

Research Article

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Persicaline, an alkaloid from *Salvadora persica*, inhibits proliferation and induces apoptosis and cell-cycle arrest in MCF-7 cells

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Abstract: Cancer is the second largest cause of mortality worldwide. Many natural bioactive chemicals generated from plants have favorable impacts on health, including cancer chemoprevention, compared to their manufactured counterparts. Persicaline, a novel sulfur-containing imidazoline alkaloid derived from *Salvadora persica*, has been shown to display promising antioxidant activity. In this study, the antiproliferative activity of persicaline was tested against different cancer cells using (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay. The cell death mode and cell-cycle arrest were examined using flow cytometry analysis. In addition, the proapoptotic and molecular mechanism effects of persicaline against mammary MCF-7 cell line were explored. Furthermore, the impact of persicaline on apoptotic genes markers, generation of reactive oxygen species (ROS), and mitochondrial membrane potential were monitored. It was found that persicaline inhibits cell proliferation in a dose-dependent manner. Persicaline-treated MCF-7 cells also showed initiation of apoptotic events and G1 cell-cycle arrest. In addition, persicaline treatment led to an increase in ROS generation, Bax

and caspase upregulation while the Bcl-2 was downregulated. Hence, for the first time, this study showed that persicaline causes G1 phase arrest and apoptosis induction in MCF-7 cells. Increased proapoptotic genes and ROS levels were required for the antiproliferative and apoptotic effects of persicaline.

Keywords: apoptosis, *Salvadora persica*, reactive oxygen species, cell cycle

1 Introduction

Cancer is one of the leading causes of death in the world. The occurrence rate of cancer is globally growing due to spread of several risk factors including lifestyle behavior, overweight, smoking, physical inactivity, and aging [1]. Based on GLOBOCAN estimates, new cancer cases reached 19.3 million and around 10.0 million deaths occurred in 2020 worldwide [2]. Surgical intervention, radiotherapy, and chemotherapy are the most well-known choices in cancer treatments [3]. Due to the drastic side effects associated with these types of treatments, compounds derived from natural products have shown hopeful outcomes against numerous malignant tumors [4]. The use of compounds of natural origin for the treatment of various diseases have played a key role in drug discovery. These bioactive chemicals found in plants are what give them their medicinal value since they can prevent the occurrence of diseases through various processes [5–9]. Interestingly, several preclinical studies have shown that various dietary agents play a promising role in cancer treatment and prevention of cancer progression [10]. In the past few decades, many antitumor agents obtained from medicinal herbs have been characterized, developed, and introduced into clinical use. These include camptothecin derivatives, vinblastine, and paclitaxel (Taxol), which were purified from the

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Chinese *Camptotheca acuminata* Decne, *Catharanthus roseus*, and *Taxus brevifolia* Nutt, respectively [11,12]. In addition, several preclinical and clinical studies of promising anticancer agents of plant source are presently undergoing further investigation [13]. *Salvadora persica*, the synonym of the species called “miswak” or “Arak,” belongs to the *Salvadoraceae* family and is widely used as a natural toothbrush [14]. The inhibitory effect of *S. persica* against pathogenic bacteria has previously been reported [15,16], and extracts of *S. persica* showed inhibitory effects against oral squamous cell carcinoma [17]. Moreover, several reports had shown that *S. persica* has different phytoconstituents that display various pharmacological properties [18,19], including antiproliferative activity against a panel of cancer cell lines [20]. It has been found that different parts of *S. persica* are rich in alkaloids, phenols, tannins, and flavonoids [19,21]. Among these phytocomponents, alkaloids represent a good source for anticancer drugs [22,23]. Recently, it was found that ethanolic extract from *S. persica* fruits has a chemopreventive activity by suppressing 7,12 dimethylbenz[a]anthracene-induced mammary carcinogenesis. The fruit extract also prevented breast cancer cell proliferation, causing apoptosis and having anties-trogen and antioxidant properties [24]. Moreover, the prepared gold and copper nanoparticles from the fruit extract of *S. persica* have shown a promising anticancer property when applied to MCF-7 and MDA-MB-231 breast cancer cells [25]. In the current study, we demonstrated the potential cytotoxic and apoptotic effects of persicaline (Figure 1), a new alkaloid from *S. persica* roots that was initially isolated by Farag et al. [26]. This is the first report that we are aware of that demonstrates the growth inhibitory effect of persicaline in MCF-7 cells through apoptosis induction and G1-phase cell-cycle arrest.

