



Cytotoxic Activities of Certain Medicinal Plants on Different Cancer Cell Lines

Bazı Tıbbi Bitkilerin Farklı Kanser Hücre Hatlarında Sitotoksik Aktiviteleri

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ABSTRACT

Objectives: In recent years, the use of plants for the prevention and treatment of cancer is gaining more attention due to their diverse range of phytochemical constituents and fewer adverse effects. In this study, four medicinal plant species from the Kars province of Turkey were investigated for their cytotoxic potential against six different cancer cell lines and one normal cell line.

Materials and Methods: MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-dipenyltetrazolium bromide] assay was performed to assess cytotoxic activity and apoptotic effect was determined using flow cytometry and caspase-3 analyses.

Results: Significant cytotoxicity ($\geq 70\%$) was observed with the leaf extract of *Artemisia absinthium* on A-549, CCC-221, K-562, MCF-7, PC-3 cells, whereas seed extracts caused significant cytotoxicity ($\geq 70\%$) on CCC-221, K-562, MCF-7, PC-3 cells. Selective cytotoxicity was obtained with leaf extract on A-549 and K-562 cells; and with seed extract on K-562, MCF-7 and PC-3 cells compared with normal Beas-2B cells. The levels of cytotoxicity for both extracts were time- and dose-dependent at lower concentrations. Moreover, selective cytotoxicity (78%) was detected on A-549 cells with the seed extract of *Plantago major*. Cytotoxicity of extracts from *Hyoscyamus niger* and *Amaranthus retrosa* ranged between 10% and 30%.

Conclusion: *A. absinthium* extracts and *P. major* seed extract have potential for development as therapeutic agents for cytotoxicity on certain cancer cells following further investigation.

Key words: Cytotoxicity, cancer cells, medicinal plants, apoptosis, *Artemisia absinthium*

ÖZ

Amaç: Son yıllarda kanserin önlenmesi ve tedavisi için, farklı sınıfta fitokimyasal içerikleri ve daha az yan etkileri nedeniyle bitkilerin kullanımı oldukça önem kazanmaktadır. Bu çalışmada, Türkiye'nin Kars ilinden toplanan dört farklı tıbbi bitki türünün altı farklı kanser hücre hattı ve bir normal hücre hattı üzerindeki sitotoksik etkileri incelenmiştir.

Gereç ve Yöntemler: Sitotoksik aktivite MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-dipenyltetrazolium bromide] yöntemiyle belirlenirken, apoptotik hücre ölümü akış sitometri ve kaspaz-3 analizleriyle tespit edilmiştir.

Bulgular: *Artemisia absinthium* yaprak özütü A-549, CCC-221, K-562, MCF-7, PC-3 hücre hatları üzerinde anlamlı sitotoksik etki ($\geq 70\%$) gösterirken, tohum özütü ise CCC-221, K-562, MCF-7, PC-3 hücreleri üzerinde anlamlı sitotoksik etki ($\geq 70\%$) göstermiştir. Normal hücre hattı Beas-2B hücresine kıyasla, seçici sitotoksik aktivite yaprak özütüyle A-549 ve K-562 hücreleri üzerinde gözlenirken, tohum özütüyle K-562, MCF-7 ve PC-3 hücreleri üzerinde gözlenmiştir. Her iki özütün sitotoksikite seviyesi düşük konsantrasyonlarda zaman ve doza bağlı olarak değişmektedir. İlave olarak, *Plantago major* bitki özütüyle A-549 hücreleri üzerinde seçici sitotoksikite (%78) elde edilmiştir. *Hyoscyamus niger* ve *Amaranthus retrosa* özütlerinin sitotoksikite aktivitesinin %10 ve %30 olduğu gözlenmiştir.

Sonuç: *A. absinthium* özütleri ve *P. major* tohum özütü gelecek çalışmalarda bazı kanser hücrelerinin sitotoksikite için tedavi edici ilaç geliştirilmesi potansiyeline sahiptirler.

Anahtar kelimeler: Sitotoksikite, kanser hücreleri, tıbbi bitkiler, apoptozis, *Artemisia absinthium*

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INTRODUCTION

Cancer is the most common and lethal disease in the modern era.¹ New strategies or compounds need to be discovered because most known cancer treatments have adverse effects and all tumors do not react in the same way to treatment. Plant-based medicines have good potential as a primary source for chemotherapeutic drugs. Some of the currently-used chemotherapeutic agents such as paclitaxel for breast cancer, vinca alkaloids for leukemia, and flavopiridol for colorectal cancer were initially derived from plants.²⁻⁵ In addition, herbal products are still used as primary health care products in most third world countries.⁶ Accordingly, plants as herbal medicines are good sources to search for antitumor compounds.

Traditional studies of plants use two parts, subterranean and surterranean. While almost all subterranean parts of the plant consist of root, surterranean parts of the plant have the bark, leaves, flowers, and seeds. All of these are derived from shoot apical meristem, but they are highly differentiated and consist of different secondary metabolites in different compositions.⁷ In addition, medical uses of such plants are also specific to different parts of the plant, or in some cases, highly specific to certain regions of some parts. Therefore, it is important to clarify the part of the plant to be used for the source of therapeutic agents.

Studies have demonstrated that most phytochemicals act by interfering with several cell-signaling pathways and lead to cell cycle arrest and/or differentiation induction, apart from their apoptosis-inducing potential.⁸ Apoptosis is a central event essential in the maintenance of tissue homeostasis for all organ systems in the human body.⁹ Suppression of apoptosis in carcinogenesis plays a central role in the development and progression of cancer. Tumor cells use a variety of molecular mechanisms to suppress apoptosis.¹⁰ Hence, induction of apoptosis in tumor cells is a specific therapeutic approach towards cancer chemotherapy.

Artemisia species belong to the *Asteraceae* family and are widely distributed in Asia, Europe, and North America. Medicinal values of *Artemisia* species have been approved throughout the world. Pharmacologic studies of *Artemisia annula* indicated the presence of novel biologically active compounds such as monoterpenes, sesquiterpenes, lactones, flavonoids, and coumarins.¹¹ The extracts of some *Artemisia* species have been reported to exhibit cytotoxic effects on breast cancer cells.¹² Moreover, *Plantago* species have been used against cancer and Gálvez et al.¹³ showed cytotoxic activities of luteolin-7-O-beta-glucoside, a major flavonoid against human renal adenocarcinoma and human melanoma. In contrast to *Artemisia* and *Plantago* species, not much information is available for *Hyoscyamus niger* and *Amaranthus retroso* on cytotoxic activities against cancer cells.

