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Cytotoxicity Evaluation of Methanol Extracts of Some Medicinal Plants on P19 Embryonal Carcinoma Cells

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ABSTRACT

Strategies for conventional cancer treatment including chemotherapy and radiation therapy decrease the bulk of tumor cells but a population of cancer stem cells (CSCs) remains. CSCs comprise a very small population in many cancers with ability of self-renewal and differentiation. Drug resistance of CSCs lead to their survival after treatment which finally result in cancer recurrence. It has been shown that many phytochemicals and plant extracts are able to target CSCs and may be a true therapeutic strategy for eradicating cancer. In this study, we evaluated the in vitro cytotoxic activity of methanol extracts of four medicinal plants against P19 embryonal carcinoma cell which is considered as a CSC. Trypan Blue exclusion assay and MTT assay were used for evaluation of plant extract cytotoxicity. *Morus alba* showed the highest cytotoxic effect on P19 cells which is followed by *Eucalyptus camaldulensis* and *Zataria multiflora* and the least cytotoxicity was found in *Circhorium intybus*. Although *Morus alba* methanol extract showed the most potent anti-proliferative activity and could be investigated for finding pure anticancer compounds, but IC50 values> 100 of mentioned plant extracts after 48, 96 and 144 h incubation period indicates their weak cytotoxicity against P19 cells.

INTRODUCTION

Cancer is the second reason of death worldwide. Conventional therapies for cancer include surgery, cytotoxic chemotherapy, immunotherapy and radiation therapy which are used as a single or combinatorial therapy have some side effects. Furthermore, a small subpopulation of cancer cells called cancer stem cells (CSCs) are resistant to conventional cancer therapy. Therefore, it is essential to use alternative method for cancer treatments.

In the last decades of the 20th century, the cancer stem cell hypothesis has attracted much attention. According to this concept, CSCs as rare sub-population of tumor cells have been recognized with some similarity to normal stem cells such as capacity of self-renewal and differentiation. These kinds of cancer cells are also resistant to apoptosis and have long lifetime. Some properties of CSCs such as cell cycle quiescence, increased ability to repair damaged DNA, as well as increased expression of anti-apoptotic proteins, detoxifying enzymes and transporters responsible for drug efflux make them main agents for cancer initiation, progression, metastasis, relapse and resistance to conventional cancer therapies such as chemo and radiotherapy (Al-Hajj *et al.*, 2003, Dick, 1997, Li *et al.*, 2007, Schatton *et al.*, 2008, Yang *et al.*, 2008, Dalerba *et al.*, 2007). Thus, antitumor therapies that do not target CSCs may lead to a reduction of the tumor mass, but finally lead to cancer recurrence (LaBarge, 2010, Lacerda *et al.*, 2010). Therefore, targeting CSCs in tumor could improve quality of life of cancer patients, reduce relapse of cancer and increase their lifespan (Mukherjee, 2010, Reya *et al.*, 2001, Gupta *et al.*, 2009).

Today, many plants-derived compounds "phytochemicals" have been identified that have anti-tumor properties, for example, induction of apoptosis and inhibition of cell proliferation which finally decrease the risk of cancer (Amin *et al.*, 2009, Tan *et al.*, 2006). Low or non-toxicity and their availability in an ingestive form make these components useful for cancer treatment. Furthermore, up to now many phytochemicals have been known that can inhibit tumor progression by targeting

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CSCs via different mechanisms such as reversing drug resistance (Kim *et al.*, 2008, Zhang *et al.*, 2011), inducing cell death (Shin *et al.*, 2011, Pandey *et al.*, 2011, Takeuchi *et al.*, 2011), inducing differentiation of CSCs (Massard *et al.*, 2006, Li *et al.*, 2011) and inhibiting cell proliferation and self-renewal ability of CSCs (Qi *et al.*, 2016, Di and Zhao, 2015, Li *et al.*, 2011, Dai *et al.*, 2010, Li *et al.*, 2010, Casagrande *et al.*, 2011).

