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Anticancer activity of ethanolic extract of propolis on AGS cell line

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ABSTRACT

Introduction: Propolis is a natural product derived from various plant resins collected by honeybees, and has been used as a folk medicine for centuries. Propolis has been reported to exhibit a broad spectrum of activities including antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, hepatoprotective, and anticancer properties. The aim of this study was to investigate the effect of ethanolic extract of propolis (EEP) obtained from Dinaran area (Iran) on AGS human gastric cancer cell line.
Methods: The ethanolic extract of samples was obtained by ethanol 96% and pure extract was dissolved in dimethyl sulfoxide (DMSO) and used for experiments. The cytotoxic effects of various concentrations of EEP on AGS cells were investigated by MTT assay test after 24, 48, and 72 hours and compared with control cells.
Results: The EEP inhibited the growth and proliferation of AGS human gastric cancer cell line. The antiproliferative effects were revealed in a dose and time-dependent manner. The IC_{50} values were recorded as 60, 30, and 15 (µg/ml) in treatment times of 24, 48 and 72 hours, respectively.
Conclusion: These findings indicated that the native EEP has strong antiproliferative effects against cancerous AGS cells. Thus, propolis and related products may provide a novel approach to the chemoprevention and treatment of human gastric cancer.

Implication for health policy/practice/research/medical education:
Ethanolic extract of propolis prevents growth and proliferation of AGS cells due to strong cytotoxic effects against tumor cells. Therefore, this compound of honey bee might be useful in developing chemotherapeutic agents for treating human gastric cancer.
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Introduction

Propolis is a natural, resinous and strongly adhesive substance that is collected from blooms and leaves of trees and plants by honeybees and is combines with pollen and enzymes secretions of bees (1). Bees use propolis as a general-purpose sealer to smooth out the internal walls of the hive and also as a protective barrier against intruders (2). Overall, propolis is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances, including organic debris (2). Wax and organic debris are removed throughout biological processes that are usually done by ethanol extracts, and balsam thus obtained, contains the bulk of propolis bioactive constituents. Over 300 compounds among which polyphenols, terpenoides, steroids, sugars and amino acids have been identified in raw propolis. The frequency of these compounds is influenced by botanical and geographical factors, as well as by the collection of season (1,3). Propolis is considered responsible for the low presence of bacteria and moulds within the hive. The action against microorganisms is an important characteristic of propolis and humans have used it for centuries for its pharmaceutical properties (4,5). Besides its antibacterial, antifungal, antiviral characteristics, propolis presents many other biological activities including antioxidant,

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antiinflammatory, antitumor, hepatoprotective, local anesthetic, immunostimulatory and anti-mutagenic (2,6-8). For this, propolis is used as a generic remedy in folk medicine, as a constituent of biocosmetics and healthy foods, and for numerous other purposes (4,5,7). Propolis is relatively non-toxic, with a non-observed effect level (NOEL) of 1400 mg/kg body weight/day in a mouse study (2).

Due to geomorphologic characteristics, the plants of Dinaran area (Iran) exhibit high biodiversity with many endemic plants, so the propolis collected from this region is likely to contain biologically active compounds. In literature scarce data can be found about the composition, antibacterial, anticancer and antioxidant properties of Iranian propolis extracts and no data at all about propolis from Dinaran in the literature.

Gastric cancer is the fourth most important cancer and the second leading cause of cancer-related deaths worldwide (9), including one million mortalities per year worldwide (10). Cancer is one of the most important healthcare problems in Iran. After road accidents and cardiovascular diseases, cancers are the third leading cause of mortalities in Iran. More than 30 000 individuals die per year because of cancer, and it is estimated that more than 70 000 individuals per year develop cancer in Iran. Out of all cancers that have been registered to date, skin cancer, breast carcinoma, gastric cancer, colorectal cancer, bladder cancer, hematopoietic system cancer, prostate cancer, esophagus cancer, lymph nodes cancer, and lungs cancer are the most prevalent. In Iran, over 50% of prevalent cancers are gastrointestinal tract-related, among which gastric cancer is the most prevalent. Most gastric cancers are developed in the elderly, and since Iranian population is relatively young, the incidence rate of and the mortality due to this fatal disease may be increase rapidly in the near future as life expectancy increases. Therefore, in light of the importance of fighting with this fatal disease, a program for controlling and treating it seems necessary (11). In the present study, anticancer activity of the ethanolic extract of propolis collected from Dinaran area (Iran) was investigated on AGS human gastric cancer cell line.

Materials and Methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were purchased from Gibco Co (Invitrogen, Carlsbad, CA, USA). Dymethyle sulfoxide (DMSO) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay Kit was purchased from BIO IDEA Co (Tehran-Iran).

