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Cytotoxicity evaluation of hydro-alcoholic extract of *Prangos pabularia* Lindl root on breast cancer MCF-7 cell line

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ABSTRACT

Introduction: Breast cancer has the second worldwide rank and is expected to reach more than 3.2 million new cases by 2050. In Afghanistan, *Prangos pabularia* plant is used for many purposes, including cancer treatment with no scientific evidence. This study aimed to evaluate the cytotoxic effects of root extract of *P. pabularia* collected from Afghanistan on MCF-7 breast cancer cell line.

Methods: The hydro-alcoholic extract of *P. pabularia* roots at the concentrations of 50, 100, 200, 400, 600, 800, and 1000 µg/mL was studied on MCF7 breast cancer cell line after 24, 48, and 72 hours. The cytotoxicity assessment was done by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) assay, spectrophotometry, and Annexin-A5 apoptotic-flow cytometry.

Results: The cytotoxic effect of the extract exhibited dose-dependent at 24 hours exposure time with IC50 of 756 µg/mL. However, at 48 and 72 hours exposure, the cytotoxicity effects were observed in high extract concentrations with IC50 of 682 and 450 µg/mL, respectively. The extract concentration at 600 µg/mL after 72 hours decreased the cell viability below 50%. This concentration was selected for apoptosis assay. The flow cytometric analysis indicated that the extract had significant cytotoxic effects ($P < 0.01$) by inducing early apoptosis on the cell line.

Conclusion: This research revealed that *P. pabularia* extract represented cell toxicity against the MCF-7 cell line. In addition to the claimed therapeutic uses, proliferative activity was observed in some conditions. Further studies are necessary to confirm this plant's efficacy and safety.

Implication for health policy/practice/research/medical education:
The findings from our study as well as similar research could be integrated into medical education by updating curricula to include information on the benefits and potential applications of plant extracts in cancer treatment. This can help educate future healthcare professionals to consider alternative and complementary therapies.

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Introduction

Cancer is considered one of the main causes of death in the world and is predicted to be the first and most important reason for human death by 2060 (1). To date, various types of cancer have been identified, among which, according to the World Health Organization report, breast cancer has the 2nd worldwide rank (2.3 million cases) (2), and it is expected to reach more than 3.2 million new cases by 2050 (3). The factors that contribute to the risk of breast cancer in patients can vary in significance depending on the region of the world. Generally, these risk factors can be categorized as demographic, reproductive, hormonal, hereditary, lifestyle, and breast-related (4).

There are several methods, including surgery, chemotherapy, radiotherapy, hormone therapy, and immune therapy for the treatment of breast cancer. However, these methods can have severe adverse effects. Chemotherapy is accompanied by drug resistance, physical side effects, and a reduction in cognitive dysfunction that adversely affects the quality of life (5). Radiotherapy

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is accompanied by cardiotoxicity, a hypoxic tumor microenvironment, which increases cell proliferation, apoptosis resistance, and radiotherapy resistance (6). Hormone therapy is accompanied by vasomotor symptoms that negatively affect health-related quality of life (7). Immune therapy is accompanied by high cost, modest response rates, pneumonitis, hepatitis, colitis, and endocrinopathies (8). Consequently, the development of new treatments or the achievement of pharmacological agents with fewer/no side effects need to be explored.

The low side effects of medicinal plants compared to chemical drugs, their affordability, and widespread accessibility have contributed to the growing global utilization of these plants, their extracts and essential oils. Therefore, anti-cancer agents from natural sources have been progressively considered (3). Some herbal medicines contribute to the inhibition of the progression of cancer but many of them have not been tested (9,10).

In traditional medicine, *Prangos pabularia* is widely used in Asia as a crucial herbal remedy (11-13). *P. pabularia* is used for the induction of immune response, hemorrhage treatment, stomach or digestive disorders, wounds, scars, and leukoplakia (13-15). More than thirty species of the *Prangos* genus have been studied around the world (16-18). *P. pabularia* is distributed from Afghanistan to Central Asia, Pakistan, Iraq, Iran, India, Turkey, Russia, and Caucasian. Although some studies have demonstrated that the synthesis of secondary metabolites and their composition could be affected by ecological factors (19-21), phytochemical studies have indicated that *P. pabularia* mainly contains umbelliferone, 6-hydroxycoumarin, meranzin, oxypeucedanin, imperatorin, heraclenin, and osthole, which are derivatives of coumarin and furanocoumarin (as phenolic compounds) (22). It was shown that some compounds, especially osthole and its derivatives, have stimulated apoptosis and inhibited the proliferation of cancerous cell lines (22,23).