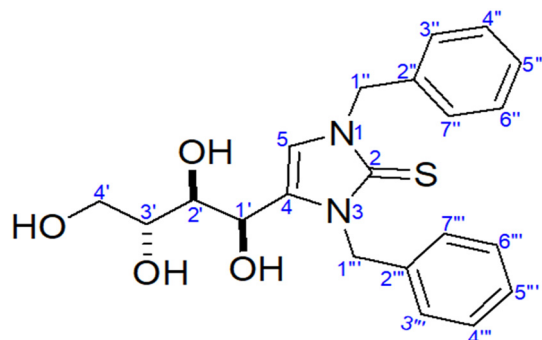


Figure 1: The chemical structure of persicaline, which was isolated from *S. persica*.

2 Materials and methods

2.1 Persicaline preparation

In the spring of 2015, *S. persica* L. roots were harvested from the garden of medicinal plants at the Pharmacognosy Department of King Saud University. Dr. Mohammed Yusuf verified the plant's authenticity, and voucher number (15616) was deposited in the department's herbarium. The air-dried roots of *S. persica* were extracted with alcohol, dried, and then subjected to solvent fractionation and acid–base treatment to extract the alkaloids. Petroleum ether and water were used to complete the solvent fractionation process. To get rid of the nonbasic material, the aqueous layer was agitated with chloroform and mixed with 0.2 N HCl for 16 h. Persicaline was produced by passing the chloroform fraction over a silica gel column using a CHCl_3 -MeOH gradient. The detailed isolation of the compound and its characterization have been clearly reported in the study of Farag et al. [26].

2.2 Cell culture

The breast (MCF-7), colon (LoVo), and HepG2 (Liver) cancer cell lines were obtained from the German Microorganisms and Cell Cultures Collection (DSMZ) (Braunschweig, Germany). Cells were grown in Dulbecco's Modified Eagle media (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin and streptomycin, and kept at 37°C in a humidified CO_2 incubator.

2.2.1 Measurement of cellular viability

2.2.1.1 Cell viability assay

The viability of breast (MCF-7), colon (LoVo), and liver (HepG2) cancer cells treated with persicaline was assessed using (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay as described elsewhere [27]. The cells were initially cultured in a 96-well plate at a density of 1×10^5 cells per well for 24 h. Thereafter, various concentrations of persicaline (40, 20, 10, 5 $\mu\text{g/mL}$) were added as well as dimethyl sulfoxide (DMSO) as vehicle for 24 h, and doxorubicin served as a positive control. Afterward, the MTT solution (10 μL of 5 mg/mL) was added to each well and incubated at 37°C for 4 h. Formazan product was dissolved by acidified isopropanol and the absorbance

of both treated and untreated wells was measured using a microplate reader (Biotek Elx-800, USA) at 570 nm. From the dose-dependent curve, the half inhibitory concentration (IC_{50}) was determined.

2.3 Flow cytometry studies

2.3.1 Cell-cycle analysis

Using flow cytometry, cell-cycle distribution analysis in untreated and persicaline-treated MCF-7 cells was carried out using Flow Cytomics FC 500 (Beckman Coulter, CA, USA) as described previously [27]. Cells grown on a 6-well cell culture plate were treated with both vehicle (DMSO) and persicaline at ($IC_{50} = 30 \mu\text{g/ml}$) concentrations. After 24 h, the MCF-7 cells were collected by trypsinization, washed in cold phosphate-buffered saline (PBS), and cell pellets were fixed in ice-cold 70% (v/v) ethanol. Thereafter, the collected cell pellets were stained with $100 \mu\text{g/mL}$ propidium iodide (PI) for 30 min in the dark. The cell percentage in various phases G0/G1, S, and G2/M was calculated using CXP software V. 3.0. (Beckman Coulter, CA, USA).

