

# EVALUATION OF THE THERAPEUTIC ANTI-CARCINOGENIC EFFECT OF FAGONIA ARABICA ON ORAL SQUAMOUS CELL CARCINOMA (AN IN VITRO STUDY)

Alyaa S. Abou El-nil<sup>1\*</sup> BDS, Fatma H. Eldidi<sup>2</sup> PhD, Salama M. El-Darier<sup>3</sup> PhD,

Enas M. Omar<sup>4</sup> PhD

## ABSTRACT

**INTRODUCTION:** Despite the fact that surgery, with or without adjuvant radiation and chemotherapy can be an effective therapeutic approach for malignancies, drug resistance and side effects are the major treatment hurdles. The platinum-based chemotherapy drug, cisplatin, has been a cornerstone in the treatment of cancer since it boosts the survival rate. So, the use of natural products has become one of the most promising therapeutic modalities to lessen the toxicity that cisplatin causes. Fagonia arabica plant is a new herbal product that assumes an outstanding performance as an analgesic, antioxidant, anti-inflammatory and anti-carcinogenic. Herein, this study assessed the anti-carcinogenic effect of Fagonia arabica on oral squamous cell carcinoma (OSCC), which accounts for 90% of all oral cancers.

**OBJECTIVES:** The study aimed to investigate the anti-carcinogenic effect of Fagonia arabica shoot ethanolic extract (FASEE) on squamous cell carcinoma-4 (SCC-4) cell line. Then, to compare its anti-carcinogenic effect with that of the conventional chemotherapeutic drug; cisplatin.

**MATERIALS AND METHODS:** SCC-4 cell line was divided into three groups. The first group was treated with FASEE, the second group with cisplatin (the positive control); while the third group received no treatment (negative control). Comparison between the groups was done in terms of cytotoxicity, cell cycle arrest, and apoptosis.

**RESULTS:** Treatment of the SCC-4 cell line with Fagonia arabica extract resulted in inhibiting cell growth through induction of apoptosis. Furthermore, the cell cycle was arrested at the G0/G1 phase.

**CONCLUSIONS:** Fagonia arabica extract has a significant anti-carcinogenic effect on OSCC.

**KEYWORDS:** Cisplatin, Fagonia arabica, OSCC.

**RUNNING TITLE:** Assessing the efficacy of Fagonia arabica extract as an anticancer agent.

1 BDS, Oral Pathology department, Faculty of Dentistry, Alexandria University

2 Professor of Oral Pathology, Oral Pathology Department, Faculty of Dentistry, Alexandria University, Alexandria, Egypt

3 Professor of Botany, Botany and Microbiology Department, Faculty of Science, Alexandria University, Alexandria, Egypt

4 Lecturer of Oral Pathology, Oral Pathology Department, Faculty of Dentistry, Alexandria University, Alexandria, Egypt

\* Corresponding Author:

E-mail: [alyaa.salah.dent@alexu.edu.eg](mailto:alyaa.salah.dent@alexu.edu.eg)

## INTRODUCTION

Surgery, radiation, and chemotherapy are the standard methods for curing cancer (1). Although they are of great benefit in the treatment of various tumors, there is a chance of adverse effects and relevant resistance (2,3). A wide range of cancers have been treated with cisplatin, a cytotoxic chemotherapeutic medication, including oral, breast, ovarian, lung, bladder, testicular, and cervical cancers (4). However, the observed side effects of cisplatin such as renal failure, cardiotoxicity, leukaemia, and neuropathy, limited its use (5). Scientific data suggests that a considerable amount of natural products (NP) may

be able to guard against the toxicity caused by cisplatin (6).

Natural products are substances secreted by plants, bacteria, or other marine species as a by-product of secondary metabolism. Notwithstanding the fact that almost all organic compounds are synthesised, NPs account for more than thirty percent of all pharmaceutical revenues (7). They have been used for a long time as active ingredients in folk medicine, dating back to African herbal medicines, traditional Chinese medicines, and ancient Indian Ayurveda. There have been found to be about two million bioactive compounds with properties resembling pharmaceutical drugs. Furthermore, they

are more affordable than traditional chemotherapy (8).

Desert plants have a special way of adapting to their surroundings. Over 20 species of plants in the Zygophyllaceae plant family belong to the genus *Fagonia*, which ranges from the Mediterranean region through Southern Africa, Chili, California, and India. *Fagonia* is popularly known as *Dhamasa* (9). Many species have been identified, including *Fagonia arabica*, *Fagonia cretica*, *Fagonia schweinfurthii*, *Fagonia bruguieri*, *Fagonia indica*, *Fagonia longispina.*, *Fagonia laevis*, and *Fagonia mysorensis* (10).

