Anticancer Activity of Anise (Pimpinella anisum L.) Seed Extract

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Abstract: Cancer incidence is much lower in India than in western countries. The reason is not fully understood, but the high spice consumption could be one of the contributing factors. Anise is one of the plants grows in India and people from our region believe that anise seeds are helpful in cancer prevention and treatment. In this study, anticancer activity of ethanol extract of anise (Pimpinella anisum L.) seed was investigated. MTT and LDH assays revealed that ethanolic extract have cytotoxic activity on human prostate cancer cell line [PC-3] at concentrations found safe to normal cells (rat skeletal muscle cell line [L6]). Treatment with anise seeds extract caused anti proliferative and apoptotic effects, with IC50 value of 400 μg/mL to cancer cells. Thus, anise could be one of the foods that attribute to cancer prevention and treatment. It could also be a natural source of novel anticancer compounds with anti proliferative and/or apoptotic properties and it is worth to work on for isolation and identification of novel anticancer drug candidates.

Keywords: Pimpinella anisum L., Anise, Natural product, Anticancer activity, Nutraceutical.

INTRODUCTION

The majority of the currently used cosmetics and drugs are natural products-based compounds or their derivatives. This could add weight to the argument that natural based products are inherently better tolerated in the body than synthetic chemicals and have higher chance to be approved as drugs [1, 2]. Natural product-based medicines, particularly, herbal- based drugs represented about 60-80 percent of all drugs in use by 1990 [3, 4]. During the past couple of decades, after the introduction of high throughput synthesis and combinatorial chemistry, natural products became less significant source of leads and drugs. Although global expenditure on drug research has doubled since 1991, the number of new drug entities approved annually decreased by 50% or even more [5]. To change this situation, the players in the pharmaceutical industry shifted their interest back to natural or natural-based products [6]. It becomes commonly accepted that natural based products are inherently better tolerated in the body and have innate advantages for drug discovery and development over synthetic chemicals [7]. As well, it is well accepted that nutritional factor is playing significant role in prevention and therapy of many diseases [8, 9].

Cancer is not a simple disease but a complex interaction between multiple signaling pathways with various target molecules [10]. Despite significant progress in the treatment of certain forms of cancer, it remains a major cause of death throughout the world [11-13]. So we are asking the question, why are we losing the war against cancer? what is the future of cancer research?

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Some people claim that the main reason of very limited success in the anticancer era is due to focusing on targeting a single target, usually a single gene, gene product, or signaling pathway. Such strategy could have very little therapeutic impact against complex diseases (e.g., cancer).

The World Cancer Research Foundation 2007 report estimates that 35% of the cancer incidence worldwide could be referred to lifestyle factors such as nutrition, food and physical activity. Increasing evidence indicates that consuming fruits, vegetables, spices and nuts could protect against cancer. Studies indicate that individuals who consume more fruit, vegetables and spices are associated with lower incidence of cancer [14-17]. In 2000, the United States had 356 colon cancer cases reported compared to 40 cases in India. Why cancer incidence is so much lower in India than in USA and western countries is not fully understood, but the high spice consumption in India compared to the west could be one of the contributing factors [18, 19].

In this short paper, we will focus on beneficial effect of anise seeds on prevention and treatment of cancer. Anise seeds could be one of the spices contributing to low incidence of cancer in India and Mediterranean region compared to USA and Europe. A short survey among people in our region revealed that most of them believe that anise seeds is helpful in cancer prevention and treatment. Searching the PUBMED we have not found any report on effects of anise extracts on cancer. In this study we aim to check this claim and verify it scientifically.

Pimpinella anisum L. is an annual herb and a grassy plant with white flowers and small green to yellow seeds, which grows in the Mediterranean region, India and many other worm regions in the world. Pimpinella anisum L. is primarily grown for its fruits (seeds) that are currently used for fla-

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voring and for different purposes. The essential oil from Pimpinella anisum L. seeds is used in food preparation, traditional medicine and perfumery industry. It was reported that anise seeds has several therapeutic effects [20, 21] on several conditions such as digestive, neurologic, cough [22] and respiratory disorders. Anise seeds are used in middle east as appetizer and is specially known for its digestive properties [23]. Anise seeds' extract of water when consumed after meals helps in the process of digestion. Among the reported pharmacological effects we can find anise extracts active as anti-ulcer [24, 25], Antispasmodic [26], Antibiotic [27], Performance enhancement (immunomodulation) [28], Insecticidal [29]. However, to our best of knowledge, anticancer activity of anise extract was not reported. In the present study we will evaluate the anti cancer activity of ethanolic extract of Pimpinella anisum L. seeds and determine its anti proliferative and apoptotic properties.

