International Journal of Phytomedicine 4 (2012) 582-588
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

In vitro inhibition of the growth of glioblastoma by *Teucrium polium* crude extract and fractions

Zohreh Mahdinia[1], Rana Eftekharvaghefi[2], Mitra Mehrabani[3], Fatemeh Nabipour[4], Vahid Hemayatkhah-jahromi[5], Seyed Noureddin Nematollahi-mahani[1, 2, *]

***Corresponding author:**

Seyed Noureddin Nematollahi-mahani

[1] Neuroscience Research Centre, Kerman University of Medical Sciences, Kerman, Iran.
[2] Department of Anatomy, Afzalipour School of Medicine, Kerman University of Medical Sciences, Kerman, Iran.
[3]Department of Pharmagnosy, School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran.
[4]Department of Pathology, Afzalipour School of Medicine, Kerman University of Medical Sciences, Kerman, Iran. Department of
[5]Developmental Biology, Azad University-Jahrom branch, Jahrom, Iran.

Abstract

The cytotoxic effects of *Teucrium polium* (Lamaceae) have been shown in some studies. However, the antitumor activity of the fractions of this herb has not been studied, neither has the pattern of cell death been evaluated yet. The inhibitory effect of *Teucrium polium (T. polium)* on U87 cells was evaluated by treatment of U87 cells with various concentrations of crude extract (CE), a petroleum ether (PE) fraction and a diethyl ether (DE) fraction of *T. polium* for 24 and 48 h. Trypan blue and differential staining were also used to assess the cell viability and the pattern of cell death. CE, PE and DE inhibited cell growth in a dose-dependent manner, and this inhibition was accomplished by necrotic cell death, as assessed by differential staining. The highest inhibitory effect (IC_{50}=64.47) was demonstrated by the PE fraction of *T. polium* followed by the DE fraction and the CE. Cell death was also higher when U87 cells were treated with PE ($P<0.001$ compared with other treated groups). Necrotic cells were predominant in all treated groups including CE, PE and DE. The results suggest that the PE fraction of *T. polium* is the most potent against U87 cells. The antitumor effect of the PE fraction of *T. polium* is probably executed through the necrotic cell death mechanism.

Keywords: *T*eucriu*m polium*, Glioblastoma multiform, Cytotoxicity, Differential staining

Introduction

It is widely held that the solutions to natural problems are hidden in nature [1, 2], and many of the anticancer drugs (over 60%) applied in cancer chemotherapy have been derived from natural sources [3].

Teucrium polium (Lamiaceae) is a wild-growing flowering plant, found abundantly in many regions of the world including the Middle East and Iran. *T. polium* is well known for its antipyretic, antispasmodic, hypolipidemic [4], anti-inflammatory, antihypertensive, analgesic [5,6] antibacterial [7,8] and antidiabetic effects [9]. One of the most interesting features of *T. polium* is its antitumor properties as reported in several studies [10,11];. Recent investigations have shown that a crude extract of *T. polium* prevents the proliferation of cancer cell lines such as PC12, MCF7 and A549 [10]. These findings have been confirmed by other studies [11,12]. The combination of *T. polium* with some known anticancer drugs including vincristin, vinblastin and paclitaxel has also strengthened the cytotoxic effects of these anticancer drugs [13].

Glioblastoma multiforme (GBM) is the most common and most aggressive type of primary brain tumour in humans [14]. Tumours that are caused by this type of cancer cell are highly aggressive and invade the surrounding normal brain tissue far from the tumour area in the form of microsatellites. This property of GBM makes their therapy problematic, and current treatments are relatively ineffective in the prevention and cure of this type of brain tumour. In the present study we used the U87 malignant glioma multiforme cell line as a model to investigate the antitumor properties of *T. polium* crude extract (CE) along with petroleum ether (PE) and diethyl ether (DE) fractions of this herb. In addition, we investigated the mode of action of *T. polium* crude extract and its fractions on U87 cells by differential staining (for a graphical abstract refer to Figure 1). We also studied the effects of *T. polium* extract and fractions on a primary noncancerous mesenchymal cell obtained from human umbilical cord matrix (Wharton jelly; hUCM). These cells have shown high proliferative capacity in vitro and have properties similar to embryonic stem cells [15, 16].