*Fagonia arabica* is found in abundance in the Horn of Africa. This plant shows marvellous biological effects, such as anti-inflammatory, analgesic, anti-pyretic, androgenic, anti-allergic, and neuroprotective. Other benefits such as antibacterial, cytotoxic, and anti-tumor activity have also been demonstrated (9). By reducing lipid peroxidation and eliminating free radicals, *Fagonia arabica* strengthens the body's natural immune system and antioxidant capability which has noticeable impacts on cancer cells (11). Furthermore, *Fagonia arabica* was found to be a potent thrombolytic which suggests that it could be an alternative and cheaper anticoagulant. Yet, it should be used with caution in haemophilia patients and those taking heparin for liver failure since it might cause severe bleeding (12). To date, no proven serious side effects have been reported for *Fagonia arabica*. However, consuming it on an empty stomach could cause gastric issues (13).

The protein kinase B (Akt/PKB) cascade is crucial for cell growth and survival throughout carcinogenesis and development. Phosphatidylinositol 3-kinase (PI3K) triggers the activation of Akt/PKB (14). By stimulating pro-survival transcription factors and blocking the fork head box transcription factor O class 3a (FOXO3a), activation of this cascade alters the balance among cell cycle and apoptosis (15). FOXO3a promotes growth arrest and apoptosis signaling by either promoting the production of pro-apoptotic Bcl2-family members, boosting the expression of death receptor ligands, or increasing certain cyclin dependent kinase inhibitors levels. Loss of FOXO3a has been found in several malignancies (16). P21, a cyclin dependent kinase inhibitor, directly regulates the cell cycle. Cell cycle arrest in the G1 phase is brought about by p21, which suppresses the activity of cyclin D-CDK2/4 complexes (17).

In a recent study, considerable elevation of FOXO3a and Akt was observed in the untreated mice bearing Ehrlich carcinoma. However, after administration of the FASEE, there was a significant decline in the levels of FOXO3a and Akt. This reveals that FASEE may be a potent anticancer drug for breast cancer (11).

To the best of our knowledge, not enough is known about the anti-carcinogenic effect of FASEE on

cancer of the oral cavity. Oral cancer, also known as oral squamous cell carcinoma (OSCC), is the most prevalent form of malignant oral cancers (18). It is one of the most common cancer forms that is more of an issue worldwide. As compared to industrialized nations, oral cancer is significantly more common in poor nations (19).

Therefore, the current study evaluated the anti-carcinogenic effect of FASEE on SCC-4 cell line and compared the anti-carcinogenic effect of FASEE versus cisplatin.

The null hypothesis of this research denoted that FASEE would exert no statistically significant anti-carcinogenic effect on OSCC.

## MATERIALS AND METHODS

### I. Cell line and cell culture

Squamous cell carcinoma-4 (P 9-11), an authenticated human oral squamous cell carcinoma cancer cell line was used in the present research. It was ordered from American Type Culture Collection (ATCC). This cell line was a primary culture of squamous cell carcinoma of the tongue. Cells were allowed to proliferate in DMEM in addition to 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum. Then incubated at 37°C until confluent enough for work. The research was carried out in the Centre of Excellence for Research and Regenerative Medicine and its Applications, CERRMA, Faculty of Medicine, Alexandria University.

### II. Harvesting and preparation of *Fagonia arabica* extract

*Fagonia arabica* was gathered from El-Dabaa area. The *Fagonia arabica* shoot was cleaned and dried in a shaded, reasonably dark environment. Wiley mill was used to grind the entire plant since smaller particles were thought to be better suited for ethanolic solvent extraction. The standard plant extract preparation (maceration) procedure was used to prepare the ethanolic extract. In more detail, 200 g of the plant material was stirred continuously for three days while being suspended in one litre of ethanolic solvent. Thereafter, filtering of the solution and evaporation of solvents using a rotary evaporator under reduced pressure were done. Until use, the dried extract was preserved at 21°C (20).

### III. Grouping

The SCC-4 cell line was randomly divided into 3 groups. Group I served as the study group and were treated using different concentrations of FASEE. Group II served as the positive control and treated with cisplatin alone. Group III served as the negative control with no treatment received.

