Pharmacogn. Mag.
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ORIGINAL ARTICLE

Anti-Cancer Activity of Ethanolic Leaf Extract of *Salvia officinalis* against Oral Squamous Carcinoma Cells *in vitro* via Caspase Mediated Mitochondrial Apoptosis

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Submitted: 15-Mar-2020 Revised: 05-May-2020 Accepted: 15-Jul-2020 Published: 30-Nov-2020

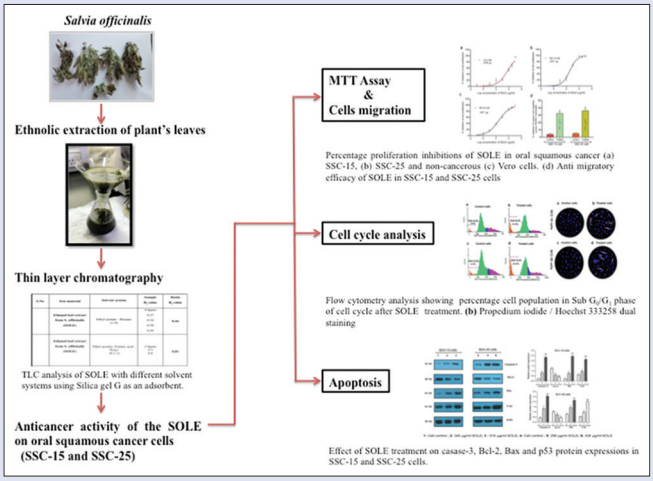
ABSTRACT

Aim: Need for novel agents that fight oral squamous cancer is on constant demand. The current study aims in the identification of active phytochemical ingredients from *Salvia officinalis* leaf extract (SOLE) and evaluation for anticancer properties in oral squamous carcinoma cells. **Materials and Methods:** Soxhlet method was used for SOLE extraction. Phytochemical tests and thin-layer chromatography (TLC) were performed for active compounds identification. Oral squamous cancer cells (SSC-15 and SSC-25) were used to assess anticancer efficacy. MTT analysis was utilized for the viability of cells. The utilization of flow cytometry was done to assess the changes in cell cycle and apoptosis induction. Western Blotting method was used to analyze the expressions of apoptotic protein. **Results:** Preliminary phytochemical screening showed the presence of sterols, flavonoids, and tannins. TLC study revealed the presence of rutin in SOLE. The extract inhibited cell proliferation of SSC-15 and SSC-25 cells with GI_{50} values of 340.7 μg/ml and 287.7 μg/ml, respectively. SOLE inhibited the migration of these cells across the endothelial membrane and induced nuclear fragmentation in cancer cells. Analysis of the cell cycle revealed SOLE to increase the sub G_0/G_1 population in SSC-15 and SSC-25 cells. SOLE increased both early and late phase apoptosis in both oral squamous cancer cell lines. Apoptotic markers such as caspase-3, Bax and P-53 were found to be dose-dependently increased with SOLE treatment in both the tested cell lines, while the anti-apoptotic Bcl-2 protein was decreased. **Conclusion:** In summary, SOLE demonstrated excellent anticancer and anti-migration efficacy in oral squamous cancer cells through caspase-mediated mitochondrial apoptosis. More than one compound might be responsible for the activity, which deserves further research.

Key words: Anticancer, apoptosis, oral squamous carcinoma, phytochemicals, *Salvia officinalis*

SUMMARY

- *Salvia officinalis* leaf extract (SOLE) had excellent anticancer and anti-metastasis activity against the oral squamous cancer cells at a reasonably safer therapeutic window.
- The mechanism of SOLE action was driven caspase-mediated mitochondrial apoptosis in the cancer cells.
- More than one active compound present in SOLE could have been responsible for the anticancer effects, which needs further elucidations.



Abbreviations used: SOLE: *Salvia officinalis* leaf extract; TLC: Thin layer chromatography; OSCC: oral squamous cell carcinoma; ATCC: American Type Culture Collection; HGF: Hepatocyte growth factor; ECGS: Endothelial cell growth supplement; PBS: Phosphate Buffer Solution; SDS-PAGE: Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis; DNA: Deoxyribonucleic acid.