In this study, we aimed to evaluate cytotoxicity of methanol extract of four medical plant that were gathered from Kerman, Iran, including; *Circhorium intybus, Zataria multiflora* , *Morus alba* and *Eucalyptus camaldulensis* on embryonal carcinoma (EC) P19 cells. EC cells which derived from teratocarcinomas are recognized as pluripotent cells with cancerous and stem cells properties, so they could be considered as the archetype of CSCs (Andrews *et al.*, 2005). Therefore, P19 cell line is evaluated as a good system to assess the effect of different component on cell viability and differentiation induction of EC cells as a model of CSCs (Seeley and Faustman, 1998).

MATERIALS AND METHODS

Plant extracts' preparation

Samples of *C. intybus*, *M. alba*, *E. camaldulensis* and *Z. multiflora* leaves were collected from different parts of Kerman, Iran. Plant material were dried at room temperature and finely ground with a hammer mill to obtain fine particles. Then, 300 g of plant powder was soaked and shaken with 1L of 80% methanol for 48h at room temperature before filtration. The product was then filtered and concentrated in a rotary evaporator (EYELA SB-1100, JAPAN) at 40°C to obtain semisolid extract under reduced pressure. Extract were kept at -20 °C until cytotoxicity tests were carried out.

Cell Line and Culture Medium

P19 cell line obtained from pasture, Tehran, Iran. The cells were cultured at 37°C in 5% CO2 and maintained in minimum essential medium alpha (a-MEM; Sigma-Aldrich) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco). In order to subculture cell line, after washing with Phosphate Buffer Saline (PBS), cells were incubated with 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA) for a few minute. Finally trypsin was inactivated by adding fresh serum-containing medium to detached cells.

In Vitro Assay for Cytotoxic Activity

A stock solution of each plant extracts was prepared by dissolving 30 mg of extract in 95 µl of dimethyl-sulfoxide (DMSO, Merck, Germany) and 2905 µl of cell culture medium to a final stock concentration of 10mg/ml and then diluted with complete culture medium to reach the desired concentrations. For doing the test, at first, detached cells were counted with a neobar lam. Then, cells were seeded (400 cells per well) into 96-well plates, a density that allowed the untreated control to grow exponentially for 144h. Twenty-four hours after seeding, cells were treated with different concentrations of plants extracts including 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 µg/ml at the selected times (48, 96 and 144h). The medium was changed every 48 h. For MTT test, control samples were incubated with equivalent amount of DMSO as a solvent of plant extracts. There were three replicates for each concentration of plants extracts.

MTT assay

The effect of methanol extract of the plants on the viability of cells was determined using MTT assay. For MTT assay, at the end of incubation period (48, 96 and 144h), 20 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (Atocel) (5 mg/ml) was added into each well. After 3 h incubation at 37 °C, media was removed and 100 µL of DMSO was added to each well. Finally, optical densities (ODs) were measured by an enzyme-linked immunosorbent assay (ELISA) reader (BioTek-ELx800, USA) at 490 nm. ODs were used to calculate the viability of cells. Percentage of viable cells was obtained by dividing the mean absorbance of treated cells (for each concentration of extract) to the mean absorbance of its control cells. Finally IC50 values (The concentration of extract that inhibited the growth of cells to the level of 50% of control) were determined by incubating P19 cells with increasing concentrations of each extract (Haghighitalab *et al.*, 2014, Arghiani *et al.*, 2014). MTT assay were carried out in three independent experiments done in triplicate.