2.3.2 Apoptosis detection (Annexin V FITC/PI staining)

Apoptosis induction by persicaline was studied using the Annexin V & Dead Cell kit (Invitrogen, CA, USA) as described earlier [27]. MCF-7 cells were treated with persicaline at $30 \mu\text{g/ml}$ concentration or without (DMSO) for 24 h. Detached and adherent cells were collected, washed, and resuspended in $100 \mu\text{L}$ of Annexin V binding buffer. Annexin V-FITC and PI ($5 \mu\text{L}$ each) were added and incubated for 20 min. The events of stained cells (viable, early, and late apoptotic) were calculated using Cytomics FC 500 flow cytometry (Beckman Coulter, CA, USA). Data analysis was performed using CXP software V. 3.0. (Beckman Coulter, CA, USA).

2.3.3 Cellular reactive oxygen species (ROS)

The effect of persicaline as an inducer of the ROS production was monitored in MCF-7 cells by activation of the acetylated forms of 2',7'-dichlorofluorescein dye (DCFH-DA) as described previously [28]. MCF-7 cells were seeded in a 6-well plate (1×10^5 cells per well) for 24 h and treated with persicaline or in the presence of vehicle

(DMSO) for 24 h. On the day of detection, cells were collected, washed with $1 \times$ PBS, and $10 \mu\text{M}$ of DCFH-DA was added and further incubated at 37°C for 30 min in the dark. Thereafter, cells were washed ($2 \times$) with PBS at a higher speed (500 g). The fluorescence intensity was analyzed by FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA) with excitation of the blue laser at 488 nm where emission was detected at 535 nm.

2.3.4 Mitochondrial membrane potential (MMP)

The effect of persicaline as a disruptor of the mitochondrial membrane of the MCF-7 cells was measured using fluorescent Rhodamine 123 (Rh123) as described previously [19]. Following treatment, cells were harvested and washed by PBS ($1 \times$). Rh123 ($2 \mu\text{M}$) was added to each well and incubated in the dark at 37°C for 40 min. Next, cells were washed ($2 \times$) with $1 \times$ PBS at a high speed (500 g) and fluorescence intensity was analyzed using FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA) by the excitation of the blue laser at 488 nm where emission was detected at 535 nm.

2.4 Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from MCF-7 cells cultured in a 6-well plate (1×10^5 cells per well) from both vehicle- and persicaline-treated ($IC_{50} = 30 \mu\text{g/mL}$) cells. One microgram of extracted RNA was reverse transcribed to cDNA by Reverse Transcription System (Promega Inc., Madison, WI, USA) according to the manufacturer's guidelines. Thereafter, the expression of five genes (P53, Bax, Bcl-2, Cas-9, and Cas-3) was measured as previously described [29] using specific primers for each gene. The levels of target genes expression were normalized to GAPDH, and the fold change in the relative expression of each gene was calculated using the comparative Ct ($2^{-\Delta\Delta C_T}$) method.

2.5 Statistical analysis

Origin Pro version 8.5 (Massachusetts, USA) was used for statistical data analysis. The data were analyzed via a *t*-test, all experiments were performed in triplicates, and the results were considered significant at $*p < 0.05$ and $**p < 0.01$.

3 Results

3.1 Persicaline decreased cancer cell proliferation

With the goal of finding new compounds that can prevent the proliferation of cancer cells, the cytotoxic effect of a new alkaloid (persicaline) isolated from *S. persica* roots was explored. We first tested the impact of persicaline on different cancer cells including breast (MCF-7), colon (LoVo), and liver (HepG2) cancer cells by measuring the survival of these cells at different concentrations of persicaline (0–40 µg/ml). The MTT assay was executed after treating the cells with increasing concentrations of persicaline. We found that exposure of cancer cells to persicaline resulted in the decrease of cell growth with increasing concentration of persicaline (Figure 2). By determining IC_{50} values (Table 1), which is the concentration that causes 50% inhibition, it seems that MCF-7 exhibited the most sensitivity to persicaline with an IC_{50} of 30.5 µg/mL in comparison to other tested cells (Table 1). Therefore, MCF-7 cells were chosen to complete further experiments.