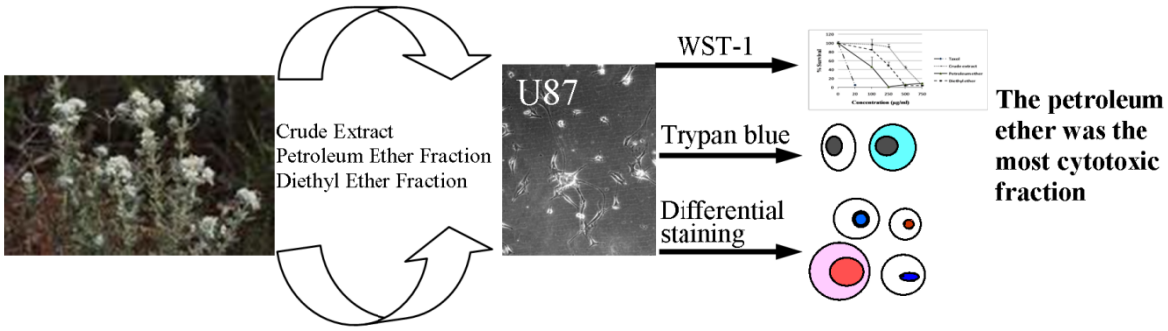

Figure 1. Graphical abstract shows the protocols used in the experiments.

Materials and methods

Plant material

The areal parts of *T. polium* were collected from Khabr (Kerman, Iran) in November 2009 and identified at the Department of Pharmacognosy, School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran. A specimen was deposited at the herbarium of the Kerman School of Pharmacy (Voucher specimen No.28125).

Extract preparation

Powdered aerial parts of *T. polium* were extracted with ethanol (80%) using the maceration method for 24 h. The ethanol extract was evaporated to near dryness by a rotary evaporator at 50 C, and the crude extract (CE) was fractionated by solvent-solvent extraction using petroleum ether and diethyl ether. These fractions were concentrated by a rotary evaporator at 50 C and lyophilized in a freeze dryer. The extracts were dissolved in a small amount of dimethyl sulphoxide (DMSO), so that the final concentration of DMSO was less than 0.05% in the culture medium.

U87 cell culture

U87 human malignant glioma cells were purchased from the Pasteur Institute (Tehran, Iran), and were maintained in a culture medium containing RPMI 1640 (Sigma Aldrich, Mo, USA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS (fetal bovine serum; Gibco, Australia) in a humidified 5% CO_2 atmosphere at 37 C. Cells in the exponential logarithmic growth phase were either used for experiments or were cryopreserved for further use.

hUCM cell culture

hUCM cells were harvested from a frozen sample in the laboratory. These cells were cultivated according to a previously reported protocol [17] with a few modifications. After thawing, hUCM cells were maintained in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were used for experiments at passages two to seven.

Cytotoxicity assay

We trypsinized U87 and hUCM cells at the growth logarithmic phase and evaluated their proliferation and viability rate after treatment of these cells with various concentrations of CE, PE and DE 24 h later for U87, and 48 h later for U87 and hUCM cells, according to a previously reported method [10]. We added 1 10^4 viable U87 and hUCM cells into each well in 96-well culture plates. The cells were incubated in a humid incubator with 5% CO_2 at 37 C for 24 h. The treatment groups received various concentrations (0, 100, 250, 500 and 750 µg/ml) of different extracts; a negative control group received 0.05% DMSO in PBS and a positive control group received 20 µg/ml paclitaxel (Stragen Pharma, Switzerland; TAX). After incubation, 10 µl WST-1 (Roche, Germany) reagent was added to each well and incubated for 1.5 h at 37 C in a humidified atmosphere. The optical density (OD) was measured using an ELISA reader (BIOTEK, USA) at 450 nm wavelength with a reference value of 630 nm. Experiments were replicated at least three times in quadruplet. The survival rate was calculated using the following formula: [OD in treatment group / OD in control group] 100. The inhibitory concentration 50% (IC50) was calculated using the Prism dose–response curve (Prism Graphpad, Prism version 5.0 for Windows, GraphPad Software, San Diego, CA, USA), obtained by plotting the percentage of inhibition versus the concentration.