### IV. Cell viability analysis

Cells were seeded in 96 well plates and allowed to form a monolayer for 24 hours in an incubator at 37°C. Various concentrations of *Fagonia arabica* (0,5,20,35,50,65,80,

and 95  $\mu\text{g/ml}$ ) and cisplatin (0,0.5,1,2.5,7.5,10,12.5,15,17.5,20,22.5 $\mu\text{g/ml}$ ) were added to the wells containing the OSCC cells. After 24 hours, the cells were monitored for their growth and morphology using the phase contrast inverted microscope and then the MTT reagent was added to the cells. The plates containing the cells and the drugs were placed in an incubator for 4 hours at 37°C. Then Dimethyl sulfoxide was added in each well and the plates were lightly rocked in the dark for 15 minutes. The results were then evaluated using a spectrophotometer at 570nm and analyzed by statistical methods. GraphPad Prism 9.0 was used to determine the IC<sub>50</sub> values (17).

#### V. Cell cycle analysis

Cells were seeded in 6 well plates and allowed to form a monolayer for 24 hrs in an incubator at 37°C. After being exposed to the extract (55  $\mu\text{g/ml}$ ) and cisplatin (9  $\mu\text{g/ml}$ ) for 24 hours, the cells were rinsed two times with 1ml PBS. Cells were then collected in pellets before being re-suspended in permeabilization buffer and kept at room temperature for 10 minutes. Cells were stained with anti-cyclin A-FITC for 45 minutes in the dark then stained with propidium iodide for 5 minutes. Cells were rinsed in permeabilization buffer before being analyzed immediately by flow cytometry (FL1 & FL3) (17).

#### VI. Analysis of cell apoptosis by annexin V/PI staining

Cells were seeded in 6 well plates and allowed to form a monolayer for 24 hrs in an incubator at 37°C. Cells were treated with the extract (55  $\mu\text{g/ml}$ ) and cisplatin (9  $\mu\text{g/ml}$ ) for 24 hrs then rinsed twice with 1ml PBS. Cells were collected in pellets then stained with annexin V-FITC and propidium iodide for 5 minutes. To minimize the possibility of false positive results, a group of cells were stained only with annexin V, another group of cells were stained only with propidium iodide, and a third group were not stained at all. Then, the cells were analyzed immediately by flow cytometry (FL1&FL3) (17).

#### VII. Statistical analysis

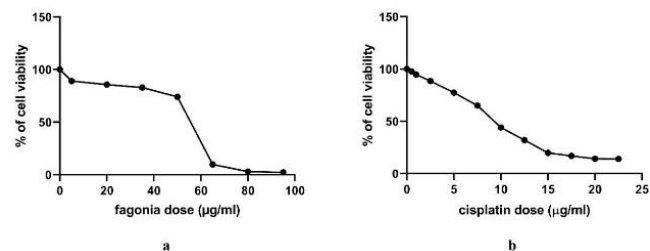
All data were collected; tabulated and statistically studied using GraphPad Prism 9.0. Two-way analysis of variance (ANOVA) test was used to analyze the data between the study groups. Tukey's multiple comparison post-test was used for pairwise group comparisons. Error bars are representative of the standard deviation.

## RESULTS

### Cytotoxicity results

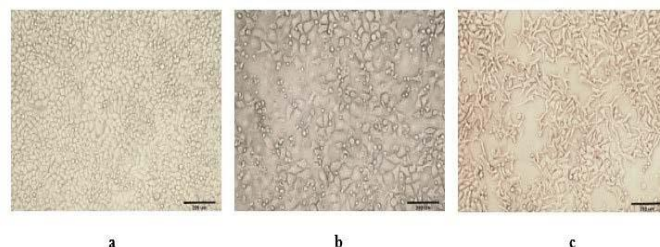
Human OSCC cell line, SCC-4 was used to study the potential cytotoxic effect of *Fagonia arabica*. The cells were treated with various concentrations of *Fagonia arabica* and cisplatin for 24 hours, whereby the impact on proliferation was dose-dependent in all of them. In other words, the reading of the cytotoxicity of the

, the IC<sub>50</sub> value for cisplatin, the positive control, was calculated at 9  $\mu\text{g/ml}$ . (Fig.1b)



**Figure 1:** Line graph showing the viable cell percentage of *Fagonia arabica* (a) and cisplatin (b) different doses. The IC<sub>50</sub> values were detected 24 hours after drugs application. Dose 55 $\mu\text{g/ml}$  for *Fagonia arabica* and 9  $\mu\text{g/ml}$  for cisplatin represent the least lethal doses.