Trypan blue exclusion assay

Cell viability was measured using the trypan blue exclusion assay. In this assay, live cells with intact cell membranes are not colored, so have a clear cytoplasm whereas; trypan blue can be absorbed by dead cells, so they have a blue cytoplasm. In each set of experiments, P19 cells were plated at a concentration of 6×10^4 (48h), 3×10^4 (96h) and 10^4 (144h) cells/well in 6-well. 24 h after plating, fresh medium containing half maximal inhibitory concentration (IC50) of extract for each time 48h, 96h and 144h was added. After exposing cells to extract for defined time, the medium was transferred to a falcon tube. Attached cells were trypsinized and pooled with cells in tube. This mixture was then briefly centrifuge and suspended in 2 ml PBS. A cell suspension was mixed with equal parts trypan blue solution, 0.4% (Merck) and placed in a haematocytometer. Numbers of viable and dead cells were counted separately in two different samples for each plate. Finally by dividing the number of viable cells by the total number of cells, percentage of viable cells was calculated.

Statistical Analysis

Experimental results are shown as mean ± SEM. MTT test were replicated three times. Data were analyzed with analysis of variance (ANOVA), Duncan test, using (SPSS) software (version 18.0), Significance level was set at $p < 0.05$. The IC50 values were calculated from linear regression analysis.

RESULTS

IC50 value determination of plants extract in P19 cell line

In this study, the cytotoxic activity of methanol extracts of 4 plants were determined using MTT assay in P19 cell line exposed to 10-2560 µg/ml of extracts at three incubation period of 48, 96 and 144 and IC50 values were determined.

Cell survival analyses indicated that *C. intybus* extract caused growth inhibition of P19 cells in dose and time dependent manners (Fig. 1-A). After 48h incubation, methanol extract of *C. intybus* showed the most cytotoxic activity at highest concentration (2560 µg/ml) with 80% of cell growth inhibition. By increasing the incubation time, cell viabilities decreased significantly at 640 µg/ml after 96 and 144 h of treatments. In conclusion, IC50 values of *C. intybus* extract were calculated as 1723, 370 and 364 µg/ml after 48, 96 and 144 h of treatments, respectively. Methanol extract of *Z. multiflora* exerts the higher cytotoxic effect than *C. intybus* at three incubation period (Fig. 2). The most severe effect was seen after 96 h treatment and IC50 value is 262 µg/ml. Longer treatment for 144 h didn't have more effect with IC50 value calculated 299 µg/ml (Fig. 1-B). Results of the cytotoxicity evaluation of methanol leaf extract of *E. camaldulensis* against P19 cells is graphically represented in Fig. 1-C. While *E. camaldulensis* extract showed significant cytotoxic effect on P19 cells at concentrations equal 417 µg/mL for 48 h and concentrations equal 219 µg/mL for 96 h, as compared to the control groups but similar to *C. intybus* and *Z. multiflora*, extract had not more cytotoxic effect after 144 h treatment with IC50 value of 244 µg/ml which is approximately equal to IC50 for 96 h treatment. Finally, inhibitory effect of *M. alba* leaves extract was tested in a time-response experiment at concentrations of 10-640 µg/ml. It was demonstrated that its extract killed 50% of the cells (IC50) at a lower concentration in three incubation period when compared to other plant extract tested (Fig. 2). The IC50 values of this plant extract were calculated as 273, 117 and 127 µg/ml after 48, 96 and 144h of treatments, respectively (Fig. 1-D).