Preparation of propolis extracts

Propolis samples were collected as fresh from Dinaran area (Iran) and kept at -20°C. Ethanolic extract of propolis (EEP) was prepared according to Kalogeropoulos and Konteles method (12). Briefly, the collected propolis samples were frozen for 24 h. Then, they were grounded by moulinex vivacio grinder, and 50 g of the obtained powder was dissolved in 500 cc ethanol solution (V/V) in a dark glass container and incubated at 37°C for 14 days. The solution was shaken twice a day throughout the incubation period. After 14 time period, the obtained extract was filtered by Whatman filter paper (No. 4). To remove waxes and less soluble substances, the suspensions were subsequently frozen at -20°C for 24 hours, then filtered with Whatman (NO.4) filter paper. The freezing-filtration cycle was repeated three times. The final filtration led to represent the balsam (tincture) of propolis and is referred to as EEP (ethanolic extract of propolis). The solutions were evaporated to near dryness on a rotary evaporator (EYELA Rotary Evaporator N-100) under reduced pressure at 40°C. The remaining extract was incubated at 37°C for two weeks till the remainder of the ethanol was evaporated and the resulting powders were kept at -20°C. For experimental treatments, EEP at 100 mg/ml concentration was dissolved in DMSO as solvent and stored at -20°C.

Cell line and culture

AGS human gastric cancer cell-line was purchased from National Cell Bank of Iran (NCBI), Pasteur Institute of Iran (NCBI, C131). AGS cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA) in an incubator with humidified air with 5% CO2 at 37°C (13). For maintaining in the exponential growth phase, the cells were passaged at 70%-80% confluence once a week by trypsin-EDTA (Gibco, USA) (14). EEP were dissolved in DMSO as solvent and were prepared as 100 mg/ml stock solution and were kept at 100 ml volumes at -20°C. Different concentrations of EEP were prepared with DMEM containing FBS 10% just prior to AGS cells treatment.

Cell proliferation assays

Cell viability was measured by MTT assay kit (BIO IDEA Co. Tehran, Iran). The viability of AGS cells was measured in different concentrations of EEP (µg/ml) within 24, 48 and 72 hours. Cells were seeded into 96 well plates at a density of 5000 cells per well and the plates were incubated at 37°C temperature in a humidified incubator containing 5% CO2 for 24 hours. After 24 hours, the medium was removed and the cells were treated for 24, 48 and 72 hours with medium containing different concentrations (0, 10, 15, 20, 30, 40, 50, 60, 80, and 100 µg/ml) of EEP. The cells that were not treated with EEP were considered as control. The same volume of DMSO was used as the vehicle control for EEP experiments at a final concentration of 0.1%. Each EEP concentration was represented by 3 wells and replicated thrice. After 24, 48 and 72 hours, AGS cells viability was measured by MTT Assay kit according to manufacture protocol. Finally,

optical density (OD) of each well was measured by an ELISA reader (AWARENESS-State Fax, USA) at 570-nm wavelength and the rate of viability (%) was calculated by the following formula:

Cell viability rate (%) = OD of treated cells/OD of control cells × 100

Statistical analysis

The statistical analysis of MTT assay data for different concentrations of EEP within 24, 48, 72 hours that calculated as viability percent was done by SPSS 19 using one-way ANOVA followed by Dennett's test. P<0.05 was considered as statistically significant.

Results

The cytotoxic effects of various concentrations of EEP on AGS cells for 24 hours are shown in Figure 1. The viability of AGS cells was decreased in a dose-dependent manner by low doses of EEP. Morphological investigation by light microscope indicated that after 4 hours treatment at 80 and 100 µg/ml concentrations, EEP induced morphological changes in AGS cells. Twenty-four hours after treatment with all studied EEP concentrations, AGS cells viability decreased significantly. The highest inhibition of the cells growth was seen after treatment with EEP at 80 and 100 µg/ml with reductions of approximately 68.4% and 80.6%, respectively. Overall, the viability of AGS cells was further decreased by higher doses of EEP (0–100 µg/ml), with an IC_{50} of approximately 60 µg/ml (Figure 1).

Similarly, 48 hours after treatment with EEP, AGS cells viability decreased in a concentration dependent manner with an IC_{50} of approximately 30 µg/ml. The highest inhibition of growth was observed after treatment with EEP at 80 and 100 µg/ml concentrations with a reduction of about 93.4% and 100% respectively (Figure 2).

