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P.oleracea induced apoptosis and inhibited the cell growth in oral epithelial cancer cell line

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ABSTRACT

Despite the progress in cancer therapies such as chemotherapy and radiation, its eradication remains as unattainable dream for the patients and doctors. Recently, using supplementary agents such as herbal medicine with fewer side effects seems efficient and attractive. Therefore, the purpose of the present study is to investigate if the ethanolic extract of P.oleracea has cytotoxicity and apoptosis induction on oral epithelial cancer cell line (KB cell line). The KB cells were cultured with different doses of ethanol extract (0, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500 µg/ml) for 24, 48 and 72 hours and the cytotoxicity and cell survival were measured through MTT and trypan blue respectively. In addition, the cells stained with Hoechst reagent to study apoptosis induction after their treatment with 4000, 4500 and 5000 µg/ml of P.oleracea ethanolic extract at 24, 48 and 72 hours. Our findings showed that P.oleracea had time and concentration dependent anticancer effects on KB oral cancer cell line ($P < 0.05$), so that there was a significant difference between all experimental and the control group. In addition, 1500 µg/ml P.oleracea extract was considered as IC50 level according to MTT assay. Also, condensation, shrinkage and breakdown of the cells nuclei confirmed the apoptosis induction at 4000, 45000 and 5000 µg/ml concentration. It seems that ethanolic extract of P.oleracea leads to inhibit cancer cells growth and induce cell death through apoptosis at all concentrations. However, several preclinical or clinical studies should be designed to prove its safety, effectiveness and mechanisms.

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Keywords
P.oleracea,
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INTRODUCTION

Recently the number of people die from cancer is increasing and this considers as a threat for health system. Therefore, focus on prevention, early diagnosis and treatment strategies in this field seem necessary.

Oral cancer is the eighth common cancer among men and the fifteenth common cancer among women. Approximately, 94% of Oral malignancies are squamous cell carcinoma. It is worth nothing that, its average annual incidence and mortality rate varies significantly in terms of race, age and sex [1]. They are treated with chemotherapy, radical surgery and radiotherapy. Despite the recent advances in cancer combination therapies; however, the survival rate in patients with oral cancer is about 50 to 59%. Therefore, it is recommended to improve therapeutics outcome with improving prevention strategies, advanced therapies induce apoptosis and senescence and various strategies for early diagnosis of the can-

cers. One of the most important approaches of cancer therapy is apoptosis induced drugs [2].

Apoptosis is a physiological or pathological process in which the cells regulate their growth and proliferation and prevent cancer. The cells that undergo apoptosis show various morphological changes including cell collapse, chromatin condensation, organized corruption of nucleus and finally cell fragmentation and forming the apoptotic bodies [3].

Application of plant-derived anti-cancer compounds has been initiated by the discovery of anti-leukemia natural alkaloids of Vincrstine and Vinblastine, as well as Phyllotoxin extract that both showed cytotoxic properties in 1950 [4] .Furthermore, alkaloids, terpenes, lignans and flavonoids that are extracted from herbal plants present different antitumor effects [5].

A body of studies are discovering natural compounds that inhibit or prevent cancer progression [6]. The P.oleracea with the scientific name of Portulaca Oleracea L. from the Portulacaceae type is one of the most valuable medicinal plants that grows both in wild and domestic forms around the world[7]. Based on traditional medicine evidence, Portulaca Oleracea L has therapeutic effects on scurvy, continuous coughing, purifiers and antipyretic. It also is helpful in healing burns, muscle relaxation as well as cancer treatment [8]. It is full of Mucilages, pectin, carbohydrates, proteins, fatty acids, especially unsaturated fatty acids omega-3 and alkaloids. Also it contains minerals including iron, copper, potassium, selenium and vitamins A, E and C [9]. The antioxidants are abundant in P.oleracea and contain alpha tocopherol, ascorbic acid and glutathione [10]. It is noted that, the cytotoxicity effects of P.oleracea have been reported on some of cancer cell lines. The aquatic extract of P.oleracea inhibits the proliferation of several tumor cell lines, including cervical cancer cells (HeLa) [11]. In Iran, the people believe this plant as a holy plant and use it fresh with food. Since, no research has ever been done on the cytotoxicity of ethanolic extract of P.oleracea on oral cancer cells. Therefore, this study has been conducted to investigate the anticancer effects of P.oleracea in in-Vitro condition on KB cell line.