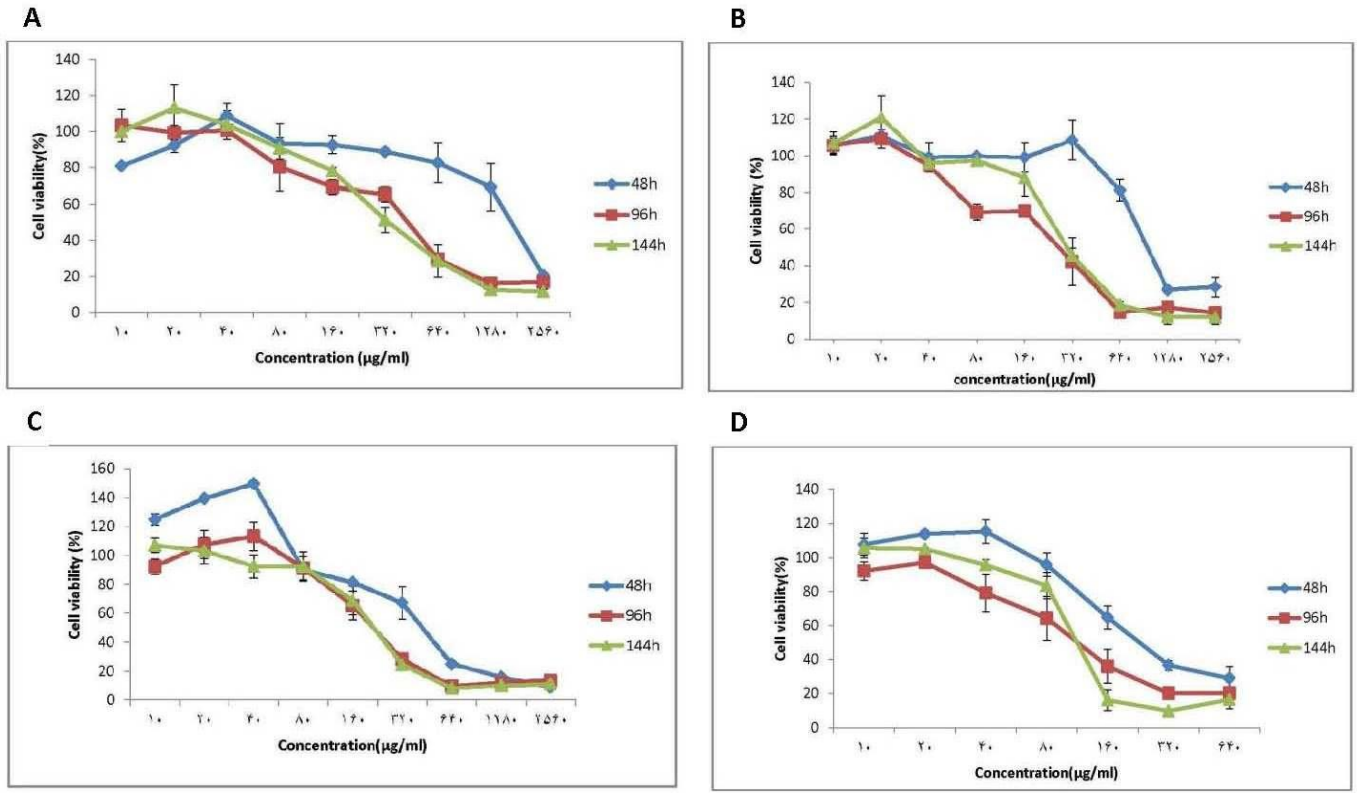

Fig. 1: Dose response curves of P19 cells to C. intybus. (A), Z. multiflora (B), E. camaldulensis (C) and M.alba (D) extract during 48, 96 and 144 h treatment. Data are shown as Mean ± standard error, n=3.