High significant inhibition of AGS cells viability was observed at 72 hours treatment with EEP. After treatment for 72 hours with EEP, the highest reduction in viability was observed at 80 and 100 µg/ml concentrations with a reduction of approximately 100%, and followed by 60 µg/ml concentration with viability reduction of approximately 99%, respectively. IC_{50} value for 72 hours EEP treatment was obtained as approximately 15 µg/ml (Figure 3).

Analysis of the data by Dunnett's test indicated that during 24, 48 and 72 hours durations, there was significant differences between control samples and all treated samples (P<0.001). In addition, at each treated concentration, there was a significant difference between 24, 48 and 72 hours durations (P<0.001). The associations between AGS cells growth and the concentrations of EEP indicated that this extract reduced viability of AGS cells in a time and dose-dependent manner (Figures 4). The IC_{50} of EEP on AGS human gastric cancer cell line in 24, 48 and 72 hours significantly decreased in time dependent manner.

Discussion

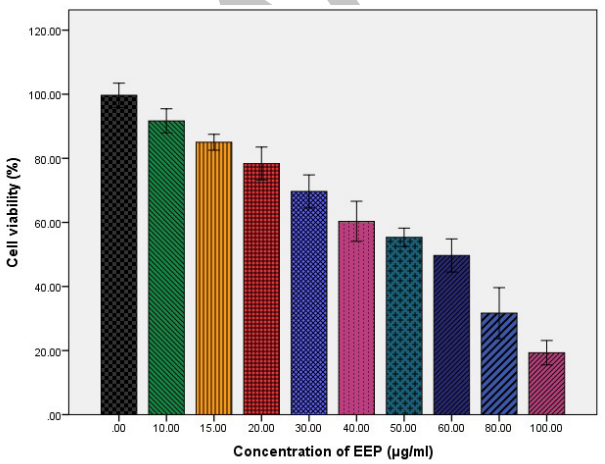

Figure 1. The effect of EEP at 0, 10, 15, 20, 30, 40, 50, 60, 80 and 100 µg/ml concentrations on AGS human gastric cancer cell line in 24 hours. Dunnett's test indicated a significant difference between different treated concentrations and control within 24 hours (P<0.001).

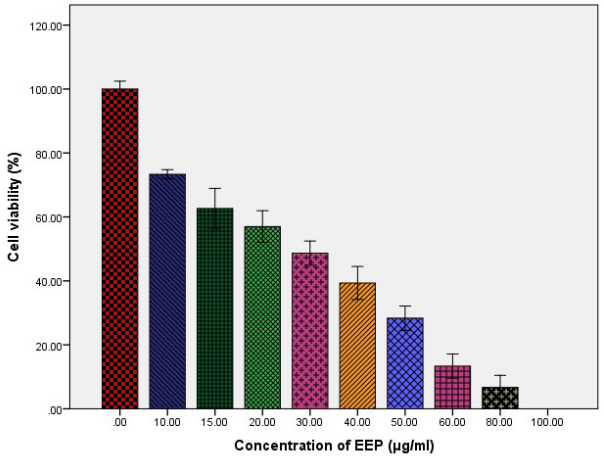

Figure 2. The effect of EEP at 0, 10, 15, 20, 30, 40, 50, 60, 80 and 100 µg/ml concentrations on AGS human gastric cancer cell line in 48 hours. Dunnett's test indicated a significant difference between different treated concentrations and control within 48 hours (P<0.001).

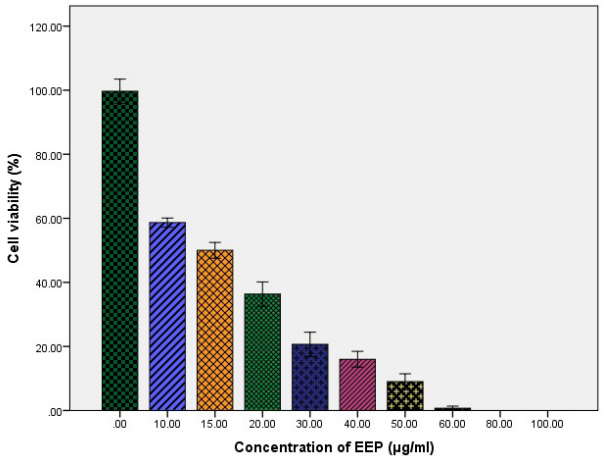

Figure 3. The effect of EEP at 0, 10, 15, 20, 30, 40, 50, 60, 80 and 100 concentrations on AGS human gastric cancer cell line in 72 hours. Dunnett's test indicated a significant difference between different treated concentrations and control within 72 hours (P<0.001).