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DOI: 10.4103/pm.pm_90_20

Access this article online
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INTRODUCTION

Cancers of oral origin account for 2%–4% of total cancer globally, with an average annual diagnosis of 300,000.[1] The incidence rate of oral squamous cell carcinoma (OSCC) proves to be constantly increasing in adults within the age range of 18–44, where the 5-year survival rate accounts for 40%–50% of total diagnosed cases.[2] Although easy to diagnose, reports indicate OSCC is usually diagnosed at advanced stages where treatments become more difficult.[3] Need for novel agents that

Cite this article as: Rajagopalan P, Wahab S, Dera AA, Chandramoorthy HC, Irfan S, Patel AA, *et al.* Anti-cancer activity of ethanolic leaf extract of Salvia officinalis against oral squamous carcinoma cells *in vitro* via caspase mediated mitochondrial apoptosis. Phcog Mag 2020;16:S546-52.

fight against OSCC is, therefore on constant demand and rewarding. Kingdom of Saudi Arabia is well-heeled with a diversity of plants, consisting of varied species of herbs, shrubs, and trees.[4] Plants are one of the major sources of drugs due to their bioactive chemical constituents, which play a vital role to cure many ailments.[5] It is very essential to make an effort toward the standardization of the plant material to be used as medicine for the reasons of therapeutic efficacy with uncompromised safety.[6]

Salvia officinalis belongs to the family Lamiaceae. Though native to the Mediterranean region, this plant is identified in many regions throughout the world for medicinal and culinary uses.[7] Conventionally, *S. officinalis* is utilized for the treatment of diarrhea, dyspepsia, colic, asthma, constipation, catarrh, bronchosis, cough, dyspnea, and depressive episodes.[8] The biological activities of this plant thoroughly studied and is delineated to use as a treatment for hyperlipidemia and Alzheimer's disease, liver diseases, gastrointestinal disorders, constipation, diabetes, as well as in gastric problems and it also affliction of the gum, mouth, and skin.[9] To the best of our knowledge, this plant extract has not been evaluated for any anti-cancer activity. This study, therefore, aims in the screening of active phytochemical ingredients from *Salvia officinalis* leaf extract (SOLE) and its biological assessment against OSCC cells *in vitro*.

MATERIALS AND METHODS

Plant's sample

Leaves of plant *S. officinalis* were collected from the Asir region of Saudi Arabia. The plant was identified and authenticated by Dr. Mahadevan Nanjaian of Department of Pharmacognosy College of Pharmacy, King Khalid University. The leaves were separated and washed to detach the superficial pollutants and then air-dried beneath the shade in room temperature. The shade dried leaves were coarsely powdered with the help of Grinder and stored in an airtight container.

Chemicals and the reagents utilized

All reagents and chemicals utilized were procured from Sigma Company (At St. Louis, MO, USA). The solvents entirely used under the research were of the analytical grade. SCC-25, SCC-15, Vero lastly HUVEC cell lines were acquired from the American Type Culture Collection (ATCC). The kit for annexin V was procured from e-Bioscience company of the USA. Other assay kits, notably trans-endothelial migration assay, Recombinant Human Hepatocyte growth factor (HGF), and Guava cell cycle reagent was obtained from the Millipore Corp., USA. Capase 3, Bcl-L, Bcl-2, and β-actin antibodies were bought from Santacruz Biotechnology, situated in Santacruz, CA, USA.

Plant extract and phytochemical analysis

Successive ethanolic extraction of the parched leaves powder substance was done with the help of the Soxhlet apparatus. Filtration of the extracts was done under hot conditions and concentrated under diminished and controlled pressure. Calculations were done for the practical and percentage products of the extracts. Then, the concentrated ethanolic decoction was subjected to qualitative chemical analysis for the recognition of various active ingredients.[10]

Thin layer chromatography

The study was carried out using precoated TLC plates by using one-way ascending technique according to the method described elsewhere.[11] The extract was dissolved in methanol, and capillary tubes were used to apply the dissolved samples on the plates uniformly and were allowed to dry. The plates were developed in a chromatographic tank using the different solvent systems. The plates were dried and visualized under normal light and iodine fumes, after which they were subjected to heating at 105°C for a period of 5–10 min. The retention factor R_f for each active compound was calculated using the formula as described elsewhere.[12]

Cell culture

SCC 25 and SCC 15 were grown in a special medium that was a mixture of equal ratio containing Dulbecco's reoriented Eagle's medium and also another medium consisting of Ham's F12 consisting of 15 mM of HEPES, 0.5 mM of sodium pyruvate, 2.5 mM of L-glutamine, 1.2 g/L sodium bicarbonate, where 10% bovine fetus' serum and 400 ng/ml of hydrocortisone were added. Growth of HUVEC cells done in F12K medium, which contained 0.05 mg/ml of a mixture of endothelial cell growth supplement and the other being 0.1 mg/ml of heparin. Penicillin (100 U/ml), streptomycin (100 U/ml), and FBS (10%) were contained in all the growth medium. 5% CO_2 incubator was used to incubate all the cells at a temperature of 37°C for optimum growing situations. Analyses were accomplished at 70% convergence of growth of cells.