METHODS AND MATERIALS

Plant collection

P.oleracea was collected from Hamadan province medicinal plants garden in late spring of 2017. The plant type identification was confirmed by the Herbarium of the Faculty of Basic Sciences of Bu-Ali Sina University. After sampling, the parts of the plant were dried for 2 weeks in shade and in the vicinity of air.

Extraction

Dried plant powdered by a cylindrical crusher. About 20g of dried powder separately added to 100ml of ethanol %70. Then solvents are placed on Shaker for 72 hours. The extracts were filtered through filter paper and centrifuged at 10000 rpm for 10 minutes, then concentrated by the Rotary and the extracts were transferred to oven at 40 °C to be dried [12]. The 50mg/ml stock was prepared and then filtered by 0.22µm sterile syringe filter to be used for final concentrations.

Cell Culture

KB cell line was purchased from the national cell bank of Pasteur Institute of Iran. It is a type of oral squamous cell carcinoma. KB cells were cultured in RPMI-1640 culture medium containing 10% of fetal bovine serum (FBS), 100 pU/ml penicillin and 100 µg/ml streptomycin at 37 ° C, 5% CO2 and 95% humidity [13]. Culture medium was replaced every three days. At about 70% confluency, the cells were passaged and subcultured using 0.25% trypsin with EDTA. Then, the Cells treated with doses of 0, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000 and 5500µg/ml of ethanolic extract of P.oleracea for 24, 48 and 72h.

Cell proliferation assay

The cytotoxic effects of ethanolic extract of P.oleracea on oral cancer cell line were assessed by MTT colorimetric methods[14]. 10^4 cells were seeded into each of the 96well plate. After 24 hours, the cells treated with 0, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000 and 5500 µg/ml of ethanolic extract of P.oleracea for 24, 48 and 72h.

Then, the medium was removed and the cells washed with PBS and replaced with 20µl of MTT solution and incubated for 4 hours. Afterward, the contents of each well were replaced with 200µl of DMSO solution and optical absorption was measured by ELISA reader at wavelength of 570 nm.

This test was repeated at least three times for each concentration and the percentage of cell viability was calculated using the following formula for each concentration:

Viability of cells = Sample OD/ Control OD×100

The concentration in which the percentage of cell viability reached to 50% was considered as IC_{50}.

Cell viability

10^6 cells were cultured in a 6 well plate. After 24 hours, the cells exposed to ethanolic extract of P.oleracea with different concentrations of 0, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, and 5500 µg/ml. After 24, 48 and 72 hours, the cells were stained with 0.4%. trypan blue. Blue color cells were considered as dead and colorless as living cells[11].

This test was repeated three times for each concentration and percentage of cell viability was calculated using the following formula:

100 × number of living cells / total number of cells = viability of cells

Apoptosis detection using Hochest

The cells were cultured in 6well plate for 24hrs and treated with 0, 4000, 4500 and 5000 µg/ml of ethanolic extract of P.oleracea for 24, 48 and 72hrs. The cells were then fixed with 4% paraformaldehyde for 1h and washed three times with PBS. Then, the cells were treated with 10µl of

0.01% Triton X-100 for 5 minutes on ice. After washing, 100µg/ml of Hoechst was applied to the cells and kept in dark for 10-15 minutes and visualized with fluorescence microscope [15].

Statistical analysis

Statistical analysis was performed using SPSS 18 software. The raw data were analyzed by one-way ANOVA followed by Tukey's test. P value less than 0.05 was considered as significance among the groups.

RESULTS

P.oleracea decreased cell proliferation and viability with same pattern

In order to know antiproliferative property of P.oleracea on KB cell line, they were cultured with different concentration of its ethanolic extract at different times. Our findings showed that after 24, 48 and 72 hours all treated groups with different concentrations of ethanolic extract decreased significantly the cell proliferation compared with the control group ($P < 0.05$). The highest level of cell toxicity was observed in 5500µg/ml in which 28.02%, 34.7%, and 41.7 % of cells were alive at 24, 48 and 72hours respectively (Fig. 1 A, B and C).

It is worth nothing that, P.oleracea exerts its antiproliferation effects on dose and time dependent, so that with increasing concentration and time, more cells were undergone cell death. Of noted, the dose of 1500µg/ml was considered as IC_{50} (Fig. 1).