Turkey is at the cross section of three phytogeographic regions, and is therefore very rich in terms of biodiversity.¹⁴ More than 12.000 species and sub-species are considered to be in the region and they are used medically, as well as in the form of food and feed.¹⁵⁻¹⁷ The Kars province in the Eastern region of

Turkey has a tradition of using herbal medicine for a variety of diseases, from headaches to cancer.¹⁵ *Artemisia* species from other countries are established to be cytotoxic in cancer^{12,18}; however, there are no reports on the cytotoxicity of other plants on cancer cells. Although the chemical composition and biologic activities of many plant species are known in the literature, the level and quality of compounds, as well as their biologic activities can change significantly according to geographic origin and growing conditions. Therefore, this is the first study to evaluate the anticancer activities of four medicinal plants from Kars, which has 1800 m altitude. Ethanolic extracts of these plant parts were examined for their cytotoxicity on six cancer cell lines and one normal cell line according to time and concentration as variables. Furthermore, the mechanism of cytotoxicity was analyzed for *Artemisia absinthium* on A-549 cells using flow cytometry and a caspase-3 assay.

MATERIALS AND METHODS

Cell lines and culture conditions

A-549 (human lung carcinoma), CCC-222 (human colorectal carcinoma), DU-145 (human prostatic carcinoma), MCF-7 (human breast carcinoma), K-562 (human leukemic carcinoma), and PC-3 (human prostatic carcinoma) cell lines were procured originally from ATCC. All cell lines were maintained in RPMI 1640 culture medium (Biochrom, Germany), supplemented with 10% fetal bovine serum (FBS) (FBS; Biochrom, Germany), penicillin (100 U/mL) and streptomycin sulphate (100 mg/mL) (Biochrom, Germany). Cells were incubated at 37°C in 5% CO₂, 95% air in a humidified incubator.

Plant material

Four different plant species, *A. absinthium* L. (*Asteraceae*), *H. niger* L. (*Solanaceae*), *A. retroso* L. (*Amaranthaceae*) and *Plantago major* L. (*Plantaginaceae*) were collected from Kafkas University Campus in July and authenticated by Dr. Fatma Güneş in the Botanic Laboratory. The herbarium numbers of plants are *A. absinthium* L. (FG 2562); *A. retroso* L. (FG 2563); *H. niger* L. (FG 2564); *P. major* L. (FG 2565). These plants are deposited in Trakya University, Herbarium of Faculty of Pharmacy.

Plant extraction

The plant samples were air-dried under shade and milled to a powder using a porcelain muller. Powdered plant materials (10 g) were extracted with 100 mL of ethanol several times by shaking at 53.1-54.6°C until the color faded. The extracts were filtered and solvent was removed in a vacuum using a rotary evaporator at 42-43°C in an Erlenmeyer bulb. After that, extracts was lyophilized to give the crude dry extract in freeze-dryer. The powdered crude extracts were stored at -20°C until used. All extracts were dissolved in 10% DMSO as stock solution and further dilutions were freshly prepared to achieve working solutions.

MTT assay

The cytotoxic activity of plant extracts was determined using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide] assay (Applichem, USA). This assay detects the reduction of MTT by mitochondrial dehydrogenase to a blue formazan product, which reflects the function of mitochondria and cell viability.¹⁹ Exponentially growing cells at 2×10^4 cells/mL were plated in triplicate into 96-well plates (Greiner, Germany) in 200 μ L of growth medium and incubated for 24 hr before the addition of extracts. Plant extracts were dissolved in 10% DMSO and added to the cell culture at a final concentration of 4 mg/mL to be tested against six cell lines. Cells were incubated for 72 hr at 37°C in a 5% CO₂ incubator. For the dose and time responsiveness of the cells, plant extracts were added at 0.1 mg/mL, 0.2 mg/mL, 0.5 mg/mL, 1 mg/mL, and 2 mg/mL final concentrations, and incubated for 24, 48, and 72 hr. After that, 10 μ L of PBS containing 5 mg/mL MTT was added into each well. After 4 hr incubation, the medium was discarded and the formazan blue crystals that formed in the cells were dissolved in 100 μ L DMSO. Reduced MTT was quantified by reading the absorbance at 540 nm on a microplate reader (Thermo Scientific Multiscan Spectrum). The cytotoxic effects of the tested extracts were determined by comparing the optical density of the treated cells against the optical density of the untreated cells. Cytotoxicity relative to controls was calculated using the following formula:

$$\% \text{ Cytotoxicity} = [(A_c - A_t) / A_c] \times 100 \quad (17)$$

Where A_c and A_t are mean absorbance of the control wells and mean absorbance of test wells, respectively.

Caspase-3 assay

Caspase-3 activity was determined using caspase-3 colorimetric assay kit (Abcam, USA). This assay measures the amount of free p-nitroanilide (pNA) in the cell produced by the cleavage of DEVD-pNA bond by activated caspase-3 during apoptosis. The experiment was conducted in accordance with the manufacturer's protocol. In brief, A549 cells were treated with 4 mg/mL of *A. absinthium* extract for 36 hr. After that, cells were trypsinized and centrifuged at 1100 g for 10 min. Cells were resuspended in 1 mL of cold PBS and centrifuged at 4000 g for 5 min at 4°C. In order to extract total protein, cell pellets were suspended and lysed with 100 μ L of lysis buffer and incubated on ice for 10 min. The cell lysates were then centrifuged at 10,000 g for 1 min at 4°C. The protein concentration was measured using a Bradford assay. Then, 200 μ g protein from each sample was mixed with 50 μ L of 2x reaction buffer containing 10 mM DTT and 5 μ L of 4 mM caspase-3 substrate (DEVD-pNA) and incubated at 37°C for 4 h. The p-NA light emission was quantified using an ELISA plate reader at 405 nm. The fold increase in caspase-3 activity was determined by comparing the absorbance of p-NA from an apoptotic sample with an untreated control cells.