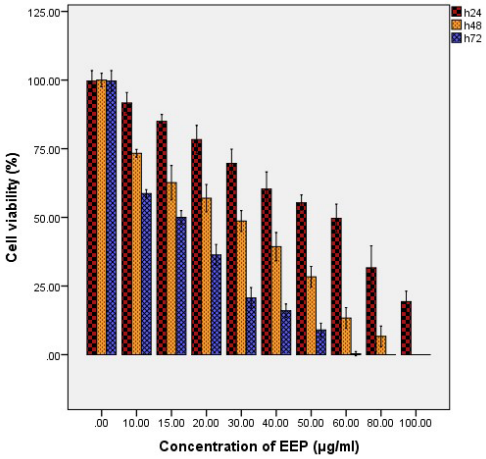

Figure 4. Comparison of the effect of EEP at different concentrations on AGS human gastric cancer cell line in 24, 48 and 72 hours. Dunnett's test indicated a significant difference between different treated concentrations and control within the periods as well as among the periods (P<0.001).

In recent years, the understanding of tumor biology has considerably changed the paradigm of cancer therapy and allowed the identification of new therapeutic targets, with development of new methods and therapeutic agents (15). Over the years, natural products have been used as promising sources for discovery of new pharmaceutical agents (16,17). Propolis, has been extensively studied for its biological properties (18), especially antitumor activity (19-25); nevertheless, there are no studies exploring Dinaran propolis with the purpose of gastric cancer therapy (24,26). In the present study we aimed to analyze the antitumoral activity of Dinaran propolis samples and we found that this EEP induced strong cytotoxic effect with decreases the viability and growth of AGS cells.

The antiproliferative activity of this extract was similar to that of propolis extracts exerting biological activities in other carcinoma cell types. For example, Dutch and Brazilian propolis exert antiproliferative activity in murine 26-L5 colon carcinoma, murine B16-BL6 melanoma, human HT1080 fibrosarcoma, and human A549 lung adenocarcinoma cell lines (27). Also, the Chilean propolis inhibited the growth of human KB mouth epidermoid carcinoma and DU145 androgen intensive prostate carcinoma cell lines (28). Therefore, findings of the presents study, in agreement with the above information, demonstrate a wide range of anticancer properties of propolis extract.

Over 300 compounds have been identified in propolis samples including flavonoids, polyphenols, phenolic aldehydes, terpenoides, coumarins, amino acids, steroids and non biological compounds (22). The propolis products obtained from European and Chinese consist of flavonoid types and phenolic acid esters (29), while Brazilian propolis consists of artepillin C, terpenoids and p-coumaric acid derivatives (30,31). Benzyl caffeate and phenethyl caffeate isolated from Chinese propolis induced antiproliferative activity in rats colon carcinoma cells (29). Ethanolic extract derived from Dutch and Chinese propolis exhibited significant cytotoxicity while Brazilian and Peruvian propolis extracts exerted low toxicity (32). One of the specifications of cancer therapies is ability to induction apoptosis and cell cycle arrest in tumor cells. Other studies suggested that propolis compounds could inhibit cell proliferation or induce apoptosis by suppressing cyclin complexes and proteins associated with cyclin, increasing the levels of cyclin-dependent kinase (Cdk) inhibitors such as p16, p21 and p27 proteins and cell cycle arrest, activation of Bax, p53 and p21 proteins, p38MAPK, JNK and ERK kinases, release cytochrome C into cytosol and activation of caspase cascades in tumor cells. These effects are not dependent on the type of tumor cell. The results of this studies also suggest the inhibition of NF-kB activation, the suppression of antiapoptotic proteins, such as IAP, c-FLIP, Akt kinase, and the initiation of extrinsic pathway of apoptosis by induction of TRAIL and Fas receptor stimulation in cancer cells (33).

Findings of present study that the EEP caused inhibition of AGS cells growth even at low concentrations, suggests that this extract contains compounds with anticancer activities. The components of propolis are associated with phytogeographical specifications including plant resources, distribution of vegetation and seasonal periods (22). Also several studies have confirmed the differences in percentages of individual components of propolis, depending on the origin of the plants from which the resin is collected and the species of bees, therefore, It is recommended to identify and isolate the active compounds of this extract to developing new drugs for gastric and other cancer therapies.

Overall, our results strongly suggest that the EEP caused dose- and time-dependent decreases in rates of AGS cell growth and proliferation. Therefore, as a strong bioactive and promising source for new drugs development, this Propolis and its compounds could be potentially useful as chemotherapeutic or chemopreventive gastric anticancer drugs.

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Authors' contributions

NAS performed the project. HT and MMD were the supervisors. NAS and SKF analyzed the data and wrote the article.

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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