vitro.

Materials and methods

Plant collection and extraction

The flower buds of S. aromaticum was purchased from a local market. Then, the genus and species of the plant were identified and confirmed in Herbarium of Medical Plants Research Center of Shahrekord University of Medical Sciences (Iran). The flower was powdered and extracted three times per maceration method. Briefly, the plant material was dissolved in 80% ethanol (Ghadir Industries, Iran) and kept at room temperature (RT) for 96 h. After that, the mixture was filtered and concentrated under nearly vacuum pressure and at 40°C using rotary evaporator. The extract was kept in sterile bottles, under refrigerated conditions, until further use. The extract was dissolved at 37°C in dimethylsulphoxide (DMSO; Merck Germany) to give a stock solution of 25mg/mL.

Cells and cell culture

HeLa (cervix adenocarcinoma) cell lines were purchased from Pasteur Institute of 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% of 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% (v/v) CO2.

Determination of total phenolic and flavonoid content

The total phenolic content of the crude extract was determined using Folin-Ciocalteu method [26]. Briefly, 0.1 ml of each of the diluted samples was added to 0.5 ml of 10% (v/v) Folin–Ciocalteu reagent and kept at room temperature (RT) 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 phenolic were calculated using a gallic acid calibration curve. The results were expressed as mg gallic acid equivalents (GAE) of dry plant matter. The total flavonoid content of the extract was
measured as previously reported method [27]. Briefly, 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 1M potassium acetate, and 2.8 ml of distilled water. Following incubation for 40 min at room temperature, the absorbance of the reaction mixture was read at 415 nm using a UV–Vis spectrophotometer (UNICO 2100: USA). 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**

Cells were seeded onto 96-well plates (SPL, Korea) at a concentration of 6000 cells per well to a final volume of 100 µl per well and incubated at 37°C with 5% CO2 for 24 h. Subsequently overlay medium removed and the cells were incubated with 100 µL/well of different concentrations of the extract (in triplicates) and incubated at 37°C with 5% CO2 for further 24, 48 and 72 hour. The number of living cells in the culture medium was determined by the MTT [3-(4, 5-dimethylthiazol-2ol) 2, 5 diphenyltetrazoliumumbromide] assay [28]. Briefly, the supernatant was removed from the wells and 60 µL of an MTT (Sigma, USA) solution (1mg/mL in PBS) was added to each well. The plates were incubated for 4 h at 37°C, and 100 µL of DMSO (Samchunkorea) 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 the extract as the percentage survival of the treated cancer and normal cultured cells was calculated according to the formula provided below:

\[
\text{Percentage of survival} = \left( \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control}} \right) \times 100
\]

The IC₅₀ was determined by regression analysis and related models with regression probit model procedure using SPSS (version 16) program.

**Flow cytometric analysis of cell apoptosis**

To determine whether the cytotoxicity of the crude extract involved the induction of apoptosis, HeLa cells were treated with 350 µg/mL 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. Briefly, HeLa cells were seeded onto 6-well plates with a concentration of 10⁵ cells/well with final volume of 2ml 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. Cells were incubated with 350 µg/mL of the extract and incubated at 37°C with 5% CO2 for further 48 hour. The cells were then collected, resuspended in 100 µL of 1× binding buffer containing 2µL of FITC-conjugated annexin-V and 1 μL (100 µg/mL) of PI and incubated for another 20 minutes in the dark. The results were then analyzed using flow cytometry (Partec, Germany).

**Results and Discussion**

**Phenolic and flavonoid compounds**

For standardization the extract, phenolic and flavonoid compounds of *S. aromaticum* extract were measured. Total phenolic and flavonoid amounts of extract were 225.6±4 mgGAE/g equivalent and 29.3±2.35mgRUT/g, respectively. According to a number of studies, *S. aromaticum* extract contains a number of polyphenols with numerous hydroxyl units attached to one or more rings that may be divided into flavonoids, phenolic acids, stilbenes, and lignans. These polyphenols have been reported to cause chemoprevention of several diseases due, at least partly, to oxidative damage because they have potent antioxidant and radical-scavenging properties [29-32].
Antiproliferative activity