3.2 Induction G1 arrest in the persicaline MCF-7-treated cells

As the inhibition of cell proliferation is associated with the regulation of cell-cycle progression, the trigger of cell-cycle arrest by persicaline was evaluated. The DNA content of both treated and non-treated cells was measured to show the phase of the cell cycle that persicaline influenced. The results displayed in Figure 3 show that the concentration of persicaline at IC_{50} caused an obvious G1 arrest. MCF-7 cells' population of G1 increased from $65.96 \pm 1.2\%$ in control to $78.51 \pm 1.5\%$ ($p < 0.01$) in persicaline-treated cells. Subsequently, there was a concomitant decrease in S and G2/M population from 14.64 ± 0.8 and $18.98 \pm 0.5\%$ in control to $7.09 \pm 0.5\%$ and $9.76 \pm 0.7\%$, respectively, for IC_{50} -treated cells. These results clearly show that persicaline prevented MCF-7 cell growth through cell-cycle arrest in the G1 phase.

3.3 Persicaline induces apoptosis in the MCF-7 cells

To establish the cell death mode initiated by persicaline, untreated and treated MCF-7 cells were subjected to flow cytometry analysis. FITC-Annexin V with PI procedure which is specifically designed for the identification of

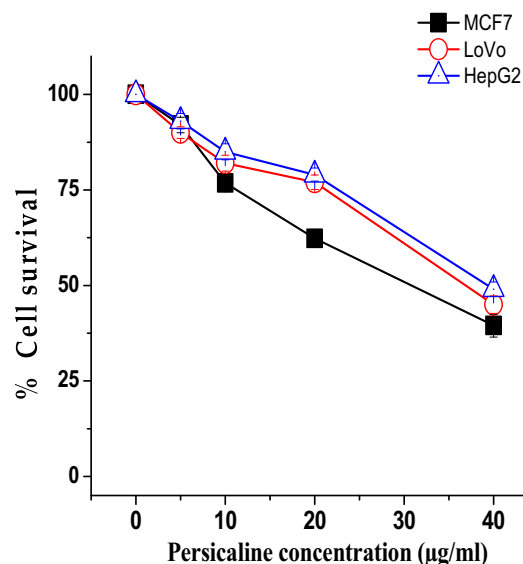


Figure 2: Cytotoxic activity of persicaline on cancer cells estimated by MTT assay. Different concentrations of persicaline were incubated with cells, and dimethyl sulfoxide served as the control. Following 24 h of treatment, cell cytotoxic was assessed by MTT assay. Data represent mean $\pm$ SD of three independent experiments.

apoptotic and necrotic cells was employed. Upon apoptosis induction, the phosphatidylserine on the inner leaflet of apoptotic cells is transferred to the outer leaflet which can bind to the FITC dye and measured using flow cytometry. Therefore, FITC-Annexin V/PI staining assay was carried out to examine the ability of persicaline to induce apoptosis. We found that persicaline at IC_{50} induced a significant increase in the late apoptosis levels (increase from 3.4% in untreated cells to 13.8% after 24 h of treatment, $p < 0.01$). Together, the sum of early and late apoptosis had increased from 3.8% in the control to 16.2% in treated cells ($p < 0.01$), which further supported apoptotic induction by persicaline in MCF-7 cells (Figure 4).

3.4 Persicaline alters the expression of proapoptotic and antiapoptotic genes in MCF-7 cells

In order to further elucidate the apoptotic mode of cell death, the activation of apoptosis-related genes (P53,

Table 1: IC_{50} values of persicaline in different cancer cells

Treatments	Cell lines and IC_{50} (µg/mL)		
	MCF-7	LoVo	HepG2
Persicaline	30.5 ± 1.5	36.5 ± 1.2	39 ± 0.5
Doxorubicin	1.2 ± 0.2	1.6 ± 0.3	1.5 ± 0.4

