



Anti-proliferative effect of the extracts and essential oil of *Pimpinella anisum* on gastric cancer cells

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ABSTRACT

Introduction: One of the most important fields to find the new drugs for combating cancers is herbal medicine and in this regard, attention to plant components against cancer has been increased nowadays. In the present study, we investigated the cytotoxic effect of alcoholic extracts and essential oil of *Pimpinella anisum* on viability of gastric cancer cell line (AGS) and angiogenesis of HUVEC cells.

Methods: The alcoholic extracts and essential oil were obtained by soxhlet and Clevenger set, respectively. The viability of cells which treated with the extracts and the essential oil were assessed by MTT assay. In vitro tube formation assay was performed to assess the effect of the extracts and essential oil on angiogenesis potential of HUVEC cells.

Results: The growth of AGS cells was inhibited by extracts and essential oil of *Pimpinella anisum*. Furthermore, the extracts and essential oil had inhibitory effects on the angiogenesis in HUVEC cells. The ethanolic extract inhibited cell proliferation of the AGS cells in 30 µg/mL at 48 hours after treatment but it had no significant effect on fibroblast cells. The inhibitory effect of methanolic extract was much better than the ethanolic extract at the same concentrations. The essential oil of the plant had the highest inhibitory effect on cancer cells compared with the alcoholic extracts.

Conclusion: Therefore, our results showed that the alcoholic extracts and essential oil of *Pimpinella anisum* have antiproliferation properties on gastric cancer cells and could be used as plant-based cures for gastric cancer.

Implication for health policy/practice/research/medical education:

Pimpinella anisum has anti-angiogenic properties which is an important aspect of this plant. Hence, the extracts and essential oil of *P. anisum* seeds possess potent anticancer effects which inhibit the growth and progression of human gastric cancer cells.

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Introduction

Cancer is an abnormal proliferation of cells which is responsible for 7.6 million deaths (around 13% of all deaths) in 2008 (1). Gastric cancer is estimated to be responsible for 8% of the total cancer cases and 10% of total cancer deaths, worldwide. Over 70% of new stomach cancer cases and deaths occur in developing countries (2). However, the side effects of chemotherapeutic agents motivated the researchers to develop alternative anticancer drugs with minimal side-effects. So, an important strategy is to search on bioactive components derived from medicinal plants which has anticancer properties (3). About 80% of the world population has been estimated to use of traditional medicine, which is predominantly based on plant materials, for their primary health care (4,5). Medicinal plants

possess secondary metabolites which are the main sources of natural agents having curative nature and effective in human health care (6).

Pimpinella anisum L. is an annual herb and a grassy plant with white flowers and small seeds which grows in different region such as Iran, India and Egypt. The seeds of *P. anisum* is used in medicine and food industries (7,8). The main constituents of *P. anisum* are volatile oil, coumarins, fatty acids, flavonoid, glycosides, proteins, carbohydrates, anethole and caryophyllene (9). *P. anisum* is grown for its seeds (aniseeds) that contain 1.5–5% essential oil. Aniseeds are traditionally used for the treatment of digestive and carminative problems and relief of gastrointestinal spasms (10-12). Despite various studies conducted on *P. anisum*, there is little information about the anti-cancer

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properties of *P. anisum*.

In this study, antiproliferative activity of ethanolic and methanolic extracts and essential oil of aniseeds on gastric cancer cells were investigated. The effects of its extracts and essential oil on angiogenesis of HUVEC cells were also evaluated.

Materials and Methods

Preparation of alcoholic extracts

Approximately, 20 g of seeds of *P. anisum* were ground to a coarse powder and placed in a soxhlet extractor containing 500 mL of each of solvents as ethanol or methanol for 8 hours and the cycle repeated for three times. Then the obtained extracts were concentrated in a rotatory evaporator under reduced pressure at 45°C for 75 minutes. The extracts were stored in refrigerator at 4°C until further use. The extracts of *P. anisum* were dissolved in dimethyl sulphoxide (DMSO) for preparation of different doses (15, 30, 60, 120, 240 and 480 µg/mL). We used 5-FU, a routine anticancer drug, in different doses (5, 10, 30, 60, 80 and 100 µg/mL) which were dissolved in culture media as a positive control.

Essential oil preparation

The shade dried seeds (100 g) of *P. anisum* were ground to coarse powder for hydrodistillation in a clevenger apparatus for 2 hours to derive the volatile constituents in the form of essential oils. Each volatile oil was dried over anhydrous sodium sulphate and then kept separately in sealed clean glass vials at 4°C until use. The essential oil of *P. anisum* was dissolved in culture media for preparation of different concentrations (15, 30, 60, 120, 240 and 480 µg/mL).