Analysis for the proliferation of the cells

Analysis for the proliferation was carried out as elucidate by Mosmann[13] with some modifications. Regular growth media was utilized to incubate 5000 cells/well overnight and 50 µl of specific concentrations from SOLE and DMSO blank was added to it. Incubation of these particular cells was done at a temperature of 37°C and 5% CO_2 for a period of 72 h. 5 mg/ml of MTT contained in 25 µl was included and incubated for a 4 h. Succeeding aspirations of the media, constituents were deliquesced in DMSO (containing 250 µl) and read the absorbance at 560 nm, and 640 nm was utilized as the reference wavelength. Ensuing subtracting of initial day values, the percentage inhibition of proliferation of the cells was figured out, and outcomes were scrutinized utilizing Graph pad Prism 6.0 software (from La Jolla, USA).

Assay for transendothelial cell migration

The analysis was implemented by utilizing QCM™ Tumor Cell Trans-Endothelial Migration Assay – Colorimetric kit from Millipore in accordance with the manufacturer's instructions. Briefly, 1×10^5 cells from HUVEC were developed on migration inserts before confluent. 1×10^5 of SCC-25/SCC-15 cells, which were starved overnight in a media-free from serum, were replaced with the media in the inserts together with SOLE treatments at desired concentrations. The inserts were further shifted to other wells having tumor cell growth media (free from serum), which contained ± 25 ng/ml HGF at eventual concentration and the cancer cells were allowed to migrate across the membrane for 12 h in CO_2 incubator, stained and eluted. Eluent was transported to a stain quantification plate containing 96-wells, and the absorbance was perused at a wavelength of 540–570 nm.

Cell cycle analysis

SCC 25 and SCC 15 cells were subjected to seeding at the particular density of 0.5×10^6 cells per well inside a plate having 6-wells and then incubated with desired concentrations of SOLE for 72 h at a temperature of 37°C within 5% CO_2 incubator. The analysis was performed with reagent from Guava® Cell Cycle in accordance with the manufacturer's directives. Ten thousand events were obtained with the help of a Guava easyCyte™ flow cytometer, and the data scrutinized with Express Pro Software procured from Millipore, USA. Calculation of percentage cell population at various stages of the cell cycle was done.

Fluorescence staining

Propedium iodide/Hoechst 333258 dual staining was done as recounted elsewhere[14] having a minor change. Growth of SCC-25 and SCC-15 cells were performed on coverslips allocated in Petri dishes, having a concentration of one million cells/plate. In all cases, sterilized Petri-plate and coverslips were utilized. Incubation of the plates was done for a duration of 48 h to acquire a sheet of cells and subsequently treated with the appropriate concentration of SOLE. Washing of the cells was done after 24 h. Utilizing cold PBS and the subsequent cell suspension was being produced with the media. 2 µl of combined dye-containing 100 mg/ml of propidium iodide and 100 mg/ml of Hoechst 333258 was appended to 20 ml of the cell suspension and then relocation of 5 ml of the stained solution to a glass slide for instantaneous analysis was done utilizing a fluorescence microscope (Nikon, Japan).

Analysis of annexin V

The analysis was carried out utilizing Annexin V detection kit procured from e-Biosciences, USA in accordance with the manufacturer's directives as follows. In brief, the growth of 0.5×10^6 SCC cells was done in 6 well plates and treated with appropriate concentrations of SOLE and then subjected to incubation in CO_2 incubator for a period of 72 h. The cells were then harvested, washed with buffer from kit and subjected to incubation with 0.25 µg/ml of Annexin V reagent for 15 min, washed once more and re-suspended in kit buffer containing 0.5 µg/ml of propedium iodide. A guava easyCyte™ flow cytometer was utilized to procure ten thousand events. Analysis of the data was accomplished with InCyte software of Millipore Company, USA. Differentiations of the healthy cells were done with various phases of apoptosis using a quadric-plot graph. Analysis of the data was performed utilizing GraphPad Prism software-6.0 version (La Jolla, CA, USA).