Viability assay by trypan blue

We trypsinized U87 cells at the growth logarithmic phase and evaluated their viability using the trypan blue dye exclusion test. A total of 3 10^5 viable cells were cultured in 3 cm culture plates containing 3 ml RPMI supplemented with 10% FBS and antibiotics. Cells were incubated overnight at 37 C in a humid atmosphere with 5% CO_2. CE, PE and DE were added to the plates at a concentration of 500 µg/ml. DMSO at a concentration of less than 0.05% and TAX at a concentration of 20 µg/ml were used as negative and positive controls, respectively. After 48 h of

treatment, all cells (adherent and non-adherent) were trypsinized, centrifuged at 2500 rpm for 3.5 minutes, mixed with an equal volume of 0.04% trypan-blue, and then were counted using an improved Neubauer haemocytometer under a light microscope (Nikon, TS100, Japan). Dead cells absorb dye and appear blue while viable cells exclude dye. The proportion of viable and dead cells was determined in duplicate. Experiments were replicated three times and the mean was used for analysis.

Differential staining

To evaluate the different types of cell damage in cultured cells we used a previously reported protocol [18], with a few modifications. Briefly, 3 10^5 viable U87 cells were cultured in 3 cm culture plates and incubated overnight at 37 C in a humidified incubator with 5% CO_2 in air. CE, PE and DE were used to examine the type of damage in U87 cells, and DMSO at a concentration of <0.05%, and TAX at a concentration of 20 µg/ml were used as negative and positive controls, respectively. After 48 h of treatment, the cells were stained with propidium iodide (20 mg/ml) for 30 minutes, washed three times with PBS, and stained with bizbensamide (Hoescht 33258, 1 µM). Following differential staining, the cells were examined under a fluorescent microscope with a UV filter (IX71, Olympus, Japan). Three types of cell damage were recorded in 200 cells: pre-apoptotic (cells with condensed irregular blue nuclei and normal cytoplasm), apoptotic (cells with condensed red nuclei and a small amount of cytoplasm), and necrotic cells (cells with less condensed red large nuclei and swollen cytoplasm), along with normal cells, according to the staining properties and cell morphology.

Statistical analysis

Cytotoxicity data are presented as mean±SD. Cell viability in the trypan blue assay was analysed by χ^2 test. A difference of <0.05 was considered significant.

Results

Cytotoxicity assay by Wst-1

We evaluated the antitumor activity of various concentrations of CE, PE and DE as well as TAX and a negative control containing 0.05% DMSO after 24 and 48 h. Our results showed that by increasing the concentration of all components from zero to 750 µg/ml, the survival rate decreased dramatically. Twenty-four hours after treatment, the survival rate was comparable between the PE and DE fractions, and the T. *polium* extract at all doses studied (Figure 2, A). After 48 h, the most active component against U87 cells was the petroleum ether fraction (IC_{50}= 64.47µg/ml) followed by the diethyl ether fraction (IC_{50}= 134.45 µg/ml) and the crude extract (IC_{50}= 176.10 µg/ml) of *T. polium* (Figure 2, B). These components were also cytotoxic to hUCM cells (Figure 2, C) with PE being the most toxic and CE the least. However, TAX was more toxic to hUCM cells than U87 cells (50% survival in U87 cells and 4% survival in hUCM cells).