There are some published reports on the therapeutic effects of different varieties of *P. pabularia* which have made it a suitable candidate for various purposes, such as antioxidant (13), cytotoxic (14), anti-vitiligo (15), antibacterial (24), antifungal (25), and anti-ulcer activities (26). In Afghanistan, this plant is known as a highly crucial herbal remedy and in traditional medicine, mainly the root and fruit of the plant are used in the treatment of GI disorders and stomachache, toothache, antihypertensive, bruises, wound healing, and cancer. The *P. pabularia* is especially widely used for the treatment of some cancers like breast and uterine cancers in Ghazni province. However, there is no scientific evidence to investigate these observations. The current study for the first time was conducted to evaluate the cytotoxic effects of hydro-alcoholic extract of the plant collected from Afghanistan on MCF7 breast cancer cell line by MTT assay for cytotoxicity and flow cytometry for the analysis of apoptosis.

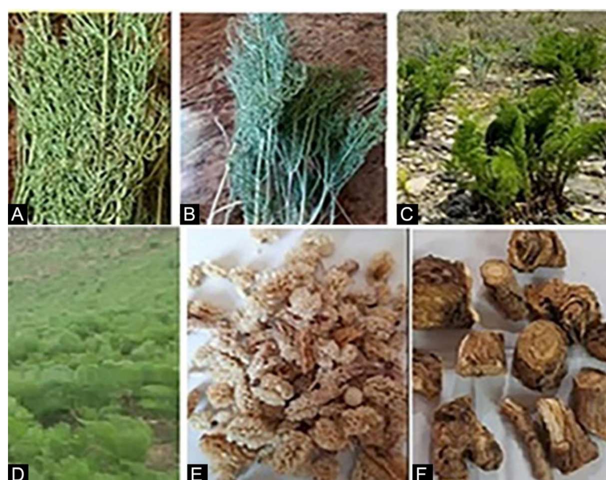

Figure 1. The collected samples of *Prangos pabularia* Lindl (A & B) from Afghanistan, its habitat in Afghanistan (C & D), flowers (E), and roots (F).

Materials and Methods

Collection of *Prangos pabularia*

Prangos pabularia Lindl. from the Umbelliferae family was collected from the slow slop of Shughla Jaghori mountain in Ghazni province, Afghanistan. The plant species was approved by Herbarium Center of Biology Department, Faculty of Science, Kabul University, Kabul, Afghanistan with voucher number KUFS 006964, before extraction (Figure 1).

Preparing the hydro-alcoholic extract from the plant roots

The plant roots were dried at ambient temperature (25 ºC). For hydro-alcoholic extraction of the plant's root, 100 g of the dried roots were powdered using an electric blender and mixed with 500 ml 95% ethanol (Nasr, Iran) and kept for 72 hours at 50 ºC. Then, the extract was concentrated in a rotary evaporator (Strike 202 model from Steroglass Private Limited, Italy).

Cell culture

To determine the cytotoxicity of the plant extract on the breast cancer cell line, MCF7 cells were purchased from the Pasteur Institute of Iran. To prepare appropriate amounts of MCF7, the cells were inoculated into Dulbecco's modified eagle medium (DMEM) (Bio-Idea Co., Iran) containing 10% FBS, 100 units/mL penicillin G and 100 mg/mL streptomycin and incubated for 3 mins (37 °C, 5% CO2), then counted with a Neobar lam (27). When the cell count reached about 110 000 cells/mL (a density of 60-70% within the flask), the culture medium was centrifuged to achieve enough cell count for the Microculture Tetrazolium Test (MTT) and apoptosis assay.