Procedure of Western immunoblotting (Pro and anti-apoptotic protein signaling)

The western blotting technique was carried out as elucidated elsewhere,[15] with minor alterations alteration. SCC-15 and SCC-25 cells were treated utilizing various concentrations of SOLE for 48 h and lysed utilizing lysis buffer. Estimation of total protein concentration was done by Coomassie plus Protein Assay Reagent kit (from Pierce; Rockford, IL, USA), and SDS-PAGE was utilized to separate 20–40 µg protein from the cell lysate, which was subsequently transferred to Nitrocellulose membrane. The addition of secondary antibodies was done after probing with appropriate primary antibodies. Then, HRP was added and the membrane was subsequently stripped, succeeding an incubation period of 30 min. Quantification of the bands was done utilizing Image J (Ver. 1.46, NIH, Betheda, Maryland, USA) and then normalized to actin to β-actin (1:5000).

Statistical analysis

The performance of all experiments was done in triplicates and consequences expressed as the mean ± standard deviation. GraphPad Prism 6.0 (from La Jolla, USA) was availed to carry out statistical analyses. Calculation of GI_{50} values was accomplished utilizing a nonlinear regression fit model with a variable slope and accordingly plotted. The differences between the two groups were analyzed using the two-tailed Student's *t*-test and $P < 0.05$ (*) were considered statistically significant.

RESULTS

Phytochemical and thin-layer chromatography analysis of *Salvia officinalis* leaf extract

We initially performed preliminary phytochemical screening to enumerate the active constituents, if any, present in SOLE. Out of the tested constituents, SOLE showed the presence of carbohydrates, sterols, flavonoids, and tannins, while alkaloids and glycosides were absent [Table 1]. Next, TLC studies of SOLE were carried out in two different solvents at room temperature using Silica gel as an adsorbent. As shown in Table 2, both solvent systems confirmed the presence of rutin as one ssof the active ingredients in SOLE.

Salvia officinalis leaf extract exhibited anti-proliferative effects and inhibited trans-endothelial migration of oral squamous carcinoma cells

Before the cancer cells, SOLE was tested for cytotoxic effects in the normal, non-cancerous epithelial (Vero) cells. The extract showed a GI_{50} value of 5708 µg/ml [Figure 1a] in the cell viability assay. Sole inhibited the proliferation of SSC-15 cell with GI_{50} value of 340.7 µg/ml [Figure 1b]. The effect was SOLE on SSC-25 cells was even improved, with a GI_{50} value of 287.7 µg/ml [Figure 1c]. We used the nearest GI_{50} value of 340 µg/ml and 290 µg/ml of SOLE in SSC-15 and SSC-25 cells, respectively, for further assays. When tested for the anti-migration potency, SOLE inhibited the migration of SSC-15 cells by 32.2% and SSC-25 cells by 36.03% across the HUVEC-trans membrane under the influence of the chemoattractant [Figure 1d].

Table 1: Phytochemical constituents identified in *Salvia officinalis* leaf extract by different methods

Tested constituent	Test method	Result
Sterols	Salkowaski test	Positive
	Libermann-burchards test	Positive
Alkaloids	Dragandroff test	Negative
	Wagners test	Negative
	Mayers test	Negative
Glycosides	Bontrager's test	Negative
Tannins	Ferric chloride test	Positive
Flavonoids	Shinoda test	Positive
Carbohydrates	Fehling test	Positive
	Molish test	Positive
	Barford's test	Positive

Table 2: Thin-layer chromatography analysis of *Salvia officinalis* leaf extract with different solvent systems using Silica gel as an adsorbent

Test material	Solvent system	Sample R_f value	Rutin R_f value
Ethanol leaf extract from *S. officinalis* (SOLE)	Ethyl acetate: Hexane (1:9)	4 spots	0.44
		0.27	
		0.34	
		0.38	
		0.44	
Ethanol leaf extract from *S. officinalis* (SOLE)	Ethyl acetate: formic acid: water (8:1:1)	2 spots	0.81
		0.9	
		0.8	