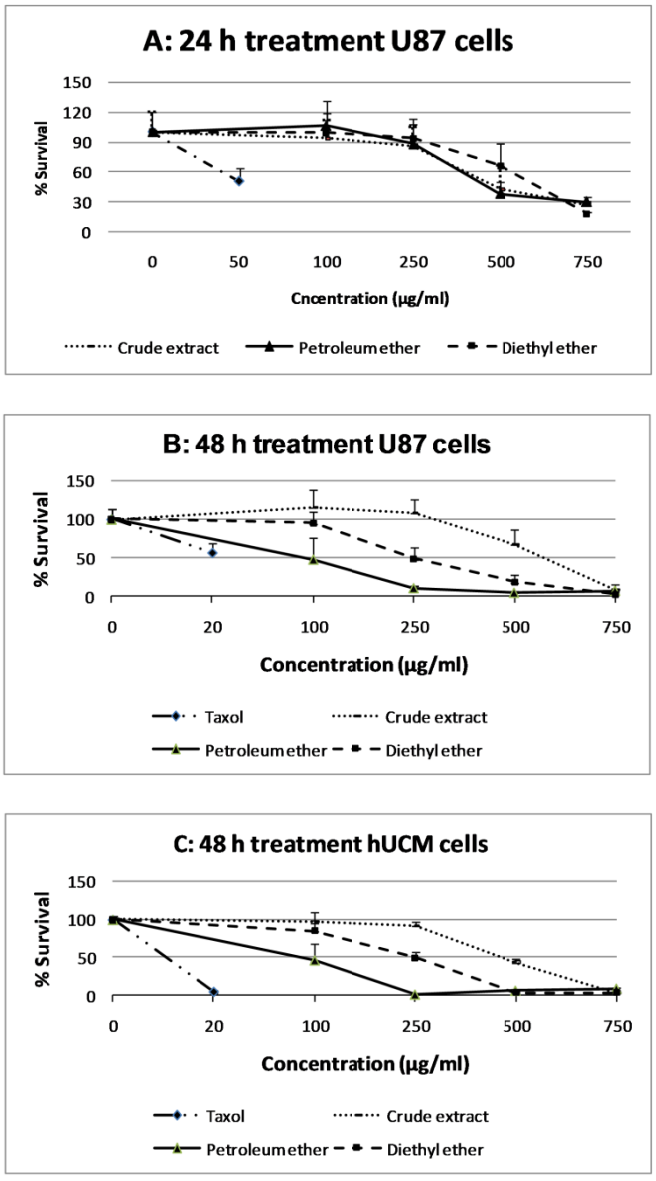

Figure 2. U87 and hUCM cells were cultured with different concentrations of crude extract, petroleum ether fraction and diethyl ether fraction of *T. polium* as well as taxol as a positive control. The petroleum ether fraction was the most cytotoxic component against U87 and hUCM cells. *A,* effect of 24 h treatment of U87 cells with different fractions of *T. polium;* B, effect of 48 h treatment of U87 cells with different fractions of *T. polium;* C, effect of 48 h treatment of hUCM cells with different fractions of *T. polium*

Viability determination

The treatment of U87 cells with PE significantly increased the number of dead cells compared with the negative control group ($p<0.001$). There was no significant difference between the PE and DE fractions but a significant difference was noted between DE and the negative control group ($p<0.01$). A significant difference was also noted between CE and the control group ($P<0.05$).