Evaluation of the extract cytotoxicity

In this study, efficiency was studied using the following methods:

Cell viability assays

The MTT assay was used to measure the live cancer cells, representing cell viability following the treatment of the cells with the plant extract.

The concentration of the plant extract that inhibited 50% of cell viability (IC_{50}) was measured for the cancer cell line.Annexin-A5 was used to detect apoptotic cells, which indicated the induction of programmed cell death by the plant extract.

Cytotoxicity assessment by MTT

To determine MCF7 cell viability, three stages including cell seeding, treatment, and MTT assay were performed using the MTT assay Kit (KiaZist Co., Iran). First, 4000 cells were seeded into a 96-well plate, then incubated at 37 ºC for 24 hours to allow the cells to adhere to the bottom of the wells. For the treatment stage, different concentrations (50, 100, 200, 400, 600, 800, and 1000 µg/mL) of *P. pabularia* hydro-alcoholic extract were added to the wells, including adhered cells, and incubated at 37 ºC for 24, 48, and 72 hours. For the MTT assay, culture media was removed from each well; then 50 µL of MTT solution (5 mg MTT in 5 mL PBS) was added to each well and incubated for 4 hours at 37 °C. The supernatant was discarded and 100 µL DMSO (Merk, Germany) was added to each well. Finally, the light absorbance of the wells was measured at 570 nm using a double-beam UV-visible spectrophotometer (Oxford instrument Co. Shamrock 500i, Model 6305) (28).

Measurement of cell apoptosis by flow cytometry

The Annexin-A5 apoptosis analytic kit (Biotin ASSAY, MyBioSource Co., USA), was used to evaluate the percentages of the cells' death. The number of 10^5 MCF7 cells was added to a 96-well plate and incubated for 24 hours at 37 °C. Then, 300 µL of *Prangos* hydro-alcoholic extract at the concentration of 600 µg/mL plus 2700 µL of the culture medium was added to each well and incubated at 37 °C for 72 hours. The supernatant was spilled away and the cells were washed by PBS. In the next step, 1 mL trypsin was added to each well and incubated for 5 minutes at 37 °C. The cells were then centrifuged in three consecutive steps: at 2000, 1500, and 2000 rpm, 5 minutes for each step. Subsequently, the cells were re-suspended at 1 mL culture medium until used in the Annexin-A5 test. The first Annexin-A5 dye plus propidium iodide dye was added to the cells. In this process, apoptotic proteins could conjugate with fluorescein isothiocyanate (FITC). In normal cells, phosphatidyl serine was located on the inner side of the membrane, while in apoptotic cells phosphatidyl serine at the cell surface reacted with Annexin-A5 dye. The normal cells did not stain, while necrotic cells were stained by propidium iodide. The late apoptotic cells were stained by FITC, while the early apoptotic cells were stained by FITC and propidium iodide. This measurement was done by flow cytometry (29).

Data analysis

The experiments were conducted in triplicate. Data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using SPSS version 21. Mean ± standard error of the mean for all data was reported. The significant levels were considered at $P < 0.05$.

Results

The efficiency of the final extract

Since 100 g of the dried plant was extracted with 500 ml ethanol to reach 16 g final extract (Figure 2), the efficiency of the final extract was calculated according to the following formula:

$$C = \frac{w2}{w1} = \frac{16}{100} = 0.16 = 16\%$$

where C is the final extract efficiency, w1 primary dried plant weight, and w2 the final extract weight. Figure 2 shows the final extract.

Cytotoxicity results obtained by MTT assay

The MTT assay results indicated that the different concentrations of hydro-alcoholic extract of *P. pabularia*, except concentration of 50 µg/mL, significantly decreased MCF7 cells viability (%) after 24 hours exposure. The reduction in the cell viability (%) at the concentrations of 100, 200, 400, 800, and 1000 µg/mL was 87.23%, 82.68%, 67.57%, 60.20%, 46.97%, and 28.66%, respectively (Figure 3A). The IC50 value of the extract was 756 µg/mL.