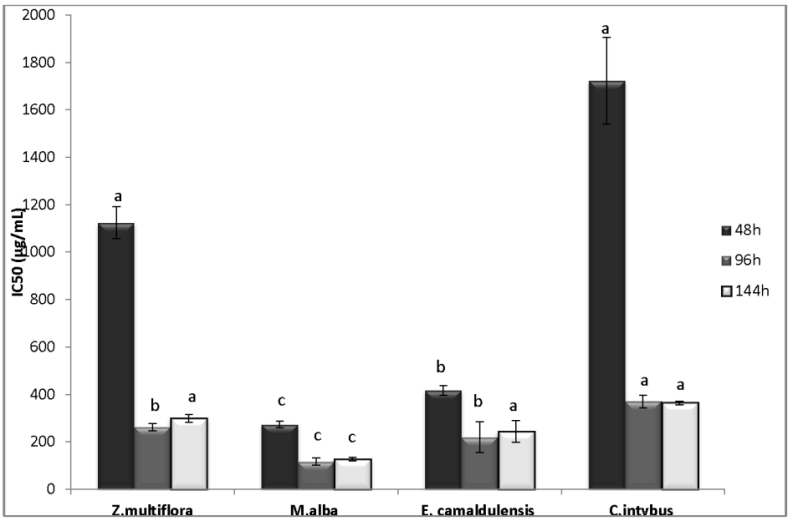

Fig. 2: Half maximal inhibitory concentrations (IC50) of C.intybus, Z. multiflora, E. camaldulensis and M.alba extracts in P19 cells following 48, 96 and 144h of exposure using MTT assays, The results represent mean ± standard error , n = 3, data were analyzed with analysis of variance (ANOVA), Duncan test, using (SPSS) software (version 18.0), Significance level was set at $p < 0.05$.

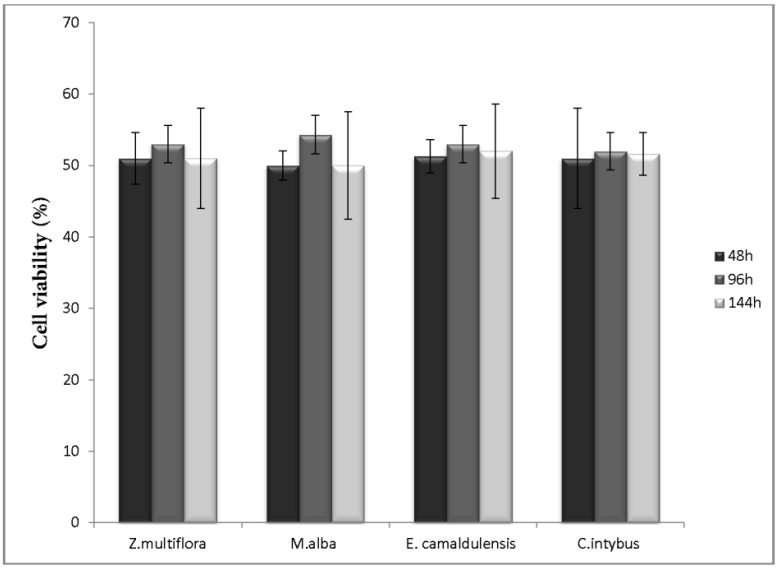

Fig. 3: P19 cells were exposed to IC50 concentration of extract of C.intybus Z. multiflora, E. camaldulensis and M.alba. Trypan blue assay was used to determine the percentage of living cells. Value are shown as mean± standard error; n=3. $P< 0.05$.

Trypan Blue exclusion assay

IC50 values of each extract were determined using the MTT assay and then trypan blue exclusion assay was used to further show effect of plant extract on cell viability reduction of P19 cells. Utilizing the trypan blue exclusion assay, it was determined that each extract induce approximately 50% reduction in cell viability at the IC50 values (Fig. 3).

DISCUSSION

Nowadays, one of the main treatments for cancer is chemotherapy. Most chemotherapeutic agents have some side effects. In recent years, anticancer effect of medicinal plants and components isolated from them is focus of many researches. In this study, our aim was to determine the cytotoxicity of the methanol extract of *C. intybus*, M. alba, *E. camaldulensis* and *Z. multiflora* leaves against P19 cell line using MTT and trypan Blue assay. Overall, this study showed cytotoxicity of examined plant extract against P19 cells, which indicate there could be some cytotoxic compounds in these extracts. Comparison of four plant extract exhibit highest significant cytotoxic activity of *M. alba* with lower IC50% and the least cytotoxicity of *C.intybus* with higher IC50%. All extracts showed maximum cytotoxic effect after 96h treatment and increasing the incubation time (until 144h) didn't decrease cell viability. trypan blue exclusion assay showed that IC50 concentration of each plant induces about 50% cell

death. Therefore reduction of cell viability after treatment with plant extract is due to cell death.