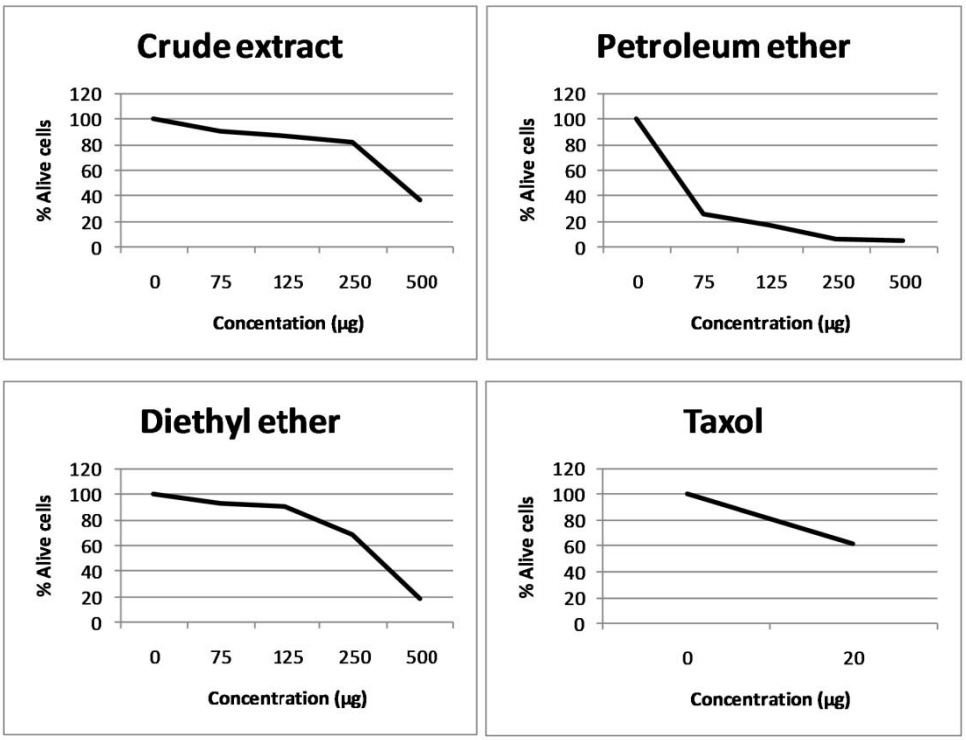

Figure 3. U87 cells were cultured for 48 h with different concentrations of the fractions of *T. polium*, and taxol as a positive control. The cell death rate in different treatments was assessed by the trypan blue exclusion dye test. The petroleum ether fraction was the most cytotoxic component.

The number of dead cells was significantly ($p<0.05$) higher in the PE and DE fractions than in the CE of T. polium (Figure 3).

Differential staining of U87 cells

We stained U87 cells with PI and Hoechst 33258 after treatment of cells with PE, DE, CE and TAX for 48 h. A group of cells received 0.05% DMSO as a control. The cell death pattern tended to follow a necrotic cell death pattern in CE as well as in PE and DE. This pattern was similar to that in TAX-treated cells. However, more pre-apoptotic cells were observed in the TAX group than in the other groups (Figure 4).

the cell membrane. Our results obtained using the trypan blue test also confirmed the results of differential staining. Both of these techniques, which evaluate cell death phenomena in treated cells, showed a necrotic pattern of cell death in treated cells, especially in the PE fraction.

Overall, our data indicate that the highest ctotoxicity was achieved by the petroleum ether fraction of T. *polium* on the glioblastoma cell line, which will now need further evaluation in an animal model of cancer. In addition, fractionation of the PE fraction into its components will elucidate the nature of the cytotoxic effects of this herb.

Discussion

The present study was carried out to evaluate the effects of an ethanol crude extract of T. polium and its fractions on the proliferation of a malignant tumour of brain origin, and a normal mesenchymal cell population with a high proliferative capacity. Our results demonstrated that all the components used in this study had antitumor activity against U87 cells. The petroleum ether fraction was the most effective, followed by the diethyl ether fraction and the crude extract. In another study we evaluated various extracts and fractions of T. polium including an aqueous extract, methanol extract, ethanol extract, petroleum ether fraction, ethyl acetate fraction, diethyl ether fraction, dichloro methane fraction and a chloroform fraction on some established cell lines including Wehi 1640 and A549 (unpublished data). CE, PE and DE were determined as the most potent fractions against some tumor cells. Therefore, in the present study we selected CE, PE and DE for our experiments. PE dissolves nonpolar components such as diterpenoids, resins, esterols and tannins. PE is the most efficient solvent for extraction of different diterpenoids [19]. The antitumor effects of T. polium diterpenoids against P388 lymphocytic leukaemia in mice [20] has been shown previously. Two new neo-clerodane diterpenoids have been isolated from a methanol extract of T. polium [21]. In addition to the diterpenoids present in T. polium, antioxidants are also present at high concentrations in T.