S. officinalis: *Salvia officinalis*; SOLE: *Salvia officinalis* leaf extract

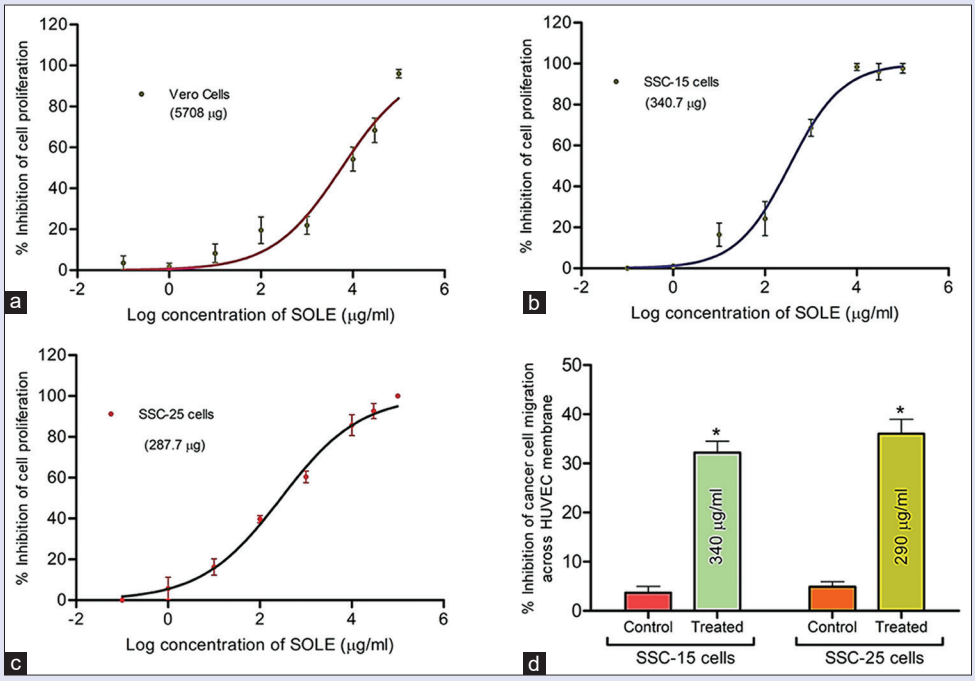

Figure 1: (a) Percentage proliferation inhibitions of SOLE in oral squamous cancer (a) SSC-15, (b) SSC-25 and normal, noncancerous (c) Vero cells. (d) Anti migratory efficacy of SOLE in SSC-15 and SSC-25 cells across the HUVEC membrane under influence of 25 ng/ml HGF which served as chemo-attractant. Results expressed as mean ± standard deviation from three independent experiments in duplicates. *$P \leq 0.05$ significant compared to untreated control. SOLE: *Salvia officinalis* leaf extract

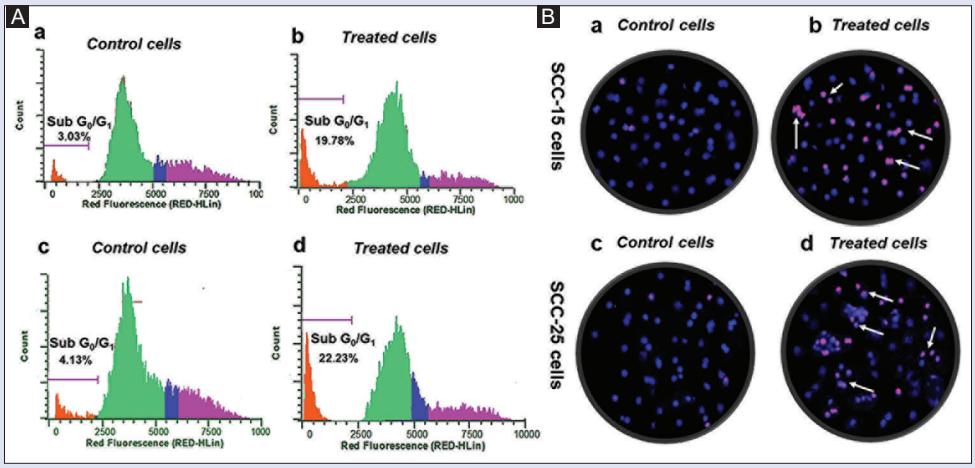

Figure 2: (A) Flow cytometry analysis of oral squamous carcinoma cells showing percentage cell population in Sub G_0/G_1 phase of the cell cycle with respect to control after respective GI_{50} dose-SOLE treatments in SSC-15 and SSC-25 cells for 72 h. Representative histograms from several repeats of the experiment are shown. (B) Propidium iodide/Hoechst 333258 dual staining of oral squamous carcinoma cells after respective GI_{50} dose SOLE treatments for 48 h. Pink color spots indicate condensed/fragmented nuclei bound with fluorescent stain. Microscopic magnification at ×200. SOLE: *Salvia officinalis* leaf extract