Flow cytometry

A-549 cells were cultured in 6-well plates (5×10^5 cells/well) and treated with 0.25 mg/mL, 1 mg/mL, 2 mg/mL, and 4 mg/mL concentrations for both *A. absinthium* leaf and seed for 24 hr. After treatment, cells were washed with PBS, trypsinized, washed, and resuspended in binding buffer. Five microliters of

propidium iodide (PI) and 5 μ L Annexin-V-FITC were added. After incubation for 15 min in the dark, 500 μ L of binding buffer was added and ten thousand cells per group were analyzed using flow cytometry (BD FACSCanto A, BD Biosciences, USA). To detect PI and AnnexinV-FITC, green solid state 488 laser was used for excitation. Filter configurations for PI and FITC were 556/LP and 585/40, and 502/LP and 530/30, respectively.

Statistical analysis

Data were analyzed using the Microsoft Office Excel (2007) SPSS software package. Multiple comparisons of treatments were performed using one-way ANOVA and Student's t-test. A difference was considered to have significance at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$. Data are presented as mean \pm standard deviation of three replicates.

RESULTS

Screening of cytotoxic effects of plant extracts on different cancer cell lines

Six different cancer cell lines were treated with 4 mg/mL leaf or seed extracts for 72 hr to distinguish the effectiveness of each part of the plant on cytotoxicity (Figure 1). The results indicated that *A. absinthium* extracts showed toxicity between 40% and 87% on all cell lines, except DU-145 cells (Figure 1a, b). Unlike *A. absinthium* extracts, other extracts exhibited either cytotoxic or mitogenic effects depending on the cell lines. The least toxic extract was the *A. retrosa* seed, which showed 25% cytotoxicity on DU-145 cells. In addition, leaf extract of *A. retrosa* was cytotoxic on DU-145 and K-562 cell lines by 22% and 28%, respectively (Figure 1e, f). With the exception of the *H. niger* seed extract, all extracts resulted in cytotoxicity between 20% and 30% on DU-145 cells.

The most effective extracts on cytotoxicity of K-562 cell lines were *A. absinthium* leaf (86%) and *A. retrosa* leaf (28%). In addition, the highest cytotoxicity (78%) for A-549 cells was obtained from *P. major* seed extract (Figure 1h). Among the other plant extracts, *P. major* seed was the best extract for selective cytotoxicity on A-549 cancer cell lines because it did not cause cytotoxicity on the normal Beas-2B cell line.

Two statistical analyses were performed to see if the effects of extracts on cell lines were significant compared with the control containing no extract (Table 1) and the normal cell line, Beas-2B (Table 2). The most significant cytotoxic effects were exhibited by *A. absinthium* leaf and seed extracts, and *P. major* seed extract (Table 1). To date, most chemotherapeutic agents do not distinguish between normal and cancer cell lines. In the interest of having less damage to normal cells after cancer therapy, it is important to discover plant extracts with selective cytotoxicity for cancer cells. Therefore, the cytotoxic effect of plant extracts on cancer cells were compared with that of normal Beas-2B cells. According to the statistical analysis, *A. absinthium* seed extracts caused significant cytotoxicity on K-562, MCF-7, and PC-3 cell lines (Table 2). In addition, significant cytotoxic effects compared with Beas-2B cells were observed with *A. retrosa* leaf extract on DU-145 and K-562 cell lines, and *P. major* seed extracts on A-549 and DU-145 cell lines.