As shown in Figure 3B, following 48 hours exposure of the cells to the plant extract, the concentrations of 50 and 100 µg/mL did not show significant effects on the viability of the cells. The extract with the concentration of 200 µg/mL increased the cell count, which might be due to an

Figure 2. The appearance of the final hydro-alcoholic extract of *Prangos pabularia* Lindl.

increase in the proliferation of the cells after 48 hours exposure. The plant extract with the concentration of 400 μg/mL reduced the cell viability ($P<0.0001$); its effect versus the control group was not statistically significant. At the concentrations above 400 μg/mL, the cell viability was significantly decreased with the increase of the plant extract: 600 μg/mL (62.99%), 800 μg/mL (31.26%), and 1000 μg/mL (9.2%). The IC_{50} value of the extract after 48 hours exposure time was 682 μg/mL.

The results obtained from 72 hours exposure of the cells to the plant extract are shown in Figure 3C. The plant extract concentrations of 50 and 100 μg/mL significantly increased the cell viability to 141.67% and 132.46%, respectively. However, the extract with concentrations of more than 400 μg/mL significantly decreased the cell viability to 60.65%, 31.88%, 10.16%, and 4.70%, respectively for 400, 600, 800, and 1000 μg/mL of the extract ($P<0.0001$). The IC_{50} value of the extract was 450 μg/mL.

Since the extract of *P. pabularia* at the concentration of 600 μg/mL after 72 hours reduced the cell viability below 50%, this concentration at the mentioned exposure time was considered for further assay through flow cytometry.

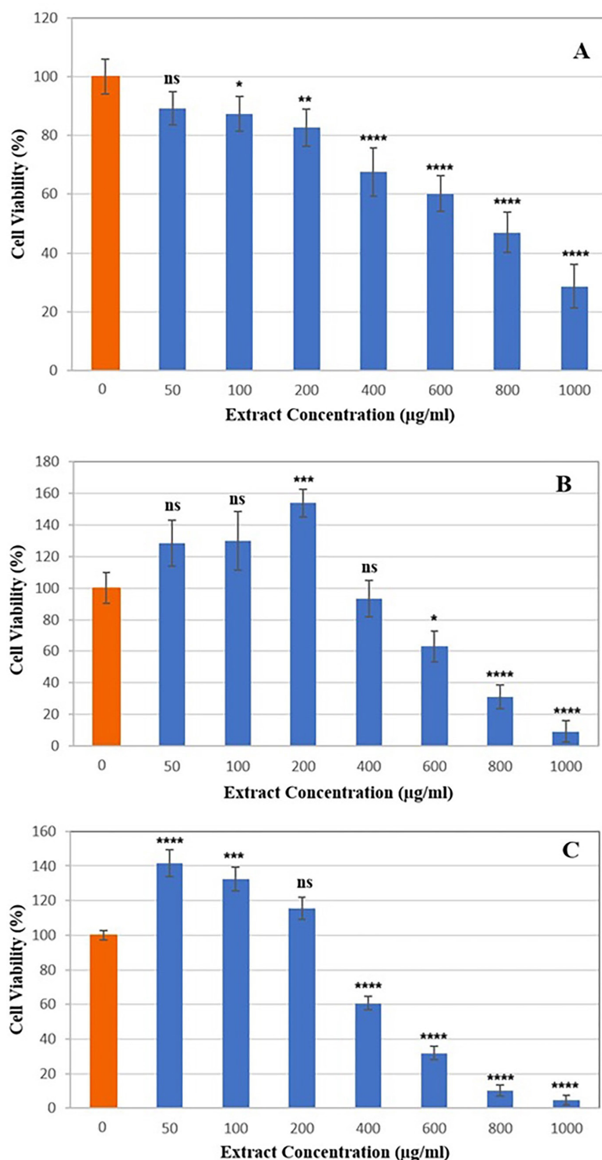

Figure 3. Percentage of MCF7 cells, 24 (A), 48 (B), and 72 hours (C) after the *in vitro* application of different concentrations of *Prangos pabularia* hydro-alcoholic extract. Values represent mean ± standard error obtained in three independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$ indicate significant versus the control group (zero concentration). ns: no significant.