There are some reports on anti-proliferative activity of plant extract that have been investigated in this research. Anticancer activity of *C.intybus* commonly known as chicory has been recognized against some cancer cell lines such as breast cancer MCF-7 (Abu-Dahab and Afifi, 2007), renal adenocarcinoma, prostat cancer LNCaP, amelanoic melanoma C32 (Conforti *et al*., 2008) and lymphoblastic leukemia jurkat cell line (Saleem *et al.*, 2014). The leaf extract of C. intybus were found to possess high values of total flavonoids and total phenolic acids. Some important anticancer compounds isolated and identified from C.intybus include Caffeic acid, Kaempferol, Quercetin, Lactucin, stigmastero and their derivatives (Street *et al.*, 2013, Al-Snafi, 2016) which show ant proliferative activity against various cancer cell line and tumors (Zhou *et al.*, 2010, Tang *et al.*, 2010, Zhang *et al.*, 2016, Choi *et al.*, 2007, Awad *et al.*, 2001, Ali *et al.*, 2015, Zheng *et al.*, 2012, Dajas, 2012, Prasad *et al.*, 2011, Jo *et al.*, 2015, Kim and Choi, 2013). Moreover, some of these components such as Kaempferol and Quercetin possess anti CSCs activity too (Zhou *et al.*, 2010, Tang *et al.*, 2010, Liang *et al.*, 2015). Z. multiflora which is belonging to the Lamiaceae family is known as Avishan-e-Shirazi (Shirazi thyme) in Iran. In addition to some biological properties such as antinociceptive, antimicrobial, spasmolytic, and anti-inflammatory effects (Sajed *et al.*, 2013), some studies showed antioxidant activity (Sharififar *et al.*, 2007, Saei-Dehkordi *et al.*, 2010) and also cytotoxic effects of Z. multiflora on some cancer cells including AGS (human gastric cancer), HepG2 (Human hepatocarcinoma), SKOV3 (Human ovary carcinoma), HepG2 (Human hepatocarcinoma) and SKOV3 (Human ovary carcinoma) cancer cell lines (JANİTERMİ *et al.*, 2015, Shokrzadeh *et al.*, 2010). According to some researches, main constituents in *Z. multiflora* are phenolic compounds such as thymol and carvacrol (Saei-Dehkordi *et al.*, 2010, Ahmad *et al.*, 1999, Malik *et al.*, 1987, Saleem *et al.*, 2004), which are well-known anti-microbial, anti-fungal (Saei-Dehkordi *et al.*, 2010, Can Baser, 2008) and antitumor agents (Yin *et al.*, 2012, Fan *et al.*, 2015). For example, suppression of mouse B16 melanomas (He *et al.*, 1997), human larynx carcinoma Hep-2 cells (Stammati *et al.*, 1999) and human colon cancer cells (Fan *et al.*, 2015) growth by carvacrol is demonstrated.

Another plant extract which is tested in this research is Eucalyptus that has many health benefits and antioxidant properties. Evaluation of cytotoxic effects of *E. camaldulensis* leaves extract showed that this extract trigger apoptosis and cell cycle changes in carcinoma cells, so can be recommended as inhibitors of the growth of tumor cells (Meshkani and Ranjbar, 2014, Islam *et al.*, 2015). Moreover, MTT assay showed in vitro cytotoxicity of *E. camaldulensis* extract against human breast and colon cancer cell lines (Hrubik and Jovin, 2012, Singab *et al.*, 2011). Some compounds including ellagitannins, flavonoids, phloroglucinol derivatives and galloyl esters which were detected in leaf extract of E. camaldulensis have anticancer activity (Singab *et al.*, 2011, Cho *et al.*, 2015, Ismail *et al.*, 2016, Gazák *et al.*, 2011).