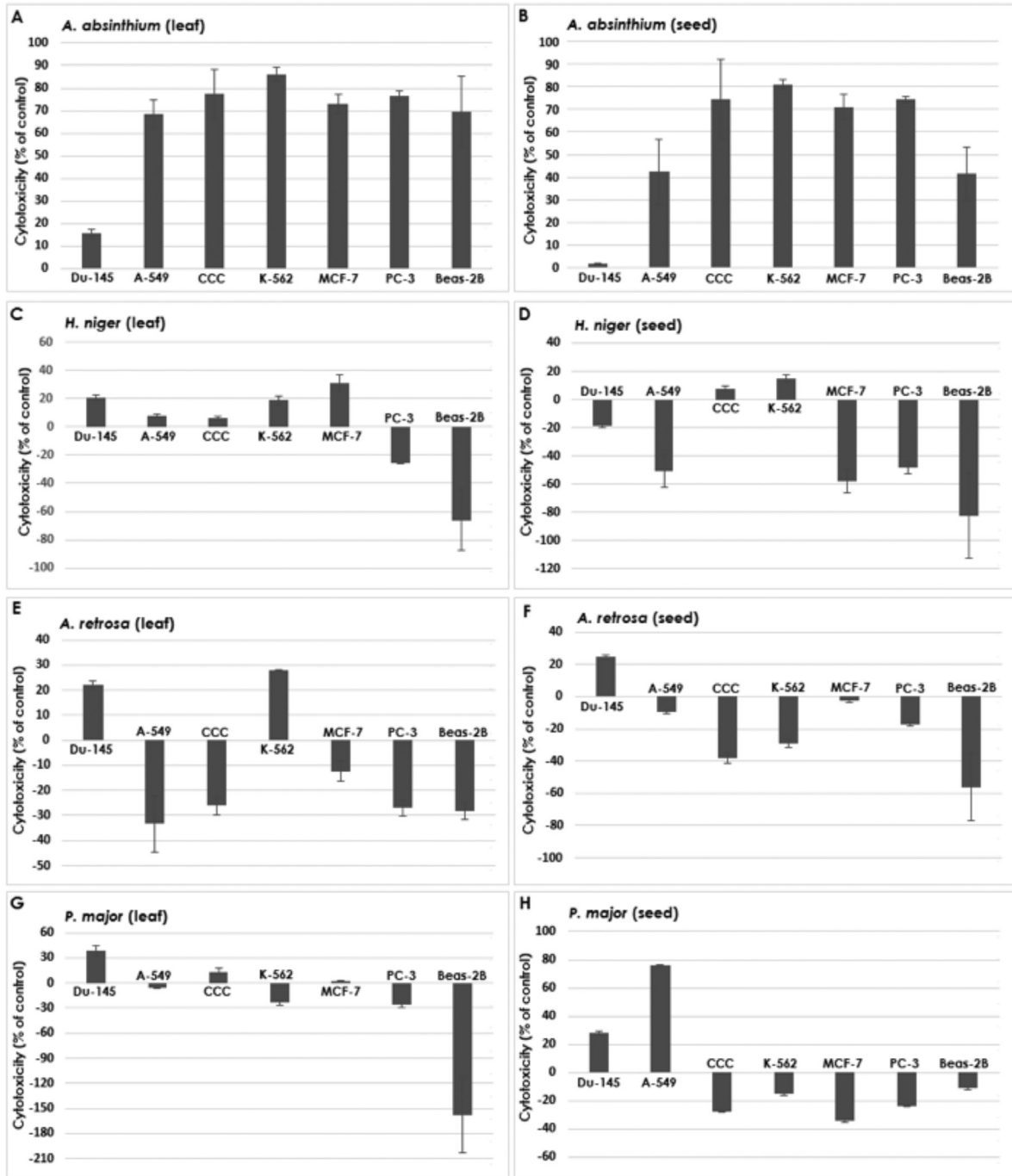


Figure 1. Cytotoxicity of plant extracts on different cancer cell lines. Cells were treated with leaf or seed extracts of plants at 4 mg/mL concentration for 72 hr. Cytotoxicity was determined using MTT assays and results expressed as percentages of cytotoxicity compared with untreated control. Data expressed as mean ± standard deviation, n=3

Time- and dose-dependence of cytotoxicity

After detecting significant cytotoxicity on A-549 and K562 cell lines treated with *A. absinthium* leaf and seed extracts, we investigated how time of treatment with varying concentrations of extracts affected the cytotoxicity. Cells were treated with 0.1, 0.2, 0.5, 1, 2, and 4 mg/mL extracts for 24, 48, and 72 hr. Extracts caused cytotoxicity in a different manner (Figure 2). For example, leaf extract above 0.2 mg/mL concentration

resulted in cytotoxicity between 60% and 70% on A-549 cells on day 1, remained the same level on day 2, and was between 60% and 83% on day 3 (Figure 2a). A similar manner of cytotoxicity was observed with seed extracts (Figure 2b). In addition, the cytotoxic effects of seed and leaf extracts on K-562 cells were proportional with the duration of exposure and the concentration of the extract (Figure 2c, d).

Table 1. Difference of cytotoxicity on treated cells compared with untreated controls

	Beas-2B	A-549	CCC-221	DU-145	K-562	MCF-7	PC-3
<i>A. absinthium</i> (leaf)	***	***	***	(-)	***	***	***
<i>A. absinthium</i> (seed)	*	(-)	***	(-)	***	***	***
<i>H. niger</i> (leaf)	(-)	(-)	(-)	*	(-)	(-)	+++
<i>H. niger</i> (seed)	(-)	+	(-)	+	(-)	+	++
<i>A. retrosa</i> (leaf)	+	(-)	(-)	*	***	(-)	+
<i>A. retrosa</i> (seed)	(-)	+	+	**	+	(-)	+
<i>P. major</i> (leaf)	+	(-)	(-)	*	(-)	(-)	(-)
<i>P. major</i> (seed)	(-)	***	(-)	***	(-)	++	+++

*Means cytotoxicity with *p<0.05, **p<0.01, ***p<0.005

†Means mitogenicity with †p<0.05, ††p<0.01, †††p<0.005

-No significant difference

Table 2. Difference of cytotoxicity on cells compared with normal cell line Beas-2B

	A-549	CCC-221	DU-145	K-562	MCF-7	PC-3
<i>A. absinthium</i> (leaf)	+	(-)	(-)	***	(-)	(-)
<i>A. absinthium</i> (seed)	(-)	(-)	*	***	***	***
<i>H. niger</i> (leaf)	*	(-)	***	*	*	(-)
<i>H. niger</i> (seed)	(-)	(-)	***	*	(-)	(-)
<i>A. retrosa</i> (leaf)	(-)	+	***	***	(-)	+
<i>A. retrosa</i> (seed)	(-)	+	***	+	(-)	(-)
<i>P. major</i> (leaf)	(-)	*	***	***	***	**
<i>P. major</i> (seed)	***	+	***	++	+	+++

*Means cytotoxicity with *p<0.05, **p<0.01, ***p<0.005

†Means mitogenicity with †p<0.05, ††p<0.01, †††p<0.005

-No significant difference

In addition, the dose-dependence experiment of *P. major* seed extract was conducted on the cytotoxicity of the A-549 cell line. Cells were treated with 4 mg/mL seed extract for 72 hr. It was observed that the level of cytotoxicity was proportional with the increasing concentration of the extract and reached around 40% at 2 mg/mL, and 73% at 4 mg/mL (Data not shown).

Flow cytometric analysis of cell death

Cellular cytotoxicity can be accounted for by various cellular processes, the most common of which is cell death. Cell death is classified as necrosis, apoptosis, and autophagy. Apoptosis is planned cell death, whereas autophagy and necrosis are considered as sudden deaths and occur when cells are under extensive stress. In this present study, flow cytometric analysis was performed in order to confirm cell death after treatment of A-549 cells with different concentrations of *A. absinthium* leaf and seed extracts. The results indicated a difference between the cytotoxicities created by seeds and leaf extracts (Figure 3). At a lower concentration of 0.2 mg/mL, seed extracts had already killed around 25% of cells, whereas the leaf extract exhibited the same cytotoxicity as the control (Figure 3b, Table 3). It took up to 2 mg/mL concentration to show a different profile than the controls for the leaf extract (Figure 3d). For both extracts, the

percentage of dead cells gradually peaked as the concentration of extract increased. At 4 mg/mL, leaf and seed extracts caused 20% and 6% survival rates, respectively (Figure 3e, Table 3). As a result, seed extract seems to kill A-549 cells through apoptosis at a lower concentration than leaf extract (Figure 3c).