Apoptosis results obtained by flow cytometry

Alongside the MTT assay, an apoptotic Annexin flow cytometry kit was exerted for the evaluation of the MCF7 cells viability for determining the percentage of necrotic, early apoptotic, and late apoptotic cells after 72 hours exposure to the hydro-alcoholic extract of *P. pabularia* at 600 μg/mL concentration. Since the extract concentration of 600 μg/mL in the 72 hours exposure time reduced the cell viability below 50%, this concentration in the mentioned exposure time was considered for further assay through flow cytometry and analysis of the cell apoptosis (Figure 4). The results revealed that the plant extract induced early apoptosis significantly ($P<0.01$), from 1.75% in the control group to 36.5% in the cells under treatment. However, it did not significantly ($P>0.05$) induce late apoptosis in the treated cells (18.5%) compared with the control group (13%). In addition, the percentages of necrotic cells in the control (13.8%) and the treatment cells (14.8%) were not significantly different ($P>0.05$). Overall, the extract of *P. pabularia* at 600 μg/mL concentration was responsible for more than 40% of cells death after 72 hours exposure upon ANEXIN apoptotic assay, which caused the percentage of the alive cells reduce significantly ($P<0.01$), from 71.5% in the control group to 30.2% in the treatment cells (Figures 5).

Discussion

In the current research, the cytotoxic activity of the root extract of *P. pabularia* was evaluated via MTT assay and flow cytometry. As the first step, hydroalcoholic extraction of the plant was carried out with an extraction efficiency of 16%. Extraction efficiency represents the proportion of the extract obtained, compared to the total amount of the original sample. This indicates the effectiveness of the extraction method in recovering the analytes from the plant samples. Extraction efficiency can be influenced by various factors, including the type and quantity of solvent used, as well as the temperature and duration of the extraction process and other extraction conditions (30).

The cytotoxicity results using the MTT assay of the current study showed that *P. pabularia* hydro-alcoholic extract was dose-dependent at 24 hours exposure time. Some studies have shown the anti-proliferative and cytotoxic effects of other varieties of this plant on other

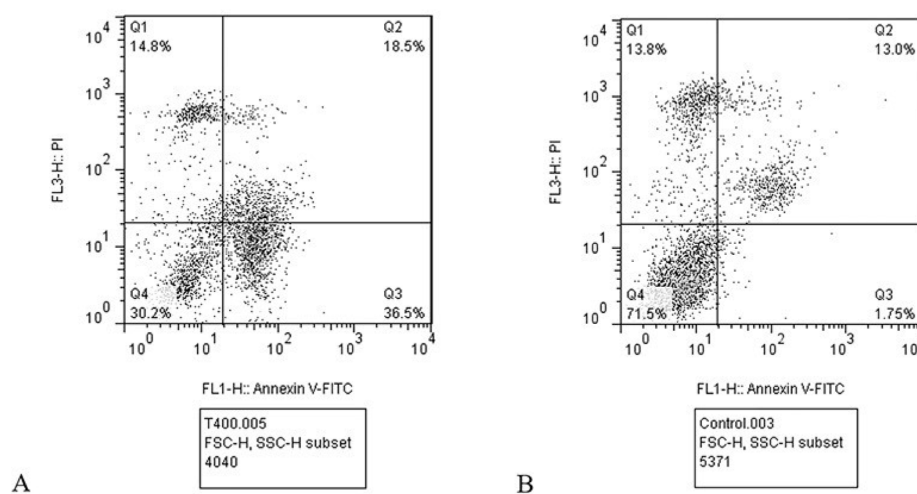

Figure 4. Flow cytometry diagram of apoptosis assay by ANEXIN kit for MCF7 cells after 72 hours exposure to *Prangos pabularia* hydro-alcoholic extract (600 µg/mL) to determine the percentages of necrotic cells, early apoptotic cells, and late apoptotic cells. A: Treatment cells; B: Control cells; Q1: Necrosis; Q2: Late apoptosis; Q3: Early apoptosis; Q4: Live cells.