Cell culture

The gastric adenocarcinoma cell line (AGS) and fibroblast cell line (HSkMc) were obtained from national cell bank of Iran (Pasteur institute of Iran, Tehran) and were cultured at 37°C in a humidified atmosphere of 5% CO₂ in RPMI1640 medium (Gibco, USA), supplemented with penicillin/streptomycin (100 U/ml and 100 µg/mL, respectively) and 10% fetal bovine serum.

Viability assay

The inhibitory effects of the ethanolic and methanolic extracts as well as essential oil of *P. anisum* on human gastric cancer cells and fibroblast cells were determined by MTT assay. The base of MTT assay is enzymatic reduction of the tetrazolium salt MTT in viable/metabolically active cells. One day before adding extracts and essence oil, the cells (2 × 10⁴ cells per well) were cultured in 96-well plate in growth medium, such that they were 40%-60% confluent, the filtered ethanolic, methanolic extracts and essential oil with final concentrations of 15, 30, 60, 120, 240 and 480 µg/mL were added. The treated cells were incubated 3 days at 37°C in a CO₂ incubator. In this study, we used the cells treated with 5-FU (Austria, Ebewe Pharma), as positive control and the ones treated with DMSO as negative

control. One, two and three days after treatment, MTT solution (20 µL) was added to the cells and incubated at 37°C for 4 hours, then the medium was removed by aspiration followed by adding DMSO (200 µL). The cells were shaken for 30 seconds and absorbance of formazan dye was read using an ELISA plate reader at 490 and 630 NM. The whole procedure was repeated for three times. The inhibitory rate of cell growth was calculated using the following formula:

$$\% \text{ growth inhibition} = (1 - \text{OD extract treated}) / \text{OD negative control} \times 100$$

Statistical analysis

The data were analyzed using SPSS 21 software and the significant difference between means was calculated. Values were expressed as mean of three samples analyzed in triplicate ± standard deviation (SD). Duncan test at P < 0.05 was used to determine significant differences among treatments. IC50s were analyzed with ED50plus v1.0 software.

In vitro tube formation assay

The tube formation assay was done by ECM gel (Sigma-Aldrich) according to the manufacturer's protocol. In brief, 100 µL of ECM gel was dispensed to wells of 24-well plates and incubated at 37°C for 2 hours to solidify ECM gel. HUVEC cells were cultured on the solidified ECM gel at the confluency of 3 × 10⁴ cells in DMEM: F12 (1:1) containing 5% FBS, treated with IC50 dose of the extracts and incubated at 37°C in 5% CO₂ humidity incubator for 24 hours. The tube formation was observed under an inverted microscope (CETI, UK).

Results

Ethanolic extract of *P. anisum* inhibited the viability of AGS cells

In this study, we investigated the antiproliferative activity of ethanolic extract on AGS cells by MTT assay. Our results revealed that the extract inhibited the growth of cancer cells in all doses between 15-480 µg/mL and its effect was in time dependent manner. The best growth inhibitory effect of ethanolic extract was observed in concentration 30 and 15 µg/mL at 48 and 72 hours after treatment respectively but not significant effect on viability of fibroblast cell as normal cells (Figure 1). So, the growth inhibitory effect of the extract was significantly much higher on cancer cells compared with its effect on normal ones.

Growth-inhibitory effect of methanolic extracts

We treated the cancer and normal cells with methanolic extract and the cytotoxicity effect of the methanolic extract on the cells was investigated. Our data showed that methanolic extract of *P. anisum* had more antiproliferative activity in comparison to ethanolic extract and its effect was not in dose and time dependent manner. We observed the methanolic extract in dose 15 µg/mL at 24 hour after treatment caused inhibition of proliferation of more than 50% of cancer cells but it had no significant effect on nor-