Investigation of apoptosis by caspase 3 assay

Apoptosis occurs through a series of cleavages of serine proteases called caspases. That serial cleavage, which is called a caspase cascade, is present in both the external and internal apoptotic pathway. In both pathways, the last protease that executes cell death is caspase-3. Cleavage of the apoptotic substrate by activated caspase-3 is a good indicator of the presence of apoptosis. Therefore, after treatment of A-549 cells with *A. absinthium* leaf extract at 4 mg/mL, the amount of active caspase-3 was measured in cell lysate. After 36 hr of treatment, caspase-3 was demonstrated to be activated in the cells and was found 4.5-folds higher than in untreated cells (Figure 4).

DISCUSSION

Use of medicinal plants as an approach in the prevention and treatment of cancer has been followed for many years and many therapeutic plants with anticancer activity are reported in

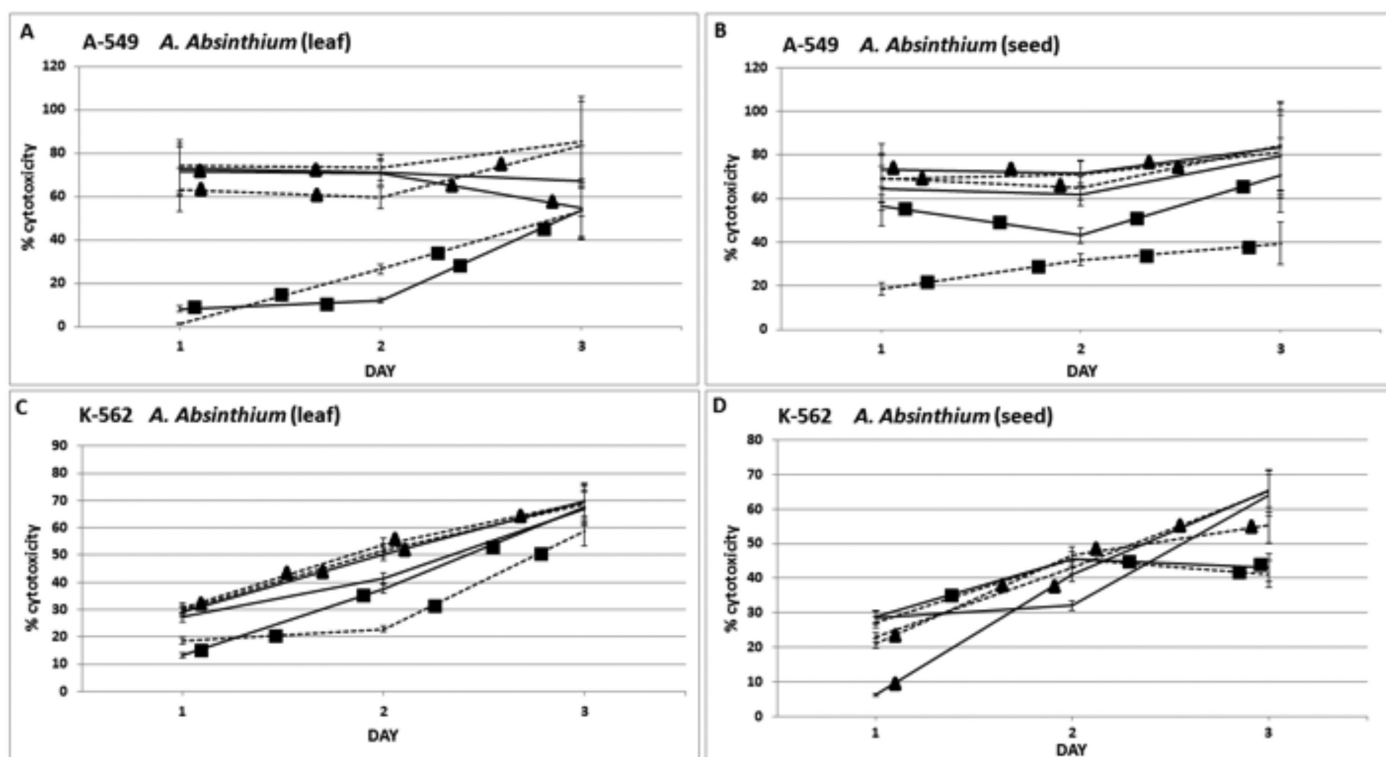


Figure 2. Time-based dose response curves of cancer cells. A-549 cells (a, b) or K-562 cells (c, d) were treated with leaf or seed extracts of *A. absinthium* at various concentrations

Data are expressed as mean ± standard deviation, n=3

---■---: 0.1 mg/mL, —■—: 0.2 mg/mL, ----▲----: 0.5 mg/mL, —▲— : 1 mg/mL, -----: 2 mg/mL, -----: 5 mg/mL

Table 3. Detection of apoptosis with flow cytometric analysis

Treatment (concentration)	Necrosis (%)	Late apoptosis (%)	Live cells (%)	Early apoptosis (%)
Untreated	1.8	9.1	87.5	1.50
<i>A. absinthium</i> leaf (0.2 mg/mL)	1.0	7.0	89.3	2.6
<i>A. absinthium</i> leaf (0.5 mg/mL)	1.1	5.9	87.8	5.2
<i>A. absinthium</i> leaf (2 mg/mL)	52.90	6.2	37.5	3.4
<i>A. absinthium</i> leaf (4 mg/mL)	47.70	23.2	21.0	8.1
Untreated	1.60	12.4	85.4	0.7
<i>A. absinthium</i> seed (0.2 mg/mL)	5.7	12.0	74.5	7.8
<i>A. absinthium</i> seed (1 mg/mL)	31.8	20.6	31.3	16.3
<i>A. absinthium</i> seed (2 mg/mL)	56.1	10.6	29.6	3.8
<i>A. absinthium</i> seed (4 mg/mL)	60.8	30.7	6.2	2.4

A-549 cells were treated with *A. absinthium* leaf or seed extract for 24 hr

the literature.^{20,21} As the interest in organic and simple lifestyles grows, the interest in plant-based medicine also increases.¹³ In addition, adverse effects and drug interactions are major restrictions in synthetic anticancer drugs; therefore, plants have been investigated across the world to exploit novel and potential sources of anticancer agents.