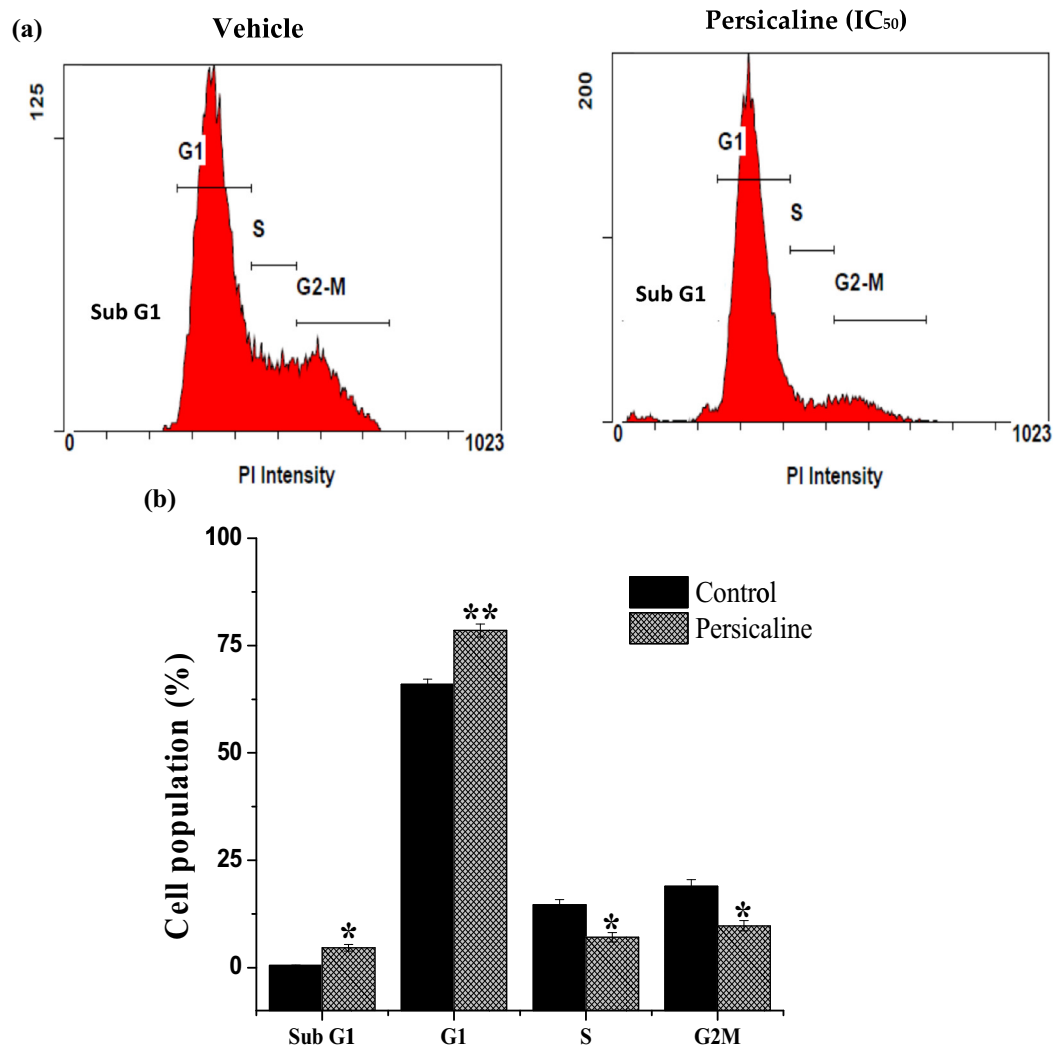


Figure 3: Persicaline-induced G1 cell-cycle arrest. MCF-7 cells were non-treated (dimethyl sulfoxide, control) or treated with IC₅₀ (30 µg/mL) and stained with PI for 24 h. (a) Histograms of DNA content in both treated and untreated cells. (b) Cell cycle phase ratio. Data are shown as mean ± SD, ** $p < 0.01$ as compared to corresponding control.

Bax, Bcl-2, caspase-3, and caspase-9) was estimated to establish the cell-death pathway induced by persicaline. We examined persicaline-induced cell death by using RT-PCR which quantified apoptotic genes in MCF-7 cells treated with 30.5 µg/mL for 24 h. We found that persicaline induces an increase in the level of P53 and Bax ($p < 0.01$), as well as a decreased level of Bcl-2 gene, as displayed in Figure 5. To clarify the initiation of apoptosis in treated MCF-7 cells, the gene expression profiles of caspases-9 and -3 were quantified after persicaline treatment at IC₅₀. As shown in Figure 5, there was a significant increase in the percentages of these genes in apoptotic cells, in comparison to control groups ($p < 0.01$).