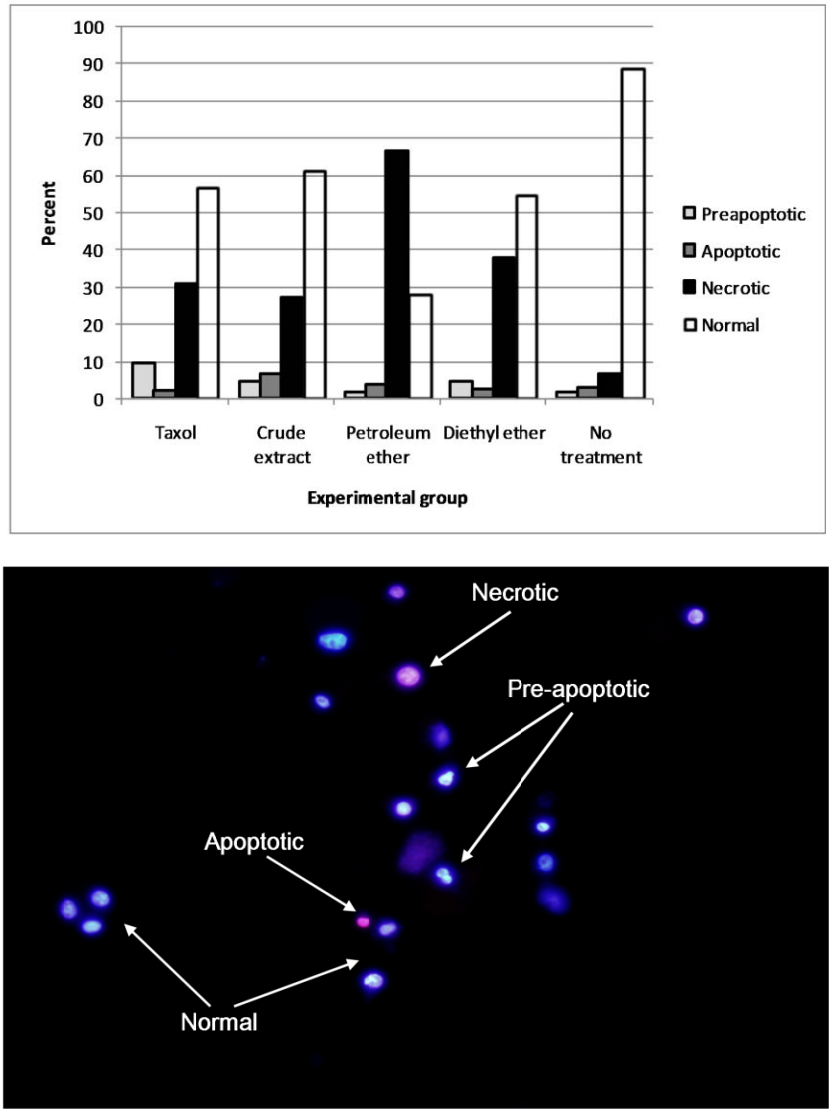

Figure 4. U87 cells were treated with the different fractions of *T. polium* for 48 h, after which the cells were stained with propidium iodide and Hoechst fluorescent dyes, visualized under a fluorescent microscope and evaluated for the type of injury. The upper figure shows the different types of cell death after different treatments; the lower figure is an example of the different types of cell death after differential staining.