Previous studies on *Artemisia* species have shown medicinal properties such as antibacterial and anticancer effects. Many

phytochemicals exert their cytotoxic effects by acting as cell cycle and apoptosis regulators, as well as anti-inflammatory agents.²² In this present study, ethanol extracts of different plant species were evaluated for their cytotoxic potentials on different cancer cell lines. Emami et al.²³ studied the anticancer activity of five species of *Artemisia* (*A. kulbadica*, *A. sieberi*, *A. turanica*, *A. santolina*, and *A. diffusa*) against Hep2 and HepG2 cell lines. The authors used extracts from aerial parts of each

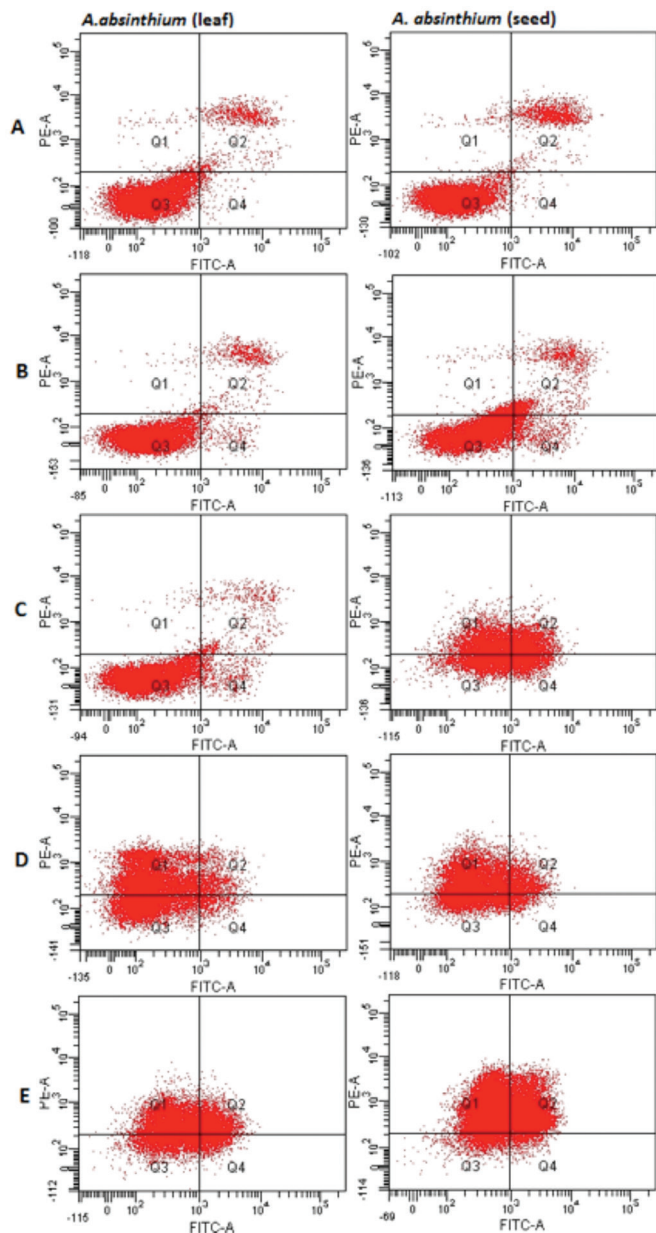


Figure 3. Flow cytometric analysis of apoptosis. A-549 cells were treated with leaf or seed extracts of *A. absinthium* at various concentrations. Results showing cells in necrosis (Q1), late apoptosis (Q2), live cells (Q3) and early apoptosis (Q4), for control (a), 0.25 mg/mL (b), 0.5 (c), 2 mg/mL (d), 4 mg/mL (e)

species at concentrations between 0.2 mg and 3.2 mg/mL. The minimum toxic dose of extracts from five species ranged from 0.4 to 1.6 mg/mL. Therefore, we used *A. absinthium* extracts at 4 mg/mL to screen the cytotoxic effect of the extracts. In order to determine the dose- and time-dependence of cytotoxicity, the extracts were used at five different concentrations starting from 4 mg/mL to 0.1 mg/mL. *In vitro* cytotoxicity of leaf or seed extracts of *A. absinthium* was observed on almost all cell lines examined, except DU-145 cells. The level of cytotoxicity on A-549 cells was time- and dose-dependent, especially for leaf or seed extracts at less than 0.5 mg/mL concentrations (Figure 2a, b). Extracts at more than 0.2 mg/mL concentrations

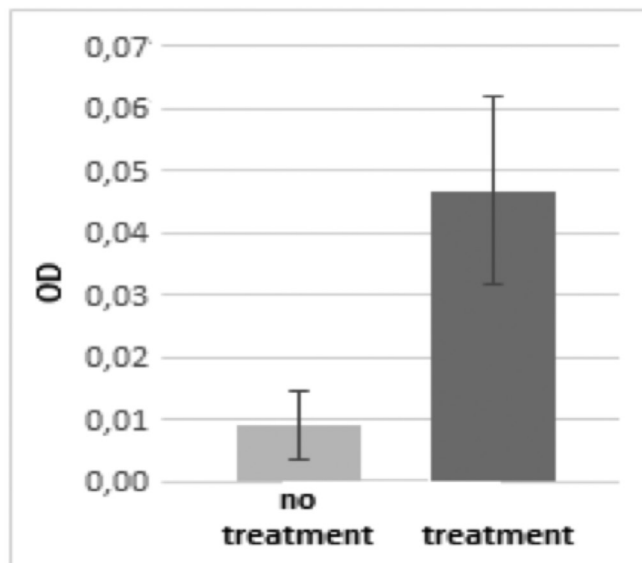


Figure 4. The effects of *A. absinthium* leaf extract on caspase-3 activity in A-549 cells as detected using a colorimetric assay. Treatment of cells with *A. absinthium* leaf extract exhibited 4.5-fold higher OD values than untreated cells. Data expressed as mean \pm standard deviation, n

caused cytotoxicity above 60% even on day one. It stayed at the same level on day two and reached to 82% by day three. These findings indicated that leaf or seed extracts of *A. absinthium* may be effective on cytotoxicity of A-549 cells in a dose- and time-dependent manner at less than 0.2 mg/mL concentrations.