Salvia officinalis leaf extract interfered with oral squamous cancer cell cycle and induced nuclear fragmentation

To substantiate the observed anti-proliferative efficacy of SOLE, we conducted the cell cycle analysis in both SSC-15 and SSC-25 cells [Figure 2A]. As observed in Figure 2A a-b, treatment of 340 µg/ml SOLE increased the sub G_0/G_1 population to 19.78% when compared to its respective control of 3.03%. Similarly, when treated with 290 µg/ml SOLE, an increase from 4.13% to 22.23% in the sub G_0/G_1 population of the cell cycle from control to treatment was observed in SSC-25 cells [Figure 2A c-d]. To augment these observations of sub G_0/G_1 increase, we carried out nuclear staining analysis in the oral squamous carcinoma cells post-SOLE treatments. Both SSC-15 and SSC-25 cells showed positivity for nuclear fragmentation in the dual staining assay after 340 µg/ml and 290 µg/ml SOLE treatments, respectively [Figure 2B a-d].

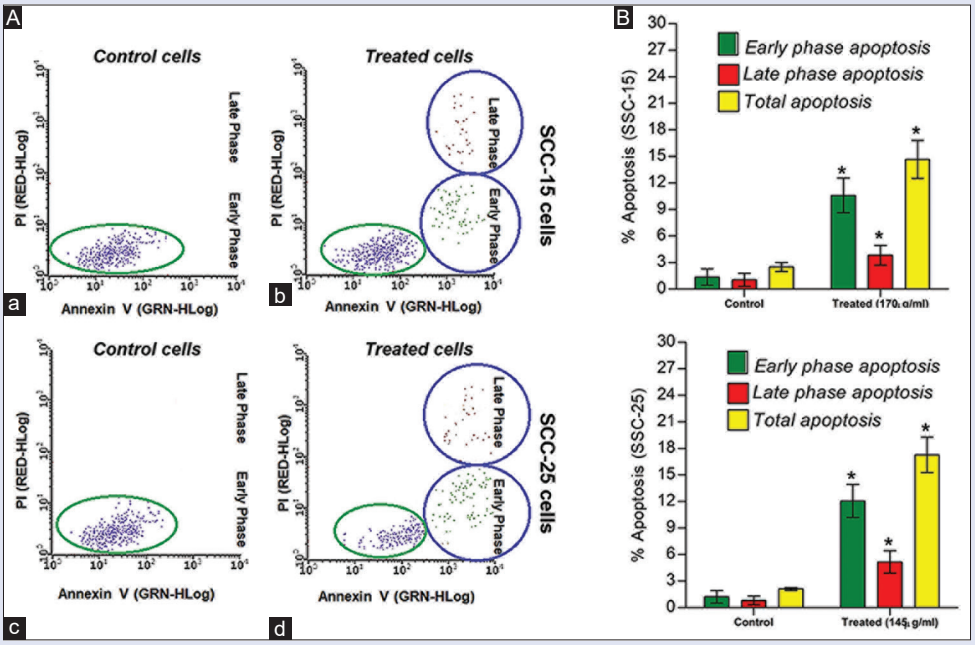

Figure 3: (A) Flow cytometry representations of apoptotic cells by Annexin V staining in SOLE treated SSC-15 and SSC-25 cells. All experiments were performed thrice, and representative plots are shown. (B) Dose-dependent increase of early phase, late phase and total apoptotic cells in SSC-15 and SSC-25 cell lines on treatment with respective GI_{50} doses of SOLE. Results are expressed as mean ± standard deviation. Significant $P \leq 0.05$ compared to * control. SOLE: *Salvia officinalis* leaf extract

Apoptosis inducing efficacy of *Salvia officinalis* leaf extract in oral squamous cancer cells