3.5 Implication of cellular MMP and ROS generation in persicaline-induced apoptosis of MCF-7 cells

Next, we explored whether ROS was involved in apoptotic cell death mediated by persicaline. ROS changes in MCF-7 cells exposed to persicaline were assessed using the cell-permeant dye chloromethyl-2',7'-dichlorofluorescein diacetate (CM-H2DCFDA) for 24 h using flow cytometry. As displayed in Figure 6a, cells treated with persicaline (green) showed increases in the concentration of ROS compared to the untreated control (purple). These increment in ROS level after persicaline treatments revealed

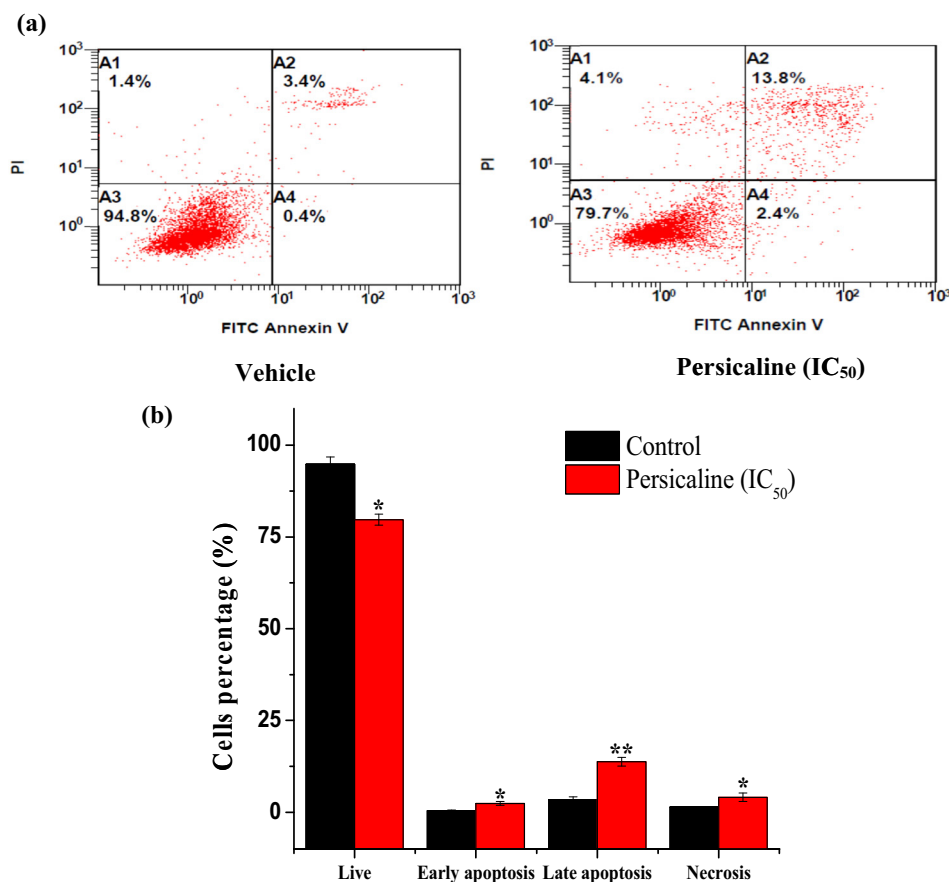


Figure 4: Persicaline-induced cell apoptosis of MCF-7 evaluated by Annexin V-FITC/PI double staining assay. (a) Annexin V/PI staining representative dot plots: A4 (early apoptosis), A3 (live), A2 (late apoptosis), and A1 (necrosis). (b) Percentage column bar graph of total cell death. Data are shown as mean $\pm$ SD, ** $p < 0.01$ as compared to corresponding control.