MATERIALS AND METHODS

Plant Extract Preparation

The plant seeds were purchased from (Al Alim - Medicinal Herb Center, Zippori,Israel), washed with distilled water and dried in shade. It was finally grinded to powder. Fifty grams of the Anise seeds powder were added to 250 ml of ethanol in a beaker with magnetic stirrer and homogenized for 15 minutes at 60°C. Then left in dark glass bottle for 24 h for complete extraction. The extract supernatant obtained was passed through a 0.2 μm filter and ethanol was evaporated to give 9.1 gram of pure extract (18% yield). GC/MS shows that the extract is composed of dozens of chemicals (data not shown since chemical composition is not the scope of this communication letter).

Cell Cultures and Treatments

PC-3 cells (human prostate cancer cell line) and L6 cells (rat skeletal muscle cell line) were purchased from American Type Culture Collection (ATCC) (Manassas, VA) though their representative in Israel, Biological Industries Israel Beit Haemek Ltd. The cells were grown in F-12K (ATCC) (PC-3 cells) medium or α-MEM (L6 cells) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 0.1 mg/ml streptomycin. All cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC. It is worth to assign that L6 is used as representatives of normal cells and is useful tool to investigate the toxicological properties of chemicals [30-32].

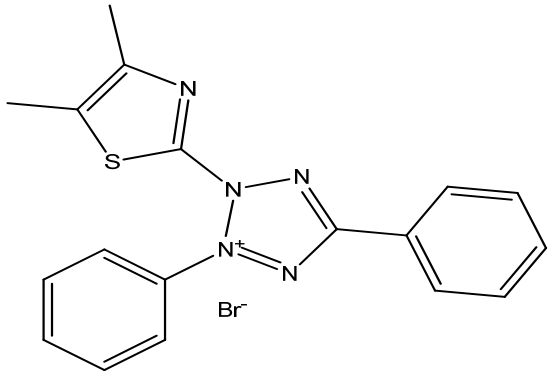
MTT Assay

The MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce MTT [3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to its insoluble formazan [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide], giving a purple color. Structures of both chemicals are shown in Fig. (**1**). Thus, MTT assay is a cell viability test often used to determine cytotoxicity following exposure to toxic substances. The MTT assay, developed by Mosmann and described for the first time by him, depends on the reduction of tetrazolium salt by living cells. The assay was optimized for the cell lines used in these experiments. MTT assay was applied to assess cell viability as described in Saad *et al* [33]. Cells ($2x10^4$/well) were plated in 100 μl of medium/well in 96-well plates (corning incorporated, USA) and were allowed to attach to the plate for 24h. *Pimpinella anisum* seeds' extract was added at increasing concentrations (0-2 mg/ml) for 24 h. The cells medium was replaced with 100 μl fresh medium/well containing 0.5 mg/ml MTT [purchased from Sigma Aldrich company] and cultivated for another 4 h darkened in the cells incubator. The supernatant was removed and 100 μl isopropanol/HCl (1mM HCl in 100% isopropanol) were added per well. The absorbance at 570 nm was measured with microplate reader (Anthos). Two wells per plate without cells served as blank. All experiments were repeated three times in triplicates. The effect of the plants extracts on cell viability was expressed using the following formula:

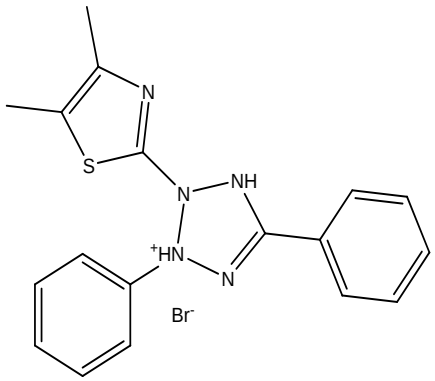
Percent of viability = (Absorbance of plant extract treated sample at 570nm wave length / Absorbance of plant extract non treated sample at 570nm wave length) * 100%