Moreover, the essential oil of the leaves was found to contain p-cymene, γ-terpinene, α-pinene, 1,8-cineole, terpinen-4-ol, α-terpineol, carvacrol and thymol as the major components (Pagula *et al.*, 2000), which some of them such as carvacrol, α-terpineol and thymol show anti-proliferative and pro-apoptotic effect on cancer cells (Yin *et al.*, 2012, Fan *et al.*, 2015, Hassan *et al.*, 2010, Kang *et al.*, 2016). M. alba, known as white mulberry, has been used as antihyperglycemic, antiinflammatory, diuretic, antiheadache, antipyretic, and anticancer treatment in the past (Naowaratwattana *et al.*, 2010). Anticancer properties of M. alba leaf polyphenols have been demonstrated in various human cancer cells of colon (Deepa *et al.*, 2013), liver (Naowaratwattana *et al.*, 2010), breast (Deepa *et al.*, 2013), and lung (Chen *et al.*, 2006). For example, it has been demonstrated that mulberry root bark and leaves extract arrest cell growth and induce apoptosis in human colorectal cancer cells, SW480 and hepatocellular carcinoma HepG2 cell line (Fathy *et al.*, 2013). Furthermore, ethanoic extract of mulberry leaf can induce differentiation of neuroblastoma stem cell-like population as a CSC, which is approved by down regulation of stem cell markers and up regulation of differentiation markers (Park *et al.*, 2012). *M. alba* leaves contain abundant varieties of polyphenols, including chlorogenic acid, rutin, isoquercitrin, quercetin, astragalin and kaempferol (Doi *et al.*, 2001), which are considered as strong anticancer agents (Zhou *et al.*, 2010, Tang *et al.*, 2010, Zheng *et al.*, 2012, Jo *et al.*, 2015, Kim and Choi, 2013, Naso *et al.*, 2014, Kang *et al.*, 2013, Amado *et al.*, 2014). Moreover, Peter Kollar et al., showed differentiation induction of THP-1 human monocytic leukemia cells by prenyl flavonoid 4´-o-methylkuwanon E (4ME) isolated from mulberry (Kollar *et al.*, 2013).

CONCLUSION

Comparing impacts of four plant extracts tested in this research show efficacy of *M.alba* extract for the cytotoxicity towards P19 cells with lower IC50 values compared to others (Fig. 2), so it can be used for isolation of pure component with antitumor potential for cancer treatment. However according to US NCI plant screening program, crude extracts and pure compounds can be considered as cytotoxic agents against carcinoma cells if after 48-72 h incubation, they show IC50 value less than 20 and 4 µg/ml respectively (Boik, 2001). So extract tested in this study didn't show significant cytotoxicity against P19 cell line, because MTT results showed that IC50 values of all extracts are more than 100 µg/ml.

However, there may be some substances in these extracts that can show effective cytotoxicity against P19 cells if they are used as pure components. So it is useful to isolate and identify these components and their anti-CSC effect. Furthermore, to confirm inhibitory effect of each plant-derived anticancer agent on CSCs, it is more accurate to define percentage of CSCs in cancer

cell line before and after treatment via evaluation of specific CSC markers and measurement of multiple drug resistance transporter activity mediated by expression and activity of ABC-transporters. If a plant-derived agent can reduce percentage of CSCs significantly, it would be effective for cancer treatment. Our future research is dedicated to find phytochemical components with anti-CSC properties.

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Authors' Contribution

Sara Soltanian: Conception and design of study, analysis and/or interpretation of data, some part of experimental work and acquisition of data, writing of manuscript, Administrative, technical, and material support.

Mahboubeh Sheikhbahaei: Many parts of experimental work and acquisition of data.

Neda Mohamadi: Cooperation in plant extraction process, statistical analysis of data and submission of manuscript.

Role of the Sponsor

The funding organization is public institution and had no role in the design and conduct of the study; collection, management, and analysis of the data; or preparation, review, and approval of the manuscript.

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