The morphological analysis was likewise consistent with the MTT results. Cells were monitored for their growth and morphology using the phase contrast inverted microscope where the untreated cells exhibited a normal growth pattern and were homogeneously distributed throughout the wells. The epithelial cancer cells were taking both the prickle and the squamoid shapes with large centrally located vesicular nuclei and prominent nucleoli (Fig.2a). *Fagonia arabica*-treated cells revealed various morphological abnormalities. The 24-hour morphological changes included cells detachment and reduction of nuclear size. The cells became rounded with some areas devoid of cells also noticed in the same culture (Fig.2b). Whereas the positively controlled SCC-4 cells treated with cisplatin showed dramatic morphological changes in comparison to the untreated SCC-4 cells, after 24-hour treatment. The cells became detached from each other and became small rounded with shrunken nuclei (Fig.2c).

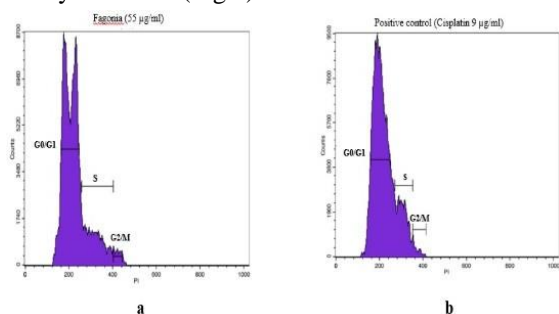


**Figure2:** Inverted light microscope photomicrographs revealing the status and morphology of the cells of the untreated group (a) showing the squamoid polyhedral shape of untreated SCC4 cells, *Fagonia arabica*-treated group (b) revealing that the cells are rounded and shrinkage with a reduction of nuclear size and cisplatin treated group (c) the dramatic morphological changes of small rounded detached cells from each other with pyknotic nuclei and thin perinuclear cytoplasmic rims.

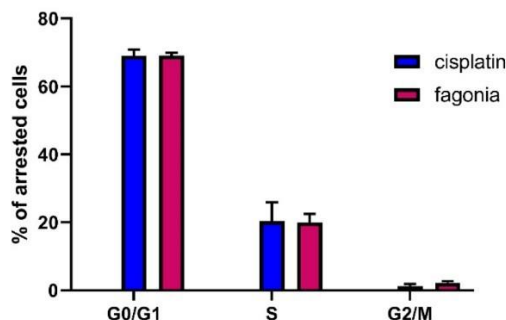
### Cell cycle flow cytometry results

Since *Fagonia arabica* showed a substantial effect on the proliferating SCC-4 cells, cell cycle analysis was

further done. The cells were treated with the calculated dosage of *Fagonia arabica* (IC<sub>50</sub>) for 24 hours. By flow cytometry, the cells revealed that their cell cycles were arrested mainly at the G<sub>0</sub>/G<sub>1</sub> phase. (Fig.3a) Additionally, the cells treated with the IC<sub>50</sub> of cisplatin, also showed cell cycle arrest at the same phase. (Fig.3b) The results of the current research comply that there is no statistically significant difference between *Fagonia arabica*-treated cells and cisplatin-treated cells in terms of cell cycle arrest. (Fig.4)



**Figure 3:** (a) *Fagonia arabica* and (b) cisplatin arrested the progress of cell cycle at G<sub>0</sub>/G<sub>1</sub> phase in the human SCC4 cell line. SCC4 cells were incubated with the IC<sub>50</sub> doses of the drugs for 24 hours and the cell cycle stages were determined by flow cytometric analysis.