Next, we checked if the cell death of oral squamous carcinoma cells due to apoptotic induction by SOLE. Annexin V assay revealed the presence of both early and late phase apoptotic cell population in both SSC-15 and SSC-25 cells after SOLE treatments [Figure 3a]. SSC-15 cells showed 10.6% of early apoptotic cells and 3.83% of late apoptotic cells compared to 1.36% and 1.05% early and late apoptotic cells, respectively, in the untreated control [Figure 3b]. Similarly, a raise in early apoptotic cells from 1.22% to 12.06% and late apoptotic cells from 0.81% to 5.16% was observed in SSC-25 cells, post-SOLE treatment. Together, total apoptosis in SSC-15 cells raised from 2.41% to 14.43% [Figure 3b] in SSC-15 cells and 2.03% to 17.22% in SSC-25 cells for the SOLE treated cells compared to their respective controls [Figure 3b].

Salvia officinalis leaf extract induced apoptosis by ameliorating key apoptosis signals in SSC-15 and SSC-25 cells

Further elucidation of the key apoptotic signals in SOLE treated oral squamous cancer cells were determined by Western blotting. We checked if these key protein signals were altered in a dose-dependent manner by SOLE. Therefore, the respective GI_{50} and GI_{75} doses of SOLE in SSC-15 and SSC-25 cells were tested. SOLE increased caspase-3, Bax, and P-53 protein expressions in SSC-15 and SSC-25 cells, while Bcl-2 protein expression was reduced in both these cells [Figure 4A]. Quantification of the expressed proteins revealed significant dose-dependent alterations in caspase-3, Bcl-2, Bax, and P-53 proteins in both cell lines tested, as observed in Figure 4B.

DISCUSSION

Documentation of bioactive ingredients from plant materials is important for reasons of easy availability and relatively less toxicity and/or less side effects compared to synthetic chemicals. The current study focused on the ethanolic SOLE to be screened for its bioefficacy against oral squamous carcinoma cells. The percent extractives in various solvents suggest the quantity and nature of components in the extract. The chromatographic portrayal may serve as a distinguishing fingerprint for qualitative evaluation of the leaves. TLC study revealed the presence of rutin-flavonoid in ethanol extract. The information obtained from the preliminary phytochemical screening could reveal useful discoveries about the chemical nature of the drugs.[16]

The present findings demonstrate the efficacy of SOLE to control the proliferation of oral squamous carcinoma cells with a wide therapeutic window against the normal Vero cells, which indicates uncompromised efficacy and safety of the extract. This observed efficacy might be from single or combinations of more than one active compound identified in SOLE [Table 1], which is in par with reported literature that effective combinations of more than one compound from natural source bring out therapeutic efficacy in controlling cancer cells.[17]

As cell invasion and metastasis are proven very vital for tumor progression,[18] We mimicked these situations to check if there was any anti-cell migratory effect by SOLE in oral squamous cancer cells. Our observations clearly indicate SOLE to inhibit the SSC-15 and SSC-25 cells in the tumor trans-endothelial cell migration assays, which proves the anti-metastasis efficacy of SOLE in addition to the observed antiproliferative effects.

The cell cycle regulation is considered as vital checkpoint to decide between survival and death for any rapidly proliferating cancer cell.[19] Results from the present investigation demonstrate an increase sub G_0/G_1 phase of cell cycle with SOLE treatments in SSC-15 and SSC-25 cells, thereby indicating the response of cancer cells to the extract treatments. The results of cell cycle alteration could also be attributed to the anti-proliferative activities of SOLE in oral squamous cancer cells. Literature reports certain anticancer agents to cause DNA damage, which results in the accumulation sub G_0/G_1 phase of the cell