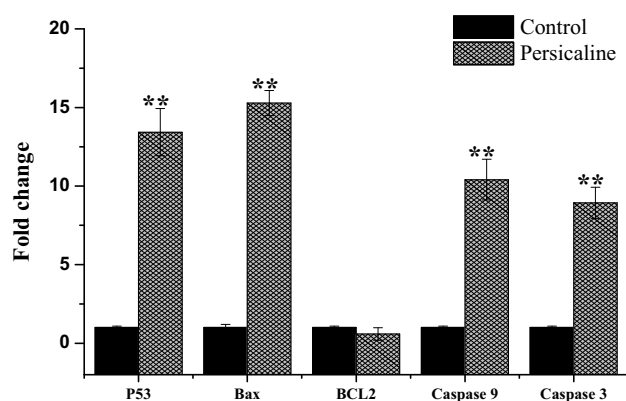


Figure 5: Effects of persicaline on the activity of apoptosis-related genes in MCF-7 cells using qRT-PCR. Cells were treated with vehicle (dimethyl sulfoxide) or indicated concentration of persicaline (30 $\mu\text{g/mL}$) for 24 h. Results are expressed as mean $\pm$ SD ($n = 3$). ** $p < 0.01$ compares the treated cell with control cell.

that apoptosis was mediated via the apoptotic pathway. MMP is also decreased as a result of various chemotherapy agents during apoptosis. Therefore, Rh123 was used to

monitor MMP loss. We found that MCF-7 cells exposed to persicaline had hyperpolarized membrane potentials indicating that persicaline can trigger the loss of cell viability and apoptosis by enhancing intracellular ROS oxidative stress (Figure 6b).

4 Discussion

Around the globe, cancer is still one of the leading causes of morbidity and mortality. Natural products, including several plant species, are becoming progressively popular and are utilized widely for their promising therapeutic effects and fewer side effects. Moreover, several scientific reports regarding the authenticity of these traditional products have confirmed their potent anticancer effects [23]. Many biologically active phytoconstituents, including sulfur compounds, flavonoids, alkaloids, steroids, terpenoids, and fatty acids, are known to be present in *S. persica*. Persicaline, salvadoreua, salvadoraside,

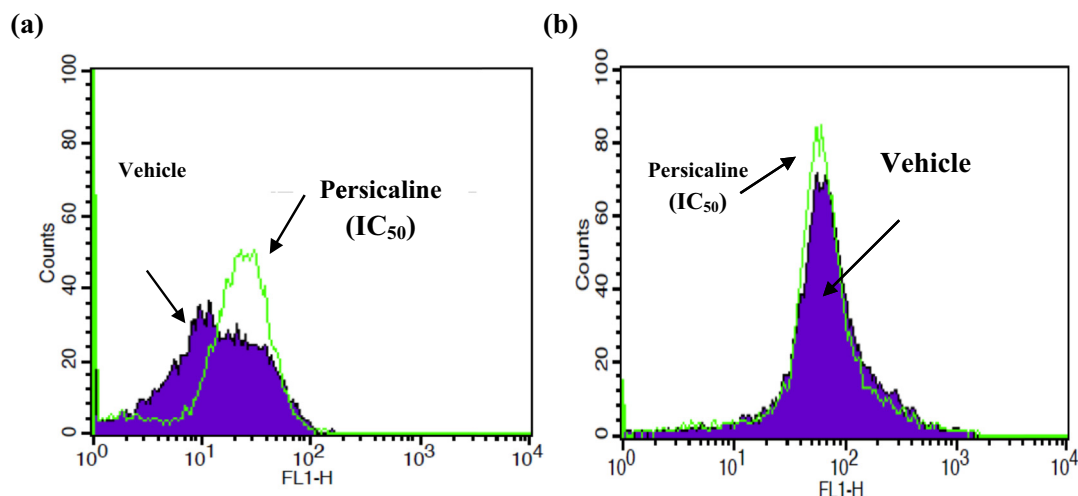


Figure 6: Persicaline-mediated intracellular ROS and MMP in MCF-7. (a) ROS generation in MCF-7 cell control (dimethyl sulfoxide) and after persicaline treatment acquired from flow cytometry experiments. (b) Histogram overlay showing a mitochondrial membrane depolarization of treated and untreated MCF-7 cells.