Viability of KB cells treated with doses of 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000 and 5500µg/ml of ethanolic extract of P.oleracea after 24, 48 and 72 hours has been shown in Figure 2 A, B and C.

As it is obvious in the figures, a large number of cancer cells treated with ethanolic extract of P.oleracea died, so that a significant difference was detected among all concentrations with the control group ($P < 0.05$) (Fig. 3). Furthermore, the cells viability pattern was dose and time dependant. The highest level of cell death was at 5500µg/ml of P.oleracea in all three 24, 48 and 72 hours in which the cells viability was respectively 26.7%, 31.8% and 42.7%.

All tests were repeated at least three times for each of the concentrations and 1500µg/ml P.oleracea was selected as IC_{50}.

Apoptotic cells were detected after P.oleracea application

The cells were stained with Hoechst to know if the apoptosis has been induced after treatment the cells with 4000, 4500 and 5000µg/ml ethanolic extracts of P.oleracea at 24, 48 and 72 hours. Condensation, shrinkage, collapse and deformation of the nucleus were compared with the control group (Fig. 4). With increasing the dose of the extract, the degree of these changes increased in the cells, which itself indicates apoptosis occurred by the ethanolic extract of P.oleracea.

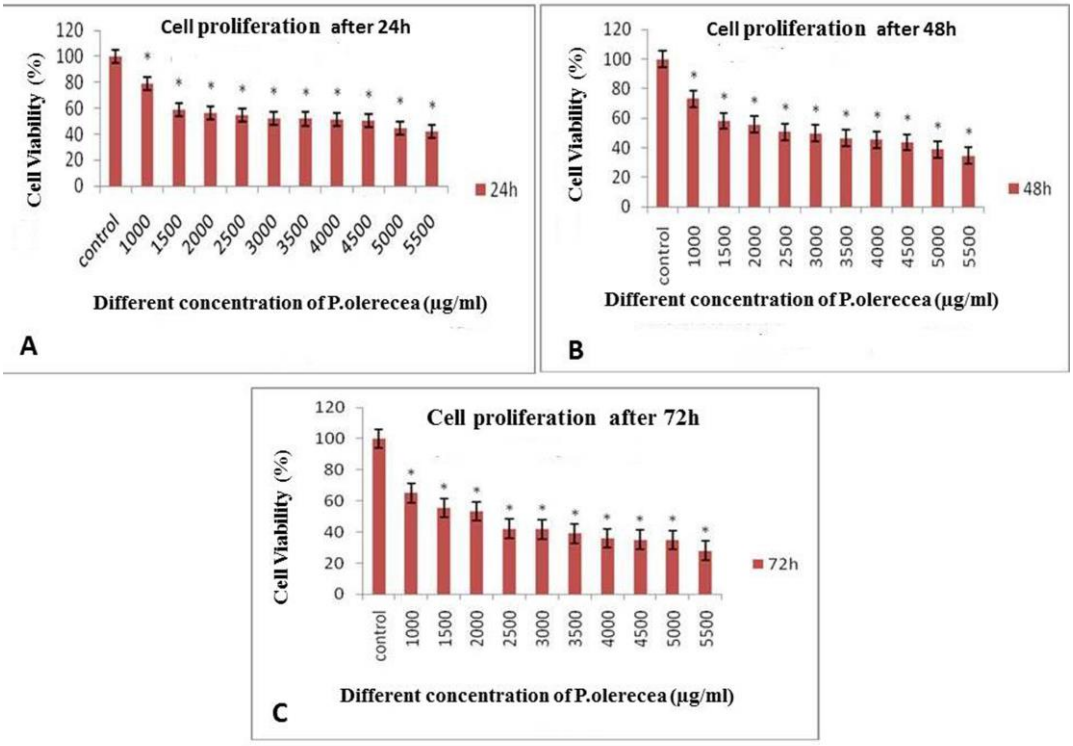

Figure 1. Cell proliferation after treatment with different concentration of P.oleracea after 24, 48 and 72 hours. P.oleracea decreased the cell growth on dose and time dependant manner.