Lactate Dehydrogenase (LDH) Leakage Assay

The permeability of cellular membranes following the exposures was determined by measuring the amount of released lactate dehydrogenase (LDH) enzyme from PC-3 and L6 cells. Activity of LDH released to the cell culture me-



3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide



3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide

Fig. (1). Chemical structures of MTT (above) and formazan (bellow). MTT is reduced to formazan by dehydrogenases and reductases.

dium was monitored following the formation of formazan by coupled enzymatic reaction at 500 nm according to the manufacture kit (CytoTox 96, Promega). Cell membrane rupture was defined as the ratio of LDH activity in the supernatant of treated cells to the LDH activity released in the control cells. PC-3 and L6 cells ($2x10^4$/well) were plated in 100 µl of medium/well in 96-well plates and were allowed to attach to the plate for 24h. After cell attachment (24h) cells were treated with increasing concentrations of the plant extracts (0-2 mg/ml). The extracellular LDH activity was measured in the medium after 24 h. Therefore, 50 µl from each well was transferred to a new 96 well plate; the enzyme reaction was carried out according to the manufacture kit. All experiments were repeated three times in triplicates. The effect of the plants extracts on cell viability was expressed using the following formula:

Percent of viability = (Absorbance of plant extract treated sample at 500nm wave length / Absorbance of plant extract non treated sample at 500nm wave length) * 100%

RESULTS AND DISCUSSION

By the end of the extraction of *Pimpinella anisum* seeds by ethanol, cytotoxicity experiments were conducted to reveal effectiveness of consuming anise in cancer prevention and treatment.

In Vitro Anticancer Activity of the Anise Seeds' Ethanolic Extract

Human prostate cancer cell line (PC-3) and rat skeletal muscle cell line (L6) were exposed to Pimpinella anisum (0–2 mg/ml) for 24 h and cytotoxicity was determined with the MTT assay and the LDH leakage assay. The EC50 values obtained by the MTT assay, is 0.74 mg/ml ± 0.008 for the L6 cell line and 0.42 mg/ml ± 0.05 for the PC-3 cell line.

Figs (**2** and **3**) present the dose response curve of the anise seeds' extract on rat skeletal muscle cell line (L6) by the MTT and LDH leakage assays, respectively. L6 cells were exposed to *Pimpinella Anisum* ethanolic extract (0–2 mg/ml) for 24 h. As shown, no Cytotoxicity is observed in L6 cells

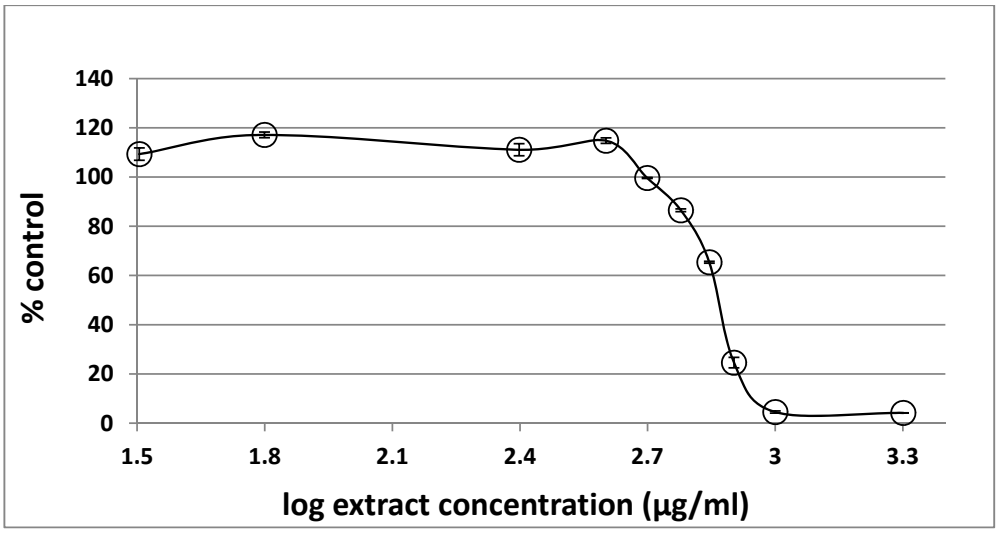


Fig. (2). MTT assay in rat skeletal muscle cell line (L6) after exposure to *Pimpinella anisum* seeds' extract for 24 h; data presented as percentage of control (n=3, each value represents the mean of triplicate) ±SEM; SEM: standard error mean.