We investigated the effect of ethanol extract of *S. aromaticum* on the proliferation of HeLa and HDFs cell lines using the MTT assay. HeLa and HDFs cells were treated with various concentrations (0, 31.2, 62.5, 125, 250, 500 and 1000 μg/mL for both cell lines) of the extract for 24, 48, and 72 h, then their viability was evaluated. The results showed that cell viability was significantly reduced in dose and time dependent manner following treatment with the extract. The IC$_{50}$ and confidence intervals (CI) 95% of the extract against HDFs was higher than that against HeLa cells (table1). Based on probit regression model, antiproliferative activity of the crude ethanolic extract on the two cell lines was significantly different (P<0.05). Results also showed cell line that treated with various concentrations of the extract, cell proliferation in a dose and time dependent manner significantly decreases after 24, 48 and 72h of incubation respectively (Figure 1). Based on probit regression model, the difference in antiproliferative activity was significant among the intervals (P<0.05). In recent years, remarkable attempts have been made to identify natural compounds and develop their synthetic derivatives 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 different types of tumor cells. These findings may contribute to confirming the role of some of the natural compounds in cell cycle inhibition of cancerous cell lines [33].

*S. aromaticum* extract has been reported to be an ideal agent for cancer treatment because it escalates apoptosis and inhibits cell proliferation [34-36]. *S. aromaticum* are also a source of oleanolic acid and betulinic acid and other triterpenes, which can serve as chemopreventive agents to treat cancer [37, 38].

The quantitative estimation of total phenolic and flavonoid content indicated the presence of phenolics up to 225.6±4mg of GAE/g extract. This significantly high presence of phenolic in the extract might be responsible for the anti-proliferation effects on HeLa cancer cell lines. The great phenolic content of the extract may be responsible for the anti-proliferative effects on HeLa cancer cell lines. Most of the phenolic compounds in plants such as gallic acid, caffeic acid, flavonoids, and their derivatives are known to exhibit different pharmacological activities such as antioxidant, free radical-scavenging, pro-oxidant toxicity, anti-proliferative activity, and apoptosis [39-42].

<table>
<thead>
<tr>
<th>Exposure time</th>
<th>HeLa (μg/ml)</th>
<th>HDFs (μg/ml)</th>
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<tr>
<td></td>
<td>IC$_{50}$ (μg/ml)</td>
<td>CI95% (μg/ml)</td>
</tr>
<tr>
<td>24h</td>
<td>&gt;1000</td>
<td>(905-4262)</td>
</tr>
<tr>
<td>48h</td>
<td>338</td>
<td>(298-383.4)</td>
</tr>
<tr>
<td>72h</td>
<td>145</td>
<td>(128.9-164.8)</td>
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HeLa (cancerous) and HDFs (normal) cell lines were treated with various concentrations of the extract and cell viability was determined using MTT assay. The IC50 was defined by regression analysis procedure using SPSS program. Hela: Cervix adenocarcinoma, HDFs: Human Dermal Fibroblast; *CI: Confidence Intervals.
**Fig. 1. Antiproliferative activity of various concentrations of S. aromaticum extract on HeLa and HDFs cell lines for 24, 48, and 72h incubations.** Cell lines, were treated with different concentrations of the extract for 24, 48, 72h and cell viability was determined using MTT assay. The data indicate mean±SEM of three independent experiments. Based on probit regression model, the difference in anti-proliferative activity was significant among the intervals (P<0.05). Hela: Cervix adenocarcinoma, HDFs: Human Dermal Fibroblast.

**Apoptosis induction mechanism**

To determine whether the anti-proliferation of the crude extract involved the induction of apoptosis, HeLa cells were treated with 350 µg/mL of 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 extract induced cell death probably via early apoptosis. The percentages of apoptotic cells treated with the extract were 66.77% on the HeLa cell lines, the percentage of necrotic cells were low (Figure 2). 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 addressing the limitations of this study should also be considered. First, the apoptosis assay was performed only at IC<sub>50</sub> concentration after 48 h treatment. The time and dose dependency of apoptosis induction should be evaluated. Secondly, Apoptotic cell death was measured using Annexin V/PI assay kit by flow cytometry, the molecular mechanisms of apoptosis should be studied as well.
Fig. 2. Flow cytometric analysis of apoptosis in HeLa cells: cells were treated with the onetime IC_{50} concentrations of crude ethanolic extract of *S. aromaticum*, stained with both Propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC), and analyzed by flow cytometry; A: Flow cytometry analysis; B: the apoptosis ratio

**Conclusion**

The ethanolic extract of *S. aromaticum* had the highest total phenolic content, and suppressed the proliferation of human cervical cancer cells probably due to induction of apoptosis. Further investigations are needed to elucidate the active compound(s) of the extract and their mechanisms of anticancer actions.

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**Conflict of Interests**

Authors certify that there is no actual or potential conflict of interest in relation to this article.
References