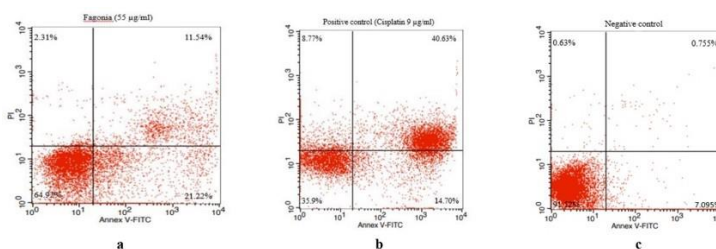


**Figure 4:** Percentages of the arrested cells in the study groups after 24 hours (mean ± S.D.)

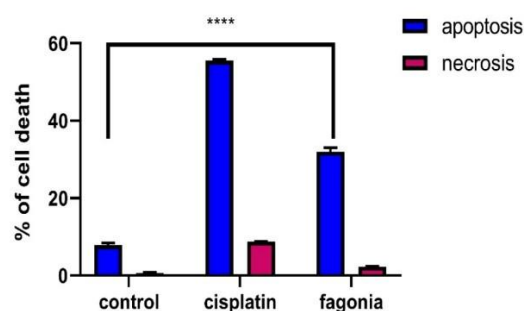
**Annexin V/PI staining results**

The cells were treated and analyzed using annexin V-FITC and PI staining to track the apoptosis and necrosis induced by *Fagonia arabica* and cisplatin. Treatment of the cells with the selected dosage 55 µg/ml *Fagonia arabica* and 9 µg/ml cisplatin for 24 hours, demonstrated a considerable percentage of cancer cells that underwent apoptosis. (Fig.5a,5b). The percentage of apoptotic cells was significantly increased in *Fagonia arabica*-treated group (32.76%) compared to the negative control group (7.85%). (Fig.5c) Also, there was statistically significant difference between *Fagonia arabica*-treated cells (32.76%) and cisplatin-treated cells (55.33%), in favour of cisplatin, in terms of apoptosis. (Fig.5) In addition, necrosis was encountered to a less degree

in both *Fagonia arabica*-treated cells (2.31%) and cisplatin-treated cells (8.77%). (Fig.6, Table 1).



**Figure 5:** Scatter plots for Annexin/PI apoptosis assay by flow cytometry. The percentages of living cells, early apoptosis, late apoptosis, and necrosis were presented in the lower left, lower right, upper right, and upper left quadrant, respectively.



**Figure 6:** Percentages of the apoptotic and necrotic cells in the study groups after treatment for 24 hours. Data denoted \*\*\*\* (p≤0.0001) are significant compared to the control.

**Table 1:** Annexin V-FITC Apoptosis Assay Results Showing the Percentages of Cell Death Modes

	Negative control	Positive control (cisplatin)	Fagonia arabica
Early apoptosis	7.095%	14.7%	21.22%
Late apoptosis	0.755%	40.63%	11.54%
Necrosis	0.63%	8.77%	2.31%

**DISCUSSION**

Radiotherapy and chemotherapy are the most favorably used cancer therapies (21). The emergence of adverse side effects from radiation and chemotherapy limits their practical application as they negatively affect patients' quality of life (22). So, shifting toward natural products is emerging and has become of top priority due to its minimal side effects, abundant drug resources, and powerful anti-carcinogenic efficacy (23). To the best of our knowledge, different natural products have been recently used for curing cancer (24). However, this study was the first to investigate the anti-



carcinogenic potential of *Fagonia arabica* in oral cancer.

Herein, the current study has shown that an ethanolic extract of *Fagonia arabica* was able to induce cytotoxicity, cell cycle arrest, and apoptosis in SCC-4 cells.

In the present study, *Fagonia arabica* was used in reference to El-benhawy et al. who studied its effect as an anti-carcinogen in treatment of breast cancer in vivo (11). *Fagonia arabica* is a member of the major group Angiosperms' Zygophyllaceae family (flowering plants) (25). It is used as a medication to cure different forms of illnesses, including cancer (17). The plant's therapeutic benefits were attributed to its various active components. Two essential classes of phytochemical substances, flavanol glycosides and terpenoid glycosides, were found in abundance among several *Fagonia* species including *Fagonia arabica*. Flavonoids have been found to possess anti-mutagenic and anti-malignant properties. Additionally, flavonoids contribute to cancer chemoprevention through their impacts on angiogenesis and signal transduction in cell growth (26). A number of trials were executed in order to detect the effective dose of *Fagonia arabica*. The cytotoxic effect in the cells increases by raising the dosage of *Fagonia arabica*. Using the dosage of 55 µg/ml concentration at a 24-hour interval, significantly reduced the SCC4 cell viability to 50%. These results are in agreement with El-benhawy et al. research which proved that the breast cancer volume, growth rate and body weight were significantly decreased after administration of *Fagonia arabica* ethanolic extract (FAEE) alone or in combination with ionizing radiation in mice bearing Ehrlich carcinoma (EC). The maximum lethal dose was calculated at a dose of 1000mg/kg of FAEE. This suggests that FAEE has a potent cytotoxic effect against Ehrlich carcinoma (11).