polium [22, 23]. The combination of diterpenoids and antioxidants from T. polium may have potentiated the cytotoxic effects of this herb against U87 cells in the PE group. The viability assay using trypan blue also confirmed the findings obtained in the Wst-1 assay. Higher proportions of cells were affected by treatment of U87 cells with the PE fraction of T. polium followed by DE and CE. Trypan blue crosses damaged cell membrane and stains the cell cytoplasm and nucleus. Damage to the cell membrane is one of the last events occurring in the cell death cascade. Differential staining of treated cells by PI and Hoechst, as a valuable method of cell death detection, showed an obvious increase in the proportion of necrotic cells in all groups. However the rate of cell death was much higher in cells treated with PE than in the other groups. TAX-treated cells also had a higher proportion of necrotic cells followed by pre-apoptotic cells. Paclitaxel has been reported to exert its cytotoxic effects in a dose-dependent manner. When a non-small cell lung carcinoma cell line, A549, was treated with various concentrations of TAX, ranging from 1–250 nM, cell death was reported to follow the apoptotic pattern of cell death at lower concentrations, and the necrotic pattern of cell death at higher concentrations [24]. We used a constant concentration of extracts to detect the cell death type, but whether T. polium, especially its PE fraction, changes the pattern of cell death at different concentrations requires further investigation. Recent studies

indicate that the serine/threonine kinase RIP1 initiates necrosis. Subsequently, calcium and reactive oxygen species (ROS) play pivotal roles during the progression and execution phases of necrosis. Any damage to proteins, lipids and the DNA content of cells results in the disruption of intracellular organelles including

Author's Contribution

Nematollahi-mahani have made design of this study, analysis and interpretation and statistical of data, and reviewed the manuscript. Mahdina and Eftekharvaghefi has made acquisition of data and drafted the manuscript, Mehrabani has designed the study and interpreted the data, Hemayatkhah and Nabipour have analyzed and interpreted the data. All authors have read and approved the final manuscript.

Acknowledgments

This study was supported by the grant No. E88/19 from Neuroscience Research Center, Kerman, Iran.

References

[1]. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, and Thun MJ, Cancer statistics, 2005. CA Cancer J Clin. 2005; 55(1): 10-30.

[2]. Cho WC, Contribution of oncoproteomics to cancer biomarker discovery. Mol Cancer. 2007; 6: 25.

[3]. Ghose AK, Herbertz T, Salvino JM, and Mallamo JP, Knowledge-based chemoinformatic approaches to drug discovery. Drug Discov Today. 2006; 11(23-24): 1107-14.

[4]. Rasekh HR, Khoshnood-Mansourkhani MJ, and Kamalinejad M, Hypolipidemic effects of Teucrium polium in rats. Fitoterapia. 2001; 72(8): 937-9.

[5]. Suleiman MS, Abdul-Ghani AS, Al-Khalil S, and Amin R, Effect of Teucrium polium boiled leaf extract on intestinal motility and blood pressure. J Ethnopharmacol. 1988; 22(1): 111-6.

[6]. Tariq M, Ageel AM, al-Yahya MA, Mossa JS, and al-Said MS, Anti-inflammatory activity of Teucrium polium. Int J Tissue React. 1989; 11(4): 185-8.

[7]. Mansouri S, Inhibition of staphylococcus aureus mediated by extracts of Iranian plants. Pharmaceutical Biology. 1999; 37: 375-377.

[8]. Atapour M, Zahedi MJ, Mehrabani M, Safavi M, Keyvanfard V, Foroughi A, Siavoshi F, and Foroumadi A, In vitro susceptibility of the Gram-negative bacterium Helicobacter pylori to extracts of Iranian medicinal plants. Pharmaceutical Biology. 2009; 47(1): 77-80.

[9]. Esmaeili MA and Yazdanparast R, Hypoglycaemic effect of Teucrium polium: studies with rat pancreatic islets. J Ethnopharmacol. 2004; 95(1): 27-30.

[10]. Nematollahi-mahani SN, Rezazadeh-kermani M, Mehrabani M, and Nakhaee N, Cytotoxic effects of Tecurium polium on some established cell lines. Pharmaceutical biology. 2007; 45(4): 295-298.