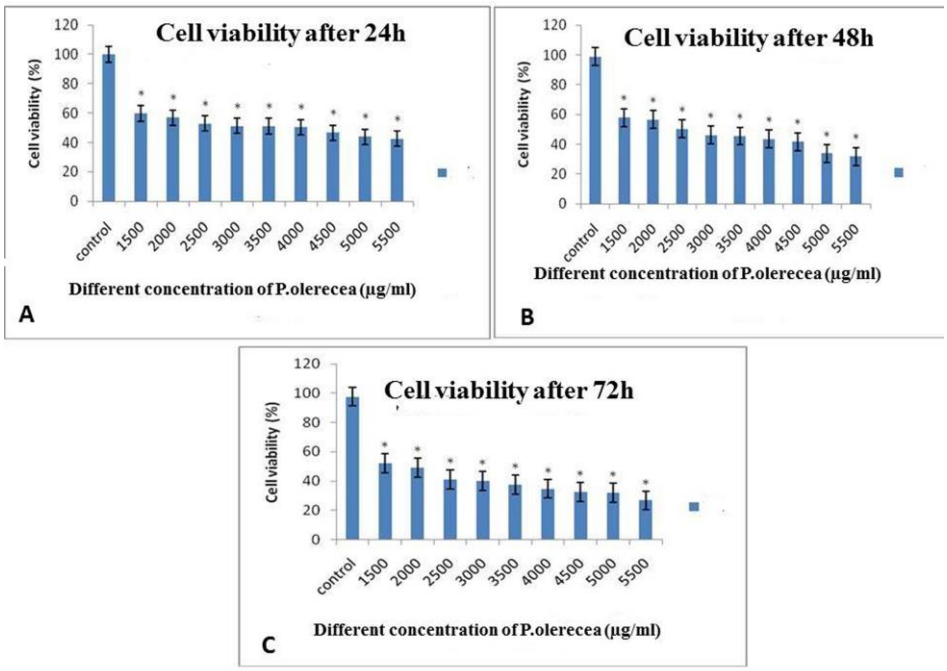

Figure 2. Survival of the cells decreased following P.oleracea treatment when detected with trypan blue staining. The more dead cells were observed at higher concentration at long time.

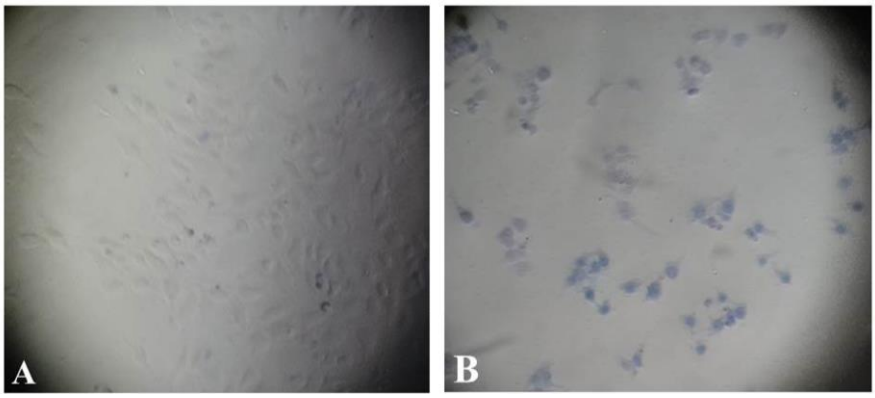

Figure 3. Photograph of KB cells stained with trypan blue. The normal cells are clear and alive (A) but the dead cells treated with P.oleracea show blue nuclei.

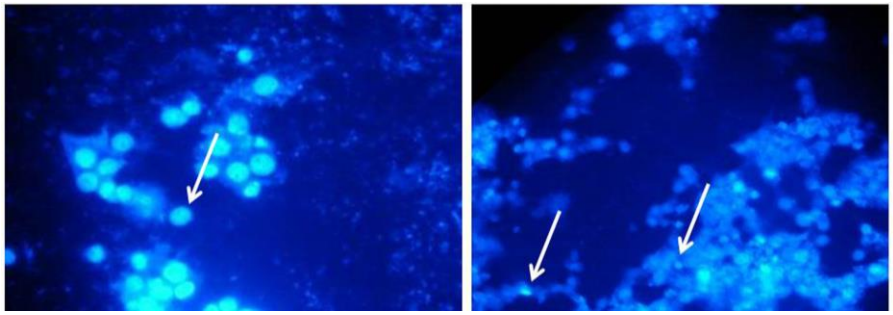

Figure 4. The cells stained with Hoechst to detect their apoptosis. The nuclei in control group are Homogenous (A) while the nuclei in the cells treated with P.oleracea are dense and irregular (B).