In contrast to the results of the current study, Alamami et al. reported that an alcoholic and aqueous extracts of *Fagonia taekholmiana* revealed no significant anti-carcinogenic effect on neither human liver carcinoma cells (HEPG2) nor human glioblastoma cells (U-251) (26). In addition, Albalawi et al. concluded that an extract of *Fagonia tenuifolia* had little anti-cancer effect on non-small cell lung cancer cells (NCI-H460) (27). Many factors are proposed to contribute to variation in the anti-cancer effect of different *Fagonia* species on different cancer forms. Such factors include diversity in the phytochemical structure of different *Fagonia* species, type of tumor, tumor characteristics, and some tumors show low sensitivity to certain *Fagonia* species as they don't show positive expression for the related molecular pathways (26).

Cisplatin is an essential chemotherapeutic agent that is considered the most commonly used drug in the treatment of OSCC (4,28,29). In the present work the

selected dosage of cisplatin (9 µg/ml) at a 24-hour interval, significantly reduced the cell viability to 50%. These results are nearly similar to those obtained by Patil et al. (30) and Bendale et al. (31). Their research reported that 10 µg/ml was the effective dose of cisplatin in the treatment of oesophageal squamous cell carcinoma, lung, ovarian, and pancreatic cell lines. On the contrary, Shabani et al. revealed that the viability of both neonatal mouse spermatogonial cells and a lymphoblastic leukaemia cell line was reduced with cisplatin at a higher dose (15 µg/ml) (32).

Indeed, other cell phenotypes of OSCC were also investigated. Cell cycle arrest is a point where the cell is no further engaged in the procedures related to cell replication and division (33). According to the present results, treatment of SCC-4 cells with *Fagonia arabica* caused cell cycle arrest in the G0/G1 phase. Consistently, a previous study done by Lam et al. on MCF-7 cells demonstrated that *Fagonia cretica* induced cell cycle arrest similarly in the G1 phase. In that work, the cell cycle arrest was found to be associated with a significant increase in P53 protein expression (tumor suppressor protein) following the treatment of the breast cancer cells using the extract. In addition, double strand DNA breaks in extract-treated MCF-7 cells were detected by comet assay which indicates evident DNA damage (17).

Within the context for verification of the anti-cancer potential exerted by *Fagonia arabica*, activation of the apoptosis pathway in contribution with the anti-proliferative effect were detected in human SCC-4 cells. The percentage of apoptotic cells after administration of 55 µg/ml of *Fagonia arabica* for 24 hours was 32.72%, which is significantly higher than the cells of the negative control. The data thus imply to the fact that *Fagonia arabica* has a good potential for triggering apoptosis in SCC-4 cells. These findings go with the research done by Lam et al., who demonstrated that breast cancer cells treated with the *Fagonia cretica* aqueous extract revealed a significant increase of Annexin V binding in PI negative cells, which is representative of apoptosis (17).

Upon assessing the expression levels of proapoptotic proteins, FOXO3a gene (a tumor suppressor gene) and BAX gene (pro-apoptotic gene) were found to be significantly increased after extract treatment in breast cancer cells. This proves that *Fagonia cretica* shows a high anti-carcinogenic potential and could be of a great benefit substituting the conventional treatments for oral cancer (17).

In contrast, Waheed et al. reported that the cytotoxic effects of a steroidal saponin glycoside extracted from *Fagonia indica* were translated mainly as necrosis in MCF-7 breast cancer cells. These results were explained by lack of caspase-3 expression in the cells after treatment, which is regarded typical for apoptosis (34).

It is worth mentioning that the present results were limited to a specific cell line and selected time interval. Hence, using different OSCC cell lines and time intervals may change the final results. Within the limitations of the current study, the null hypothesis was rejected.

Thus, exploring the clinical uses of *Fagonia arabica* alone or in conjunction with already existing antitumorogenic medications in cases of oral cancer could be required. Further techniques are also needed to detect the molecular pathways behind cell cycle arrest and apoptosis such as western blot analysis and gene expression analysis using real-time PCR. Also, additional *in vitro* in combination with *in vivo* research would be recommended to support the results of the present research.

### CONCLUSIONS

*Fagonia arabica* can effectively inhibit the growth of OSCC cells. Also, it was associated with apoptosis and arresting the cell cycle of the cells, suggesting that it could be a potent anticancer agent.

### CONFLICT OF INTEREST

The authors declared that they have no conflicts of interest.

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