In addition, the cytotoxic activity of methanol extracts from different parts of five *Artemisia* species was evaluated by Gordanian et al.¹². *A. absinthium* was found to have a greater cytotoxic effect on MCF-7 cells with a 50% inhibitory concentration (IC_{50}) value of 221.5 μ g/mL. They showed that a flower extract of *A. absinthium* was more cytotoxic than those of leaf, stem, and root extracts. Similarly, we found that seed extracts at less than 0.5 mg/mL concentrations were more effective with regards cytotoxicity on A-549 cells compared with that of leaf extracts, especially on day 1 and day 2 (Figure 2a, b). In fact, flower and leaf extracts of some *Artemisia* species contain rich sources of artemisinin, a sesquiterpene lactone Mannan et al.²⁴, and artemisinin caused *in vitro* and *in vivo* anticancer activities in a previous study.²⁵

Gordanian et al.²⁶ reported that the cytotoxic activity of methanol extract from different organs of *A. absinthium* at higher altitude was 20-30% higher as compared with those of lower altitudes. The type and level of secondary metabolites may vary in the same plant species based on different agroclimatic and geographic regions.²⁷ Gordanian et al.¹² showed that leaf extract of *A. absinthium* from Iran at 0.1 mg/mL resulted in 30% cytotoxicity on MCF-7 cell lines, whereas we found 20% cytotoxicity on MCF-7 cells at the same concentration (data not shown). However, this could be due to different geographic regions for plant origins and consequently an accumulation of different secondary metabolites, or the use of different solvents for extraction because they used different fractions of

methanol extracts in cytotoxicity assays. In addition, the greater cytotoxicity (>60%) of the same plant extracts on other cancer cell lines exhibits cell type specificity of the extracts.

Ethanol extracts from *A. absinthium* leaf and seed were evaluated for apoptotic potential using flow cytometric analysis and caspase-3 activity on A-549 cells. According to our findings, extracts induced apoptosis, and total cell death increased in a concentration-dependent manner. In other words, treatment of cells with more toxic concentration of extracts caused apoptosis and killed cells undergoing apoptosis faster through necrosis. Investigation of Bad and Bcl-2 protein expression levels in a future study will elucidate the mechanism of apoptosis in all cells lines examined here because the balance between pro-apoptotic and anti-apoptotic proteins is vital for cell survival. In a previous study, a methanolic extract of *A. absinthium* inhibited cell proliferation through apoptosis by modulating Bcl-2 family proteins and the MEK/ERK pathway in MCF-7 and MDA-MB-231 cell lines.¹⁸

P. major has been used as a remedy against stomachache, toothache, and inflammation in the Kars region.¹⁵ In addition, traditional use of *P. major* in the treatment of tumors was reviewed by Samuelsen²⁸ and Haddadian et al.²⁹; however, not much information is available for scientific validation and anticancer activities. In this present study, we investigated the cytotoxic effect of extracts obtained from leaf and seed of *P. major*. Seed extracts exhibited 78% and 28% cytotoxicity on A-549 and DU-145 cells, respectively. Even though the leaf extract did not cause cytotoxicity on A-549 cells, it gave rise to 38% cytotoxicity against DU-145 cells.

Gálvez et al.¹³ examined methanolic extracts of *P. major* against three different cancer cell lines and reported that the extract was more effective on MCF-7 cells with an LC₅₀ value of 207 µg compared with renal adenocarcinoma (TK-10) and human melanoma (UACC-62) cell lines. Unlike the finding of Gálvez et al.¹³, ethanolic extract of leaf and seed did not cause cytotoxicity on the MCF-7 cell line in the present study. However, the seed extract induced proliferation of MCF-7 cells by 38%. The differential cytotoxic effect of seed or leaf extracts on a variety of cancer cell lines may depend on differences in the fatty acid, amino acid, phenolic, and flavonoid composition of leaf or seed extracts. Kobeasy et al.³⁰ reported the differences in levels of these compounds in leaf and seed extracts of *P. major*.

Leaf and seed extract of *A. retrosa* exerted cytotoxic activity on DU-145 and K-562 cell lines. However, both extracts resulted in cell proliferation in other cell lines. Rajasekaran et al.³¹ reported antitumor effects of methanolic extracts from *Amaranthus spinosus* on Hep2, HepG2, HT-29, and Hu7 cell lines. In addition, anticancer activity of *H. niger* extract has not yet been reported in the literature. However, some studies examined the antiinflammatory and analgesic activity of the plant. It has been shown that methanol extracts of *H. niger* inhibit the activity of monoamine oxidase and lower the cellular levels of OH produced by mitochondria³², which suggests that the extract prevents oxygen damage by controlling reactive oxygen species activity and therefore prevents apoptosis. In our study, the cytotoxic effects of leaf and seed extracts on cancer cell

lines were variable. They caused a cytotoxic reaction in some cell lines but induced cell proliferation in others. The range of cytotoxicity of *H. niger* extracts changed between 7% and 30% on various cancer cell lines, suggesting that this plant does not contain anticancer compounds.

CONCLUSION

Ethanolic leaf and seed extracts of *A. absinthium* showed significant anticancer activity against five different cancer cell lines. Antitumor activities of leaf extract on A-549 and K-562 cells, and seed extracts on K562, MCF-7, and PC-3 was found to be selective compared with the normal Beas-2B cell line. In addition, seed extracts of *P. major* caused significant and selective anticancer activity against A-549 and DU-145 cell lines but not on the Beas-2B cells. Other plant extracts induced anticancer activity with various levels. Moreover, leaf or seed extract of the plant exhibited different effects on cells, suggesting that different parts of the plant may have different types and concentrations of secondary metabolites depending on the function of the plant part. In conclusion, use of crude ethanolic extracts enabled us to detect the anticancer potential of phytochemicals of medicinal plants from the Kars region of Turkey. Future studies will identify the level of chemical components of *A. absinthium* and *P. major* extracts responsible for anticancer activity and elucidate more detailed molecular mechanisms of cell death.