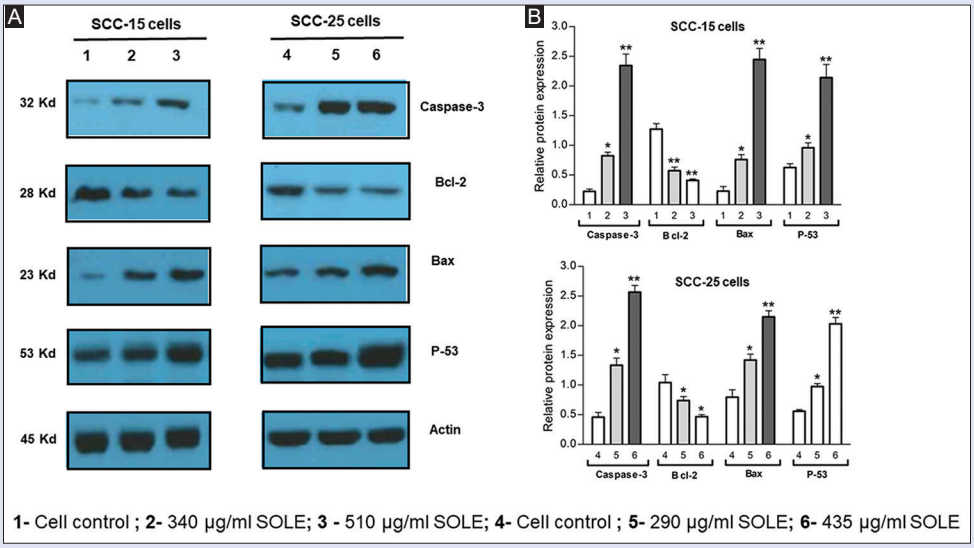

Figure 4: (A) Dose-dependent effect of SOLE treatment on casase-3, Bcl-2, Bax, and P-53 protein expressions in SSC-15 and SSC-25 cells. Representation blots are from three individual experiments are shown. Actin was used as the loading control. (B) Graphical representation of the quantified Western Blots expressed as relative protein expression in comparison to controls. SOLE increased the activation of caspase-3, Bax and P-53 proteins dose dependently while decreasing the Bcl-2 protein in both cell types. Results expressed as mean ± standard deviation from three individual experiments in duplicates and $*P \leq 0.05$/$**P \leq 0.01$ was considered statistically significant. SOLE: *Salvia officinalis* leaf extract

cycle.[20] To substantiate our observations from the cell cycle, we carried out fluorescent nuclear staining in SOLE treated SSC cells. Morphological analysis with Propidium iodide and Hoechst 333258 staining showed cell clumping and fragmented nuclei, suggesting the typical characteristic of DNA fragmentation and apoptosis as observed by other co-workers.[21]

The digestion of the cellular DNA makes them appear in the sub G_0/G_1 region of cell cycle, which is considered as a hallmark feature of apoptosis.[19] Taken together, our observations from cell cycle assay and the nuclear staining assay suggested apoptosis induction by SOLE in the oral squamous cancer cells. Increase in early, late, and consequently, total apoptotic cells with SOLE treatment in SSC-15 and SSC-25 cells established the ability of SOLE to induce apoptosis in these cancer cells. These observations were further supported by expression levels of caspase-3 protein in both cancer cell types by post-SOLE treatments.[22] Additionally, the decrease of anti-apoptotic Bcl-2 protein with relative increase in pro-apoptotic Bax protein suggests mitochondria-mediated cell death by SOLE, as reported in literature.[23,24] This hypothesis for the involvement of caspase-mediated mitochondrial apoptosis, be responsible for the SOLE activity, is further supported by the increase P-53 expression after SOLE treatment in SSC-15 and SSC-25 cells.[25]

Flavonoids, tannins, and sterols were identified in the SOLE extract. Reports indicate flavonoids to cause a rapid decline in the proliferation of leukemic cells, along with the induction of apoptosis through DNA fragmentation.[26] The presence of the flavonoid rutin was identified in the SOLE-TLC analysis. Rutin is a well-established flavonoid to possess excellent anticancer activity in breast,[27] colon,[28] and lung forms of cancer.[29,30] Evidence also indicate the beneficial effects of plant-derived sterols[31] and tannins[32] in cancer cells. We propose one or combination from any of these active compounds, be responsible for the observed anticancer efficacy of SOLE in oral squamous carcinoma cells. However, the isolation of these compounds to characterize and evaluate them individually against the cancer cells would lead to better precision on these findings and pave the way for future research on SOLE for the anticancer efficacy.

CONCLUSION

In summary, we conclude that SOLE had excellent anticancer and anti-metastasis activity against the oral squamous cancer cells at a reasonably safer therapeutic window. The mechanism of SOLE action was driven caspase-mediated mitochondrial apoptosis in the cancer cells. More than one active compound present in SOLE could have been responsible for the anticancer effects, which needs further elucidations.

Financial support and sponsorship

The authors are thankful to the Deanship of Scientific Research, King Khalid University, Abha, Saudi Arabia, for financially supporting this work through the General Research Project under grant number (G.R.P-27-1440).

Conflicts of interest

There are no conflicts of interest.

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