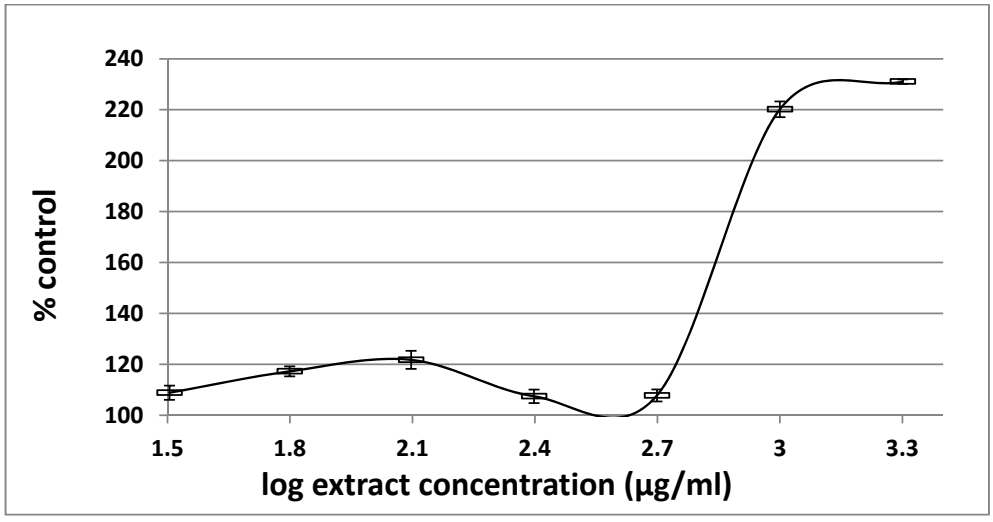


Fig. (3). LDH leakage assay in rat skeletal muscle cell line (L6) after exposure to *Pimpinella anisum* seeds' extract for 24 h; data presented as percentage of control (n=3, each value represents the mean of triplicate) ±SEM; SEM: standard error mean.

(representative of normal cells) up to 0.5 mg/ml of *Pimpinella Anisum*. No decrease in cell viability was notable even after a 24 h exposure of L6 cells up to 0.5 mg/ml *Pimpinella Anisum* ethanolic extract and higher concentrations of anise extract are required to see any notable effect.

In contrast, Figs (**4** and **5**) present the dose response curve of the extract on cancer cells measured by MTT and LDH leakage assays, respectively. The obtained results indicate that tested anise seeds' extract has an anticancer activity against human prostate cancer cell line with notable effect even at low concentration. At 0.03 mg/ml there was about 20% decrease in cell viability and at 0.5 mg/ml concentration cell viability decreased to less than 40%. The results were evaluated statistically (T-TEST) with significance set at a P value of < 0.02. The anti cancer effectiveness of anise in low concentrations could be due to targeting one or more of the signaling pathways related more to cancer cells than to normal cells. As well, anise could contain more than one chemicals bearing anti cancer activity. The findings in this report is in agreement with the hypothesis that anise seeds is helpful in cancer prevention and treatment.

CONCLUSIONS

The reported results show that ethanolic extract of anise seeds has significant anticancer effect on prostate cancer (PC-3 cell line) compared to normal cell line (L6). Thus, anise seeds could be helpful in cancer prevention and treatment. Anise could be a natural source of novel anticancer compounds with anti proliferative and/or apoptotic properties. We intend to start fractionation aiming to validate the efficacy and start the process for isolation and identification of the anticancer active ingredients. It is worth to assign that Anise seeds could contain more than one chemical active on cancer cells *via* targeting various cancer related pathways or biological targets. As well, due to its anticancer pharmacological effect, clinical trials are recommended to evaluate the beneficial effects of this plant in human models.