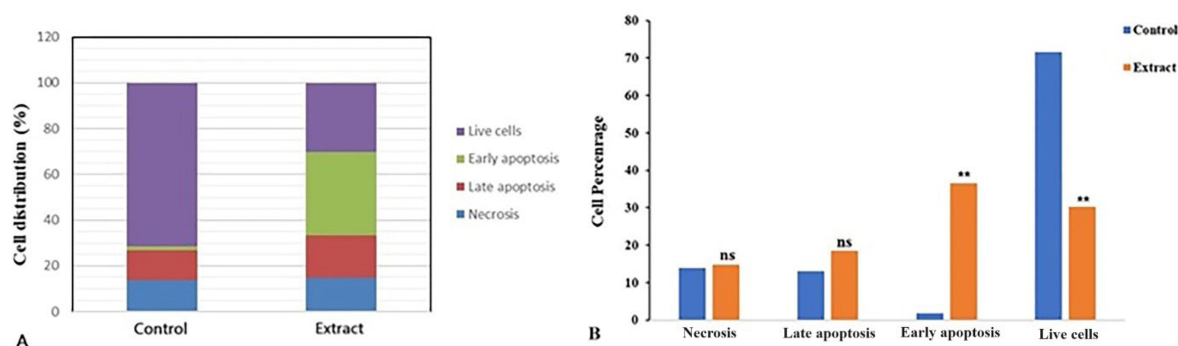

Figure 5. Flow cytometric analysis of the extract-induced apoptotic cell death in MCF7 cells. A: The percentage of MCF7 cells after treatment with 600 µg/mL *Prangos pabularia* hydro-alcoholic extract after 72 hours compared to the control; B: Statistical analysis of apoptosis versus control (B). ns: No significant; $^{**}P < 0.01$ versus the control.

cancer cells. For example, Salehi et al showed the cytotoxic effect of *P. Pabularia* extracts from Iran on the HeLa cell line by MTT assay. The plant dichloromethane extract had significant cytotoxic activity against the cells (IC_{50} = 0.526 mg/mL) in 24 hours by MTT assay, which showed lower IC_{50} compared to our results at 24 hours (31). Farooq et al. (2014) isolated the phytochemical constituents of *P. Pabularia* from India. Among the constituents, osthol revealed the highest cytotoxicity activity with IC_{50} values of 3.2, 6.2, 10.9, 14.5, 24.8, and 30.2 µM against epidermoid carcinoma (A431), melanoma (A375), lung (NCI-H322), lung (A549), prostate (PC-3), and colon (HCT-116) cell lines, respectively (14). The main constituent of the essential oil of *P. pabularia* was durylaldehyde (62.161%) (24). The cytotoxic activity was evaluated by MTT assay against several cancerous cell lines at four different concentrations. The results revealed that the concentration of 20 µg/mL had the highest activity (56.12%) against the human lung cell line (24). In another study, Shokoohinia et al isolated coumarins from *P. ferulacea* (L.) Lindl. and evaluated their potential to stimulate death on SKNMC, PC3, and H1299 cell lines by MTT method. Osthole, one of the coumarin compounds, had the highest lethal effect (up to 70.38%) on the cells; then, isoimperatorin, oxypeucedanin, and braylin induced cell death by 63.38% (32). In our research, at 48 and 72 hours exposure time, cell proliferation was increased at low extract concentrations, however, at higher extract concentrations, the cytotoxic effect was increased. Such an effect is often observed when the plant extract contains polyphenolic compounds. In this case, proliferation effects are seen at low extract concentrations, while higher concentrations cause negative effects (33). In a study by Eren and Özata, the cytotoxic effects of the roots, stems, and leaves of *Limonium globuliferum* were evaluated through MTT assay. Similar to our study, their results

showed that low concentrations of *L. globuliferum* stem and leaf had proliferative effects on cells but toxic effects at high concentrations; for leaf extract the cell proliferation increased with an increase in exposure time to 48 hours (34). In our study, many extracts treated for 24 hours, as well as those at high concentrations for 48 and 72 hours, exhibited cytotoxic effects. The effect of the extract on the cancer cells could be due to the existence of coumarin as the main component of *Prangos* species extracts (32). These compounds have various therapeutic usages because of their various effects such as antipsychotic, antiviral, antimicrobial, antioxidant, anti-diabetic, anti-inflammatory, and cytotoxic activities (32,35,36).