DISCUSSION

Cancer is the second leading cause of death after heart diseases. Understanding the key mechanisms involve in cancer development is important to promote strategies in neo-

plasms treatment [16]. It is obvious that a series of mutations escape the cells from cell cycle control and cancer cells are established. In addition, these mutations result in the cell resistance to death and apoptosis. Therefore, using apoptosis-inducing compounds w ould be the efficient modality in cancer therapy [17].

However, current pharmacological strategies not only target cancer cells but also they can damage the adjacent normal tissues. Therefore, recently using natural compounds with low side-effects and promising therapeutic properties have been considered as supplementary medicine in remedy of the cancers [18].

Nowadays, many biological compounds of herbal plants are isolated and are presented to modern medicine. P.oleracea is rich in alkaloids, flavonoids, phenolic acids and carbohydrates which have antioxidant, anti-mutagenic, anti-carcinogenic capacity and represents as free radicals scavanger.

In the present study, ethanolic extract of P. oleracea decreased cell proliferation and survival depend on dose and time manner. Furthermore, our study showed that cell death occurred through apoptosis.

It is proved that cell cycle progression and apoptosis are two main signaling mechanisms for maintaining homeostasis in healthy tissues. A large number of anticancer agents can arrest the cell cycle and then induce cell apoptosis [19].

It is worth to mention that therapeutic and anti-cancer effects of P.oleracea have been indicated in other studies. It has been shown that polyphenols derived from ethanolic and polyphenolic extracts of P.oleracea have potential to induce apoptosis in human colon cancer cells [20]. In a study carried out by Zakaria and his colleague in 2013, anti-cancer effects of ethanolic and aqueous extracts of P.oleracea on mouse breast adenocarcinoma were confirmed through inhibition of cell division [21].

In addition, our previous studies indicated that aqueous extract of P.oleracea had anticancer effects on KB and HeLa cancer cell lines more probabely through apoptosis induction [11, 15]. Furthermore, Rua Zhao et al. in 2013 concluded that polysaccharides component of P.oleracea showed anti-tumor effects by inhibition the cell cycle and inducing apoptosis in cervical carcinoma cells [22]. In another study, Portulacerebroside A induced chromatin density, nucleus fragmentation and an increase in the percentage of apoptotic cells through activation of the internal pathway of apoptosis in human liver cancer cells (HCCLM3). It indicates that this compound increased the phosphorylation of p38 MAPK and JNK proteins, thereby increasing the permeability of the mitochondria membrane, resulting in the release of cytochrome c and AIF from mitochondria to cytosol, which led to the activation of the caspases 3 and 9, and finally the activation of internal pathway of apoptosis [23]. It should be noted that inhibiting the cells growth and proliferation by inducing the internal pathway of apoptosis is dose-time-dependent.

Although, P.oleracea is a safe plant and consumed as a herb in Arabic countries and Iran, however, its safety and efficiency must be investigated in more in vitro and in vivo preclinical and clinical studies.

CONCLUSION

Our findings showed that the P.oleracea extract had potency to reduce and inhibit the growth and proliferation of cancer cells. In addition, more investigation indicated the apoptosis induction in cancer cells which were treated with ethanolic extract of P.oleracea. However, our knowledge is very scant to comprehensively realize its mechanisms. In summary, it is concluded that ethanolic extract of P.oleracea might inhibit KB cells growth in time and dose dependent, however, more unknown contents are remain and need preclinical and clinical studies.