CONFLICTS OF INTEREST

The authors confirm that this article content has no conflicts of interest.

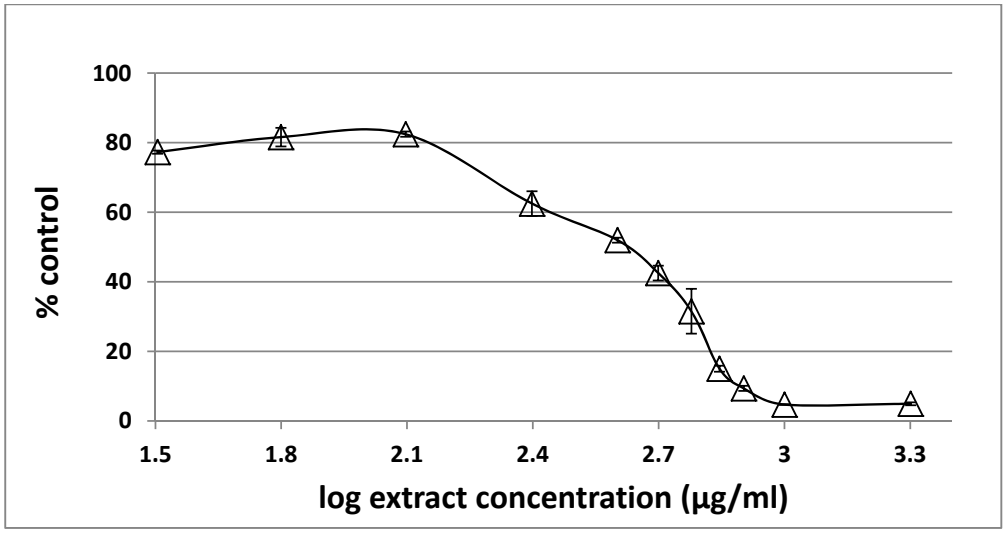


Fig. (4). MTT assay in human prostate cancer cell line (PC-3) after exposure to *Pimpinella Anisum* seeds' extract for 24 h; data presented as percentage of control (n=3, each value represents the mean of triplicate) ±SEM; SEM: standard error mean.

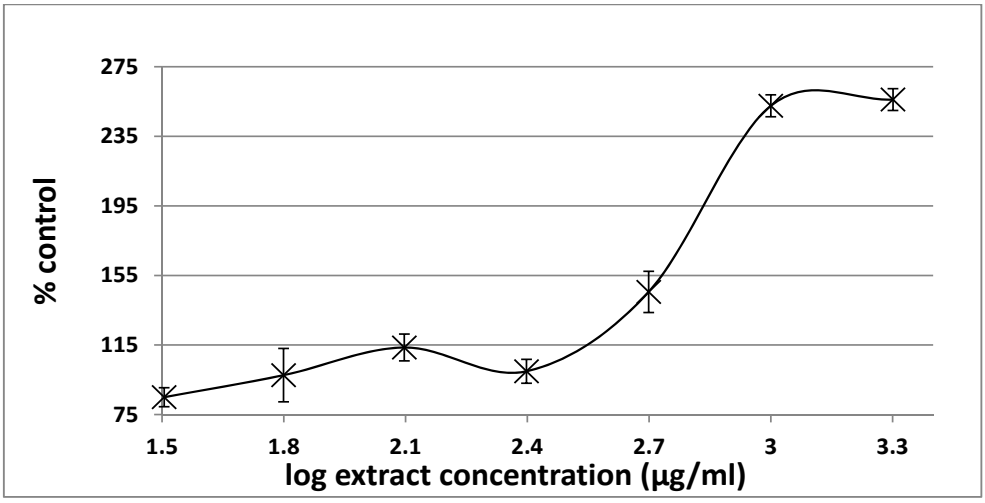


Fig. (5). LDH leakage assay in human prostate cancer cell line (PC-3) after exposure to *Pimpinella Anisum* seeds' extract for 24 h; data presented as percentage of control (n=3, each value represents the mean of triplicate) ±SEM; SEM: standard error mean.

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