[11]. Eskandary H, Rajabalian S, Yazdi T, Eskandari M, Fatehi K, and Ashraf ganjooei N, evaluation of cytotoxic efects of *Teucrium polium* on a new glioblastoma multiforme cell line (REYF-1) Using MTT and soft agar clonogenic assays. Int j pharmacol. 2007; 3(5): 435-437.

[12]. Menichini F, Conforti F, Rigano D, Formisano D, Piozzi F, and Senatore F, Phytochemical composition, anti-inflammatory and antitumour activities of four *Teucrium* essential oils from Greece. Food Chemistry. 2009; 115(2): 679-686

[13]. Rajabalian S, Methanolic extract of Teucrium polium L. potentiates the cytotoxic and apoptotic effects of anticancer drugs of vincristine, vinblastine and doxorubicin against a panel of cancerous cell lines. Exp Oncol. 2008; 30(2): 133-8.

[14]. Mahaley MS, Jr., Mettlin C, Natarajan N, Laws ER, Jr., and Peace BB, National survey of patterns of care for brain-tumor patients. J Neurosurg. 1989; 71(6): 826-36.

[15]. Nematollahi-Mahani SN, Rezazadeh-Kermani M, Latifpour M, and Salehinejad P, Biological and Biochemical Characteristics of Human Umbilical Cord Mesenchymal Cells. j reprod infertil. 2008; 9(1): 7-15.

[16]. Weiss ML, Anderson C, Medicetty S, Seshareddy KB, Weiss RJ, VanderWerff I, Troyer D, and McIntosh KR, Immune properties of human umbilical cord Wharton's jelly-derived cells. Stem Cells. 2008; 26(11): 2865-74.

[17]. Aghaee-afshar M, Rezazadehkermani M, Asadi A, Malekpour-afshar R, Shahesmaeili A, and Nematollahi-mahani SN, Potential of Human Umbilical Cord Matrix and Rabbit Bone Marrow–Derived Mesenchymal Stem Cells in Repair of Surgically Incised Rabbit External Anal Sphincter. Dis colon rectum. 2009; 52(10): 1753-1761.

[18]. Shimizu S, Eguchi Y, Kamiike W, Itoh Y, Hasegawa J, Yamabe K, Otsuki Y, Matsuda H, and Tsujimoto Y, Induction of apoptosis as well as necrosis by hypoxia and predominant prevention of apoptosis by Bcl-2 and Bcl-XL. Cancer Res. 1996; 56(9): 2161-6.

[19]. Charlwood BV and Banthorpe DV, *Methods in plants biochemistry*. Terpenoids, ed. P.M. Dey and J.B. Harborne. Vol. 7 1991: Achademic press. 278-280.

[20]. Nagao Y, Ito N, Kohno T, Kuroda H, and Fujita E, Antitumor activity of Rabdosia and Teucrium diterpenoids against P 388 lymphocytic leukemia in mice. Chem Pharm Bull (Tokyo). 1982; 30(2): 727-9.

[21]. Bedir E, Tasdemir D, Calis I, Zerbe O, and Sticher O, Neo-clerodane diterpenoids from *Teucrium polium*. phytochemistry. 1999; 51: 921-925.

[22]. Esmaeili MA, Zohari F, and Sadeghi H, Antioxidant and protective effects of major flavonoids from Teucrium polium on beta-cell destruction in a model of streptozotocin-induced diabetes. Planta Med. 2009; 75(13): 1418-20.

[23]. Ljubuncic P, Dakwar S, Portnaya I, Cogan U, Azaizeh H, and Bomzon A, Aqueous Extracts of Teucrium polium Possess Remarkable Antioxidant Activity In Vitro. Evid Based Complement Alternat Med. 2006; 3(3): 329-38.

[24]. Torres K and Horwitz SB, Mechanisms of Taxol-induced cell death are concentration dependent. Cancer Res. 1998; 58(16): 3620-6.