Coumarin compounds have the potential to inhibit the growth, proliferation, and metastasis of various tumor cells through different mechanisms. These mechanisms include the inhibition of carbonic anhydrase and disruption of PI3K/AKT/mTOR signaling pathway. In addition, coumarin compounds can affect microtubule polymerization and angiogenesis and modulate the activity of monocarboxylate transporters and hypoxia-inducible factor-1. They are also involved in influencing apoptosis-related proteins and reducing tumor multidrug resistance while regulating reactive oxygen species (ROS) levels and being involved in other relevant pathways (37). Disrupting metabolic pathways and transmitting message molecules into the cells cause cell death by apoptotic or necrotic pathways (32,38).

Numerous techniques have been designed to detect and quantify cell apoptosis; however, flow cytometry remains a unique methodology for monitoring the apoptotic cascade relative to cells' type, trigger, and time (39,40). Apoptosis, programmed cell death, in response to internal or external signals, can be induced by some biomaterials, which are characterized by blebbing, cell shrinkage, chromatin condensation, and nucleosomal fragmentation. Most cancer drugs induce apoptosis and play a critical role in eliminating tumor cells in cancer therapy (40).

To the best of our knowledge, it is the first study on the cytotoxic effects of *P. Pabularia* breast cancer using flow cytometry. The results showed 30.2% of the live cells after treatment with *P. pabularia* extract at the concentration of 600 μg/mL after 72 hours exposure time. Plant extract resulted in a 41.3% increase in cell death compared to the control group. The applied concentration of the extract in the exposure time was cytotoxic for MCF7 breast cancer cells, significantly through early apoptosis (36.5%), followed by late apoptosis and necrosis. Meanwhile, the determination of the cell survival by the MTT method showed a 31.88% cell viability (equivalent to 68.12% cell death) in the exposure to 600 μg/mL extract.

Perumalsamy et al. (2018) showed the apoptotic effect of styrene-substituted biscoumarin in human stomach cancer cell lines (AGS) using flow cytometry (FACS), with Hoechst and DAPI/PI staining (35). Abosharaf et al used flow cytometry analysis to investigate the effects of osthole, a natural coumarin extracted from Egyptian citrus fruits, on viable and apoptotic lung cancer cells. They found that induced apoptosis by osthole was increased with increasing time to 72 hours. Osthole induced early apoptosis (up to 26%), which was a coincidence with the results of the present study (36.5%), and caused late apoptosis (up to 81.7%) higher than that seen in the results of the current study (41).

Conclusion

The study for the first time contributed to understanding the cytotoxic effects of the *P. pabularia* variety from Afghanistan on MCF7 cell line. The findings provided a basis for further studies and added useful data to the existing body of research on natural compounds with potential anticancer properties. As the next plan, determination of the plant phyto-molecules, specific apoptosis genes and protein markers, and apoptosis pathway can be helpful for a comprehensive understanding of extract effectiveness. Future studies also should include assessments on normal human cells and other cancerous cell lines, as well as in-vivo studies to ensure the extract's safety and selectivity.

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Authors' contribution

Conceptualization: Masoumeh Fayazi, Sayed Yousof Mousavi.
Data curation: Masoumeh Fayazi, Meysam Sajjadi.
Formal analysis: Masoumeh Fayazi, Meysam Sajjadi, Sayed Yousof Mousavi.
Investigation: Masoumeh Fayazi, Meysam Sajjadi, Sayed Yousof Mousavi.
Methodology: Masoumeh Fayazi, Meysam Sajjadi.
Resources: Masoumeh Fayazi
Validation: Masoumeh Fayazi, Sayed Yousof Mousavi.
Writing–original draft: Masoumeh Fayazi.
Writing–review & editing: Meysam Sajjadi, Sayed Yousof Mousavi, Masoumeh Fayazi.

Conflict of interests

The authors declared no conflict of interests.

Ethical considerations

The experimental procedures were performed with the approval of Research Ethic Board of Kavosh Educational Research Organization, Kabul, Afghanistan (Approval ID: AF.KERO.REB.1400.2).

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