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CONFLICTS OF INTEREST

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article

REFERENCES

1. Kacher JE. Oral and maxillofacial pathology. Case of the month. Histoplasmosis. Tex Dent J 2013;130(3):198, 232.
2. Young-Won Chin, Marcy J. Balunas, Hee Byung Chai, A. Douglas Kinghorn. Drug discovery from natural sources. AAPS J 2006 Jun; 8(2): E239–E253.
3. Elmore S. Apoptosis: A Review of Programmed Cell Death. Toxicol Pathol 2007; 35(4): 495–516.
4. Cragg GM, Newman DJN. Plants as a source of anti-cancer agents. J Ethno Pharmacol 2005;100(1-2):72-9.
5. Urueña C, Cifuentes C, Castañeda D, Arango A, Kaur P, Asea A, et al. Petiveria alliacea extracts uses multiple mechanisms to inhibit growth of human and mouse tumoral cells. BMC Complement Alter Med 2008;8(1):60.
6. Moreno-Aspitia A, Edith A Perez EA. Treatment options for breast cancer resistant to anthracycline and taxane. Mayo Clin Proceed 2009; 84 (6): 533-545.
7. Mohamed AI, Hussein AS. Chemical composition of purslane (Portulaca oleracea). Plant Foods Hum Nutr 1994;45(1):1-9.
8. Rahbarian P, Afsharmanesh G, Shirzadi M. Effects of drought stress and manure on relative water content and cell membrane stability in dragonhead (Dracocephalum moldavica). Plant Ecophysiol 2010; 2(1): 13-19.
9. Ezekwe MO, Omara-Alwala TR, Membrahtu T. Nutritive characterization of purslane accessions as influenced by planting date. Plant Foods Hum Nutr 1999;54(3):183-91.
10. Saikia SP, Dutta SP, Goswami A, Bhau BS, Kanjilal PB. Role of Azospirillum in the Improvement of Legumes. Microbes Legume Improv 2010. p. 389-408.
11. Azarifar Z, Mortazavi M, Farhadian R, Parvari S, Roushnadeh AJPS. Cytotoxicity effects of aqueous extract of Purtulaca oleracea on HeLa cell line. Pharma Sci 2015;21(1):41-5.
12. Manojlović NT, Mašković PZ, Vasiljević PJ, Jelić RM, Jusković MŽ, Sovrlić M, et al. HPLC Analysis, antimicrobial and antioxidant activities of Daphne cneorum L. Hemijska Indust 2012;66(5):709-16.
13. Wang W, Li N, Luo M, Zu Y, Efferth TJM. Antibacterial activity and anticancer activity of Rosmarinus officinalis L. essential oil compared to that of its main components. Molecules 2012;17(3):2704-13.
14. Shokrzadeh M, Parvaresh A, Shahani S, Habibi E, Zalzar Z. Cytotoxic Effects of Lagenaria siceraria Standl. Extract on Cancer Cell Lin. J Mazand Uni Med Sci 2013;22(97):225-30.

15. Azarifar Z, Piri K, Maghsoudi H, Malati ZA, Mohammadi A. Cytotoxic Effects of Aqueous Extract of Portulaca oleracea on Oral Cancer Cell Line. IJBC 2018; 10(1): 20-24.
16. Bendjeddou D, Lalaoui K, Satta DJ. Immunostimulating activity of the hot water-soluble polysaccharide extracts of anacyclus pyrethrum, alpinia galanga and citrullus colocynthis. J Ethnopharmacol 2003;88(2-3):155-60.
17. Ait-Mohamed O, Battisti V, Joliot V, Fritsch L, Pontis J, Medjkane S, et al. Acetonic extract of Buxus sempervirens induces cell cycle arrest, apoptosis and autophagy in breast cancer cells. Plos One 2011;6(9):e24537.
18. Sun SY, Hail JN, Lotan R. Apoptosis as a novel target for cancer chemoprevention. JNCI 2004;96(9):662-72.
19. Ashokkumar P, Sudhandiran G. Luteolin inhibits cell proliferation during Azoxymethane-induced experimental colon carcinogenesis via Wnt/β-catenin pathway. Invest New Drugs 2011;29(2):273-84.
20. Mulla SK, Swamy P. Anticancer activity of ethanol and polyphenol extracts of Portulaca quadrifida linn. On human colon cancer cell lines. Int J Pharm Bio Sci 2012;3(3):488-98.
21. Zakaria A, Hazha JJIJoDD. Cytogenetic toxicity effects of local purslane (portulaca oleracea) leaf crude extracts on normal and cancer cell lines in vitro. Intl J Drug Discov 2013;5(1):173.
22. Zhao R, Gao X, Cai Y, Shao X, Jia G, Huang Y, et al. Antitumor activity of Portulaca oleracea L. polysaccharides against cervical carcinoma in vitro and in vivo. Carbohydrate Polymers 2013;96(2):376-83.
23. Zheng GY, Qu LP, Yue XQ, Gu W, Zhang H, Xin HLJPL. Portulacerebroside A induces apoptosis via activation of the mitochondrial death pathway in human liver cancer HCCLM3 cells. Phytochem Lett 2014;7:77-84.