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REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin.* 2012;62:10-29.
2. Huang T, Gong WH, Li XC, Zou CP, Jiang GJ, Li XH, Feng DP. Induction of apoptosis by a combination of paclitaxel and carboplatin in the presence of hyperthermia. *Asian Pac J Cancer Prev.* 2012;13:81-85.
3. Kintzios E. Terrestrial plant-derived anticancer agents and plant species used in anticancer research. *Crit Rev Plant Sci.* 2006;25:79-113.
4. Pandi M, Kumaran RS, Choi YK, Kim HJ, Muthumary J. Isolation and detection of taxol, an anticancer drug produced from *Lasiodiplodia theobromae*, an endophytic fungus of the medicinal plant *Morinda citrifolia*. *Afr J Biotechnol.* 2011;10:1428-1435.
5. Park HJ, Kim MJ, Ha E, Chung JH. Apoptotic effect of hesperidin through caspase3 activation in human colon cancer cells, SNU-C4. *Phytomedicine.* 2008;15:147-151.
6. Mans DR, da Rocha AB, Schwartsmann G. Anti-cancer drug discovery and development in Brazil: targeted plant collection as a rational strategy to acquire candidate anti-cancer compounds. *Oncologist.* 2000;5:185-198.
7. Fletcher JC. Coordination of cell proliferation and cell fate decisions in the angiosperm shoot apical meristem. *Bioessays.* 2002;24:27-37.

8. Lucas DM, Still PC, Pérez LB, Grever MR, Kinghorn AD. Potential of plant-derived natural products in the treatment of leukemia and lymphoma. *Curr Drug Targets*. 2010;11:812-822.
9. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*. 1972;26:239-257.
10. Frank SA. Dynamics of cancer, p.90, Princeton University Press, New Jersey, 2007.
11. Bora KS, Sharma A. The genus *Artemisia*: a comprehensive review. *Pharm Biol*. 2011;49:101-109.
12. Gordanian B, Behbahani M, Carapetian J, Fazilati M. *In vitro* evaluation of cytotoxic activity of flower, leaf, stem and root extracts of five *Artemisia* species. *Res Pharm Sci*. 2014;9:91-96.
13. Gálvez M, Martín-Cordero C, López-Lázaro M, Cortés F, Ayuso MJ. Cytotoxic effect of *Plantago* spp. on cancer cell lines. *J Ethnopharmacol*. 2003;88:125-130.
14. Avcı M. Çeşitlilik ve Endemizm açısından Türkiye'nin bitki örtüsü. İstanbul Üniversitesi Geography Department, *Geography Journal*. 2005;13:27-55.
15. Güneş F, Özhatay N. An ethnobotanical study from Kars (Eastern) Turkey. *BioDiCon*. 2011;4:30-41.
16. Kendir G, Güvenç A. Etnobotanik ve Türkiye'de yapılmış etnobotanik çalışmalara genel bir bakış. Hacettepe Üniversitesi Eczacılık Fakültesi Dergisi. 2010;30:49-80.
17. Tuzlacı E, İşbilen DFA, Bulut G. Turkish folk medicinal plants, VIII: Lalapaşa (Edirne). *Marmara Pharm J*. 2010;14:47-52.
18. Shafi G, Hasan TN, Syed NA, Al-Hazzani AA, Alshatwi AA, Jyothi A, Munshi A. *Artemisia absinthium* (AA): a novel potential complementary and alternative medicine for breast cancer. *Mol Biol Rep*. 2012;39:7373-7379.
19. Holst Hansen C, Brünner N. MTT Cell Proliferation assay, *Cell Biology: A Laboratory Handbook*. pp. 16-18, San Diego Academic Press, 1998.
20. Kumar S, Jawaid T, Dubey SD. Therapeutic Plants of Ayurveda. A Review on Anticancer. *Pharmacognosy J*. 2011;3:1-11.
21. Khazir J, Mir BA, Pilcher L, Riley DL. Role of plants in anticancer drug discovery. *Phytochemistry Letters*. 2014;7:173-181.
22. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100:57-70.
23. Emami SA, Vahdati-Mashhadian N, Vosough R, Oghazian MB. The Anticancer Activity of Five Species of *Artemisia* on Hep2 and HepG2 Cell Lines. *Pharmacologyonline*. 2009;3:327-339.
24. Mannan A, Ahmed I, Arshad W, Asim MF, Qureshi RA, Hussain I, Mirza B. Survey of artemisinin production by diverse *Artemisia* species in northern Pakistan. *Malaria J*. 2010;9:310.
25. Ferreira JF, Luthria DL, Sasaki T, Heyerick A. Flavonoids from *Artemisia annua* L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. *Molecules*. 2010;15:3135-3170.
26. Gordanian BB, Behbahani M, Carapetian J, Fazilati M. Cytotoxic effect of *Artemisia absinthium* L. grown at two different altitudes on human breast cancer cell line MCF7. *Res Med*. 2012;36:124-131.
27. Mammadov R. Tohumlu bitkilerde sekonder metabolitler. pp.412, Nobel Lt., Ankara, 2014.
28. Samuelsen AB. The traditional uses, chemical constituents and biological activities of *Plantago major* L. A review. *J Ethnopharmacol*. 2000;71:1-21.
29. Haddadian K, Haddadian K, Zahmatkash M. A review of *Plantago* plant. *Indian J Traditional Knowledge*. 2014;13:681-685.
30. Kobeasy MI, Abdel-Fatah OM, Abd El-Salam SM, Mohamed Z.E.O.M. Biochemical studies on *Plantago major* L. and *Cyamopsis tetragonoloba* L. *International J Biodiversity and Conservation*. 2011;3:83-91.
31. Rajasekaran S, Dinesh MG, Kansrajh C, Baig FHA. *Amaranthus spinosus* leaf extracts and its anti-inflammatory effects on cancer. *Indian J Res in Pharm and Biotechnol*. 2014;2:1058-1064.
32. Begum S, Saxena B, Goyal M, Ranjan R, Joshi VB, Rao ChV, Krishnamurthy S, Sahai M. Study of anti-inflammatory, analgesic and antipyretic activities of seeds of *Hyoscyamus niger* and isolation of a new coumarinolignan. *Fitoterapia*. 2010;81:178-184.