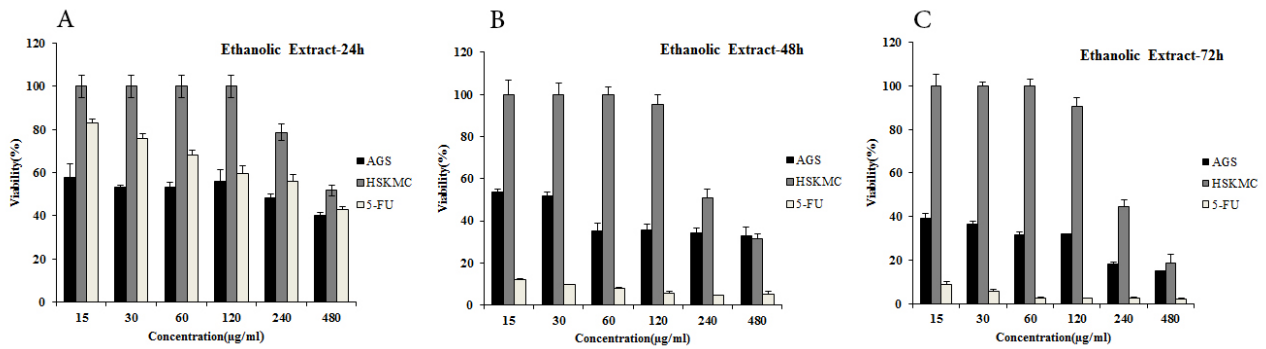


Figure 1. Gastric cancer (AGS) and human fibroblast cells (HSKMC) were treated with the ethanolic extract of *Pimpinella anisum* and viability of the cells was investigated by MTT assay one (A), two (B) and three (C) days after treatments.

mal cells (Figure 2).

Essential oil of *P. anisum* suppress the growth of AGS cells

To the same as alcoholic extracts, essential oil of *P. anisum* had cytotoxicity activity against AGS cells. Essential oil of the *P. anisum* at 24, 48 and 72 hours after treatment had inhibitory potential on proliferation of cancer cells. The best effect was observed in 15 µg/mL two days after treatment (Figure 3)

HUVE cells treated with the extracts and essential oil inhibit tube formation structures

Angiogenesis, a process of new blood vessel formation, is prerequisite for tumor growth to supply the tumor cells with oxygen and nutrients. Angiogenic process may contribute tumor progression, invasion and metastasis and is generally accepted as an indicator of tumor prognosis. Therefore, targeting tumor angiogenesis has become a high clinical relevance. So, the effect of the extracts and essential oil was evaluated on the angiogenesis process. The extracts and essential oil inhibited the formation of tube like structures in comparison to control cells (Figure 4).

Discussion

Most of the routine chemical anticancer drugs exhibit side effects, hence, there is a need for drugs that are efficient and have less side effects (13). So, the harmful side effects of chemical anticancer drugs motivated the researchers to find new, more effective and fewer side effects anticancer

drugs and this field of research known as medicinal plants is one of the most important fields in medicine and pharmacology (3). Various anticancer drugs were obtained from plants that act against proliferating of cancer cells and are useful for cancer treatment (14). Plants are rich sources for finding new drugs useful in the treatment of cancer (15).

In the first phase of our study, we investigated the cytotoxicity effect of both extracts and essential oil of *P. anisum* seeds on AGS cells. Our results showed that the alcoholic extracts and essential oil had significantly cytotoxic effect on gastric cancer cells at concentrations of 15 to 480 µg/mL but had not significant effect on fibroblast cells as normal cells. The growth-inhibitory effect of methanolic extract was much higher in comparison to ethanolic extract on prevention of growth of AGS cells but the treatments had less effects on fibroblast cells. By searching from previous papers found that aqueous extract of *P. anisum* helps in the process of digestion and is useful as antiulcer agent (16,17). *P. anisum* suspension exhibits an antiulcer potential activity through at least one or more possible mechanisms including inhibition of basal gastric secretion, stimulation of mucus secretion, endogenous gastric mucosal prostaglandin synthesis and possible antioxidative activity (18). The extracts were found to be active in motivating the differentiation and mineralization of osteoblastic cell culture (19). In the present study we indicated that the extracts and essential oil of *P. anisum* seeds had significant anticancer activity against human gastric cancer cells with

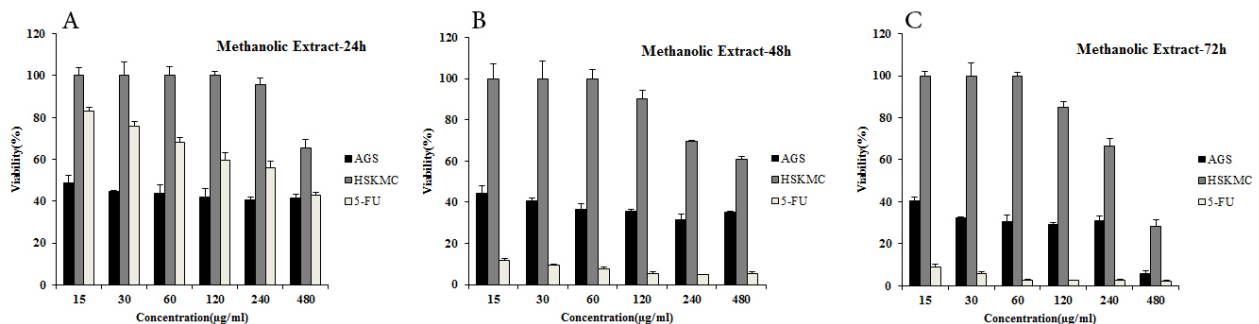


Figure 2. The growth-inhibitory effect of methanolic extract of *Pimpinella anisum* on gastric cancer (AGS) and fibroblast cells (HSKMC), one (A), two (B) and three (C) days after treatment.