and benzyl isothiocyanate are among these vital phyto-constituents that have antiviral, antioxidant, and antimicrobial effects [30]. Here, we reported for the first time that persicaline, an alkaloid isolated from the root of *S. persica*, had cytotoxic activity and induced apoptotic cell death in MCF-7 breast cancer cells. Previously, several studies reported the cytotoxic effects of *S. persica* extracts against different tumor cell lines [19,20,31]. A new acylglyceride derivative (salvastearolide) was isolated from *S. persica* seeds and it was reported to have a weak cytotoxic activity against MCF-7 cells [32]. In our study, we found that persicaline inhibited the proliferation of various cancer cells and the MCF-7 cells were more sensitive to persicaline. Furthermore, we had investigated the molecular mechanism underlying the antiproliferative effect of persicaline against MCF-7 cells *in vitro*. Apoptosis and cell cycle are well-known pathways that are highly regulated exclusively for cell proliferation and the removal of unwanted cells in physiological conditions. In addition, most chemotherapeutic treatments attempt to initiate apoptosis in cancer cells [33,34]. Therefore, new chemical substances that can induce apoptosis or inhibit cell-cycle progression might be valuable for cancer treatment. In this study, the cytotoxic effect of persicaline caused a severe damage to the treated cells as evidenced by G1 arrest and triggered apoptotic cell death as indicated by exposure of phosphatidylserine in Annexin V-FITC/PI assay.

Similar effects were also observed by Hamza et al., who demonstrated that the fruit extract of *S. persica* stimulated apoptosis and halted the cell-cycle progression of MCF-7 cells [24].

It is well known that apoptosis can happen either by the extrinsic pathway or the intrinsic mitochondria-mediated pathway. The latter is associated with permeabilization of the mitochondrial outer membrane, gene expression alteration of the antiapoptotic (Bcl2) and proapoptotic (Bax), and activation of different caspases [35,36], which were detected in the present study. Therefore, we suggest that persicaline mediated apoptosis via a mitochondrial pathway. In this study, a highly remarkable change in the expression of Bax compared to Bcl-2 gene was observed. This implies that a cell with a higher Bax to Bcl-2 ratio will be more susceptible to apoptosis initiators [37]. In addition, when the level of Bax increases this leads to the activation of initiator caspase-9 that then activates executioner caspase-3, which is a characteristic marker of apoptosis execution [38,39]. The involvement of caspase-3 and its upstream initiator caspase-9 was also determined. Here, it is evident that persicaline significantly upregulated the gene expression of caspase-9 and -3, indicating that persicaline mediated apoptosis in MCF-7 human breast cancer cells via mitochondrial pathway.

To further understand the mechanism underlying persicaline-induced apoptosis in MCF-7 cells, ROS generation and loss of membrane potential were studied using flow cytometry through DCFH-DA and Rh123 fluorescence dyes. In fact, elevated ROS levels along with MMP loss are considered among the important events in the progression of mitochondrial-mediated apoptosis. Additionally, it is well known that several chemotherapeutic agents mediate their action through MMP loss and increased ROS production [40,41]. Therefore, the ability

of persicaline to enhance intracellular ROS generations which can consequently alter the MMP was investigated. We observed that persicaline induces ROS generation and disrupts MMP in MCF-7 cells which suggests the involvement of MMP and ROS in apoptotic events mediated by persicaline. In their original work [26], persicaline showed promising antioxidant activity. In this study, we found that persicaline caused intracellular ROS increment in MCF-7 cells, which could be responsible for apoptotic cell death. This finding is consistent with numerous studies for well-known antioxidant compounds such as quercetin, gallic acids, and vitamin C, which mediated apoptosis in MCF-7 cells through elevated ROS generation [42–45].

5 Conclusions

In summary, our findings provide the first illustration of a potential role for persicaline in inhibiting MCF-7 cell growth through G1 phase arrest and induction of apoptosis. We found that the generation of ROS and upregulation of proapoptotic genes by persicaline may be considered as critical mediators in inducing the intrinsic apoptotic pathway. Further exploration is needed to determine the detailed molecular mechanisms for the potential anticancer activity of persicaline in different cancer lines. The main shortfall of this study is that the compound was not tested on an animal model. Therefore, *in vivo* study involving animal model systems for breast cancer is required to confirm its potential activity in tumor growth inhibition compared to control animals.

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Conflict of interest: All authors declare that there is no conflict of interest regarding the publication of this article.

Ethical approval: The conducted research is not related to either human or animal use.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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