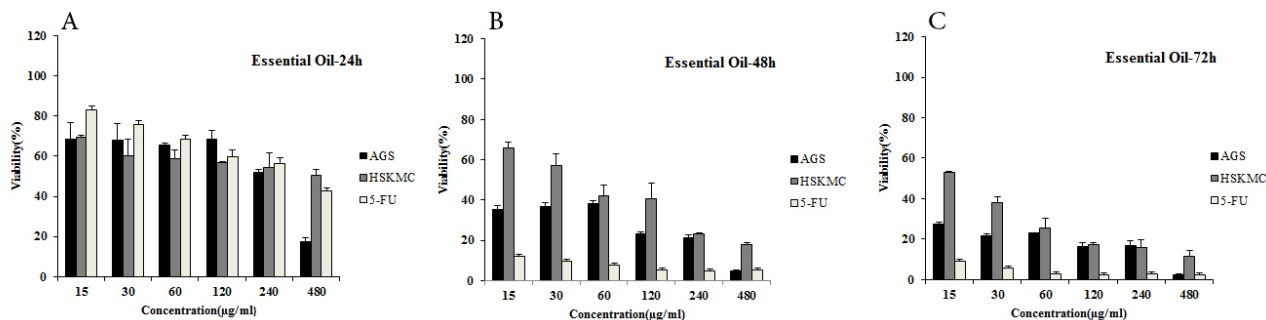


Figure 3. The essential oil of *Pimpinella anisum* has antiproliferative effect on AGS cells but do not significant effect on HSKM cells.

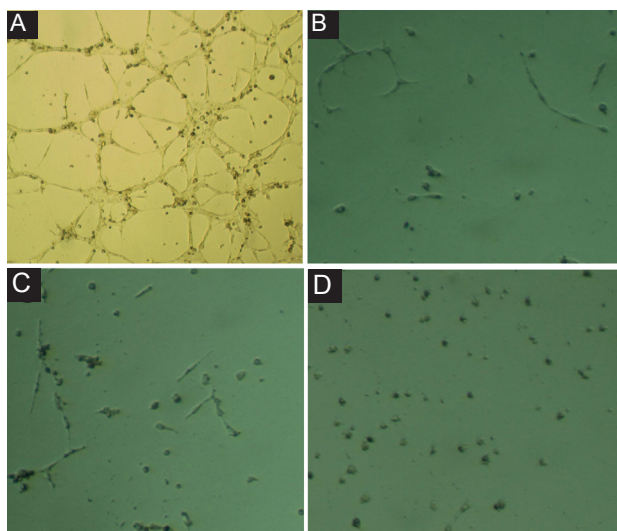


Figure 4. The tube formation assay was conducted by HUVECs which were cultured on fibrin matrices in the absence (A) or presence of ethanolic (B) and methanolic (C) extracts and essential oil (D) of *Pimpinella anisum*. After 24 hours, light microscopy images of cells showed that the extracts inhibited the angiogenesis.

notable concentration in comparison to normal cells (HSKMC). Thus, *P. anisum* could be a natural source of anti-cancer compounds with antiproliferative properties.

Angiogenesis, a process of new blood vessel formation, is a prerequisite for tumor growth to supply the tumor cells with oxygen and nutrients. Angiogenic process may contribute tumor progression, invasion and metastasis and is generally accepted as an indicator of tumor prognosis. Therefore, targeting tumor angiogenesis has become a high clinical relevance. So, the effects of the extracts and essential oil were evaluated on the angiogenesis process. Our results revealed that alcoholic extracts and essential oil had inhibitory effect on tube formation in HUVEC cells.

Altogether, our data showed that the extracts and essential oil of seeds of *P. anisum* might be considered as a potential source of metabolites which could be developed as precursors for anticancer drugs. Our results revealed that the extracts and essential oil of this plant are able to prevent the growth of cancer cells with no significant effect on normal cells. The essential oil of the plant showed high effect, the

same as the effect of 5-FU, a routine anticancer drug, at 24 hours after treatment. The results showed that the effect of essential oil on fibroblast cells was less than gastric cancer cells, so it can be conclude that this plant has medicinal value. Furthermore, anti-angiogenic properties of the extracts and essential oil of *P. anisum* seeds is an important aspect of this plant. Therefore, we might conclude that the extracts and essential oil of *P. anisum* seeds possess potent anticancer fractions which inhibit the growth and progression of human gastric cancer cells.

Authors' contributions

SRH; Data collection, drafting the article and data analysis. MHA; Design of the work, data analysis and interpretation, critical revision of the article and Final approval of the version to be published.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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