http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2026.10.23

Research Article
Salvadora persica (Miswak) Extract as a Natural Therapeutic Agent for Oral Squamous Cell Carcinoma: *in vitro* and *in silico* Evaluation

[1]Marwa Mohamed Ellithy, [1]Heba E. Tarek, [2]Abdulrahman M. Saleh, [3]Mohamed N. Ibrahim, [3]Wael Alzahrani and [3]Eman Fawzy El Azab

[1]Basic Dental Science Department, Oral and Dental Research Institute, National Research Centre, Dokki 12622, Cairo, Egypt
[2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Cairo University, KasrEl- Aini Street, Cairo 11562, Egypt
[3]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences at Al Qurayyat, Jouf University, Al Qurayyat 77454, Saudi Arabia

Abstract

Background and Objective: Oral Squamous Cell Carcinoma (OSCC), particularly tongue malignancies, remains a major global health concern due to late diagnosis and limited treatment options. *Salvadora persica* (Miswak), traditionally used by ancient Arabs for oral hygiene, contains numerous bioactive compounds with potential therapeutic value. This study aimed to evaluate the antioxidant properties of Miswak extract using different solvents, quantify its total phenolic and flavonoid content and assess its anticancer activity against an oral cancer cell line through *in vitro* and *in silico* approaches. **Materials and Methods:** Miswak extracts were prepared using water, acetone and ethanol to compare antioxidant capacity and phytochemical content. The extract exhibiting the highest activity was selected for further analysis. The ethanolic extract was subsequently tested on the human tongue carcinoma cell line (HNO97) using the Sulforhodamine B (SRB) assay to determine cytotoxicity. The ELISA was conducted to quantify TNF-α and VEGF-A levels, while real-time PCR was used to measure the expression of caspase-3 and caspase-7. Molecular docking was performed to evaluate the binding affinity of Miswak-derived compounds toward DAPK1, a key protein involved in regulating apoptosis. Statistical analyses were applied to assess the significance of the findings. **Results:** The ethanolic extract exhibited the highest antioxidant activity and was selected for anticancer evaluation. SRB assay results showed potent cytotoxicity against HNO97 cells with an IC_{50} of 260.03 µg/mL. The ELISA confirmed significant downregulation of TNF-α and VEGF-A, indicating pronounced anti-inflammatory and anti-angiogenic effects. Real-time PCR demonstrated upregulation of caspase-3 and caspase-7, suggesting activation of apoptotic pathways. Molecular docking supported these observations by revealing strong binding affinities of the extract's bioactive compounds to DAPK1. **Conclusion:** The ethanolic extract of *Salvadora persica* demonstrates promising antioxidant, anti-inflammatory, anti-angiogenic and pro-apoptotic properties, indicating its potential as a natural therapeutic candidate for oral squamous cell carcinoma. These findings provide a strong foundation for further *in vivo* and preclinical studies to validate its efficacy and safety.

Key words: Miswak, HNO97, caspase-3, caspase-7, TNF-α, VEGF-A, DAPK1 protein

Citation: Ellithy, M.M., H.E. Tarek, A.M. Saleh, M.N. Ibrahim, W. Alzahrani and E.F. El Azab, 2026. *Salvadora persica* (Miswak) extract as a natural therapeutic agent for oral squamous cell carcinoma: *in vitro* and *in silico* evaluation. Pak. J. Biol. Sci., 29: 10-23.

Corresponding Author: Mohamed N. Ibrahim, Department of Clinical Laboratory Sciences, College of Applied Medical Sciences at Al Qurayyat, Jouf University, Al Qurayyat 77454, Saudi Arabia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Free radicals have a significant and detrimental impact on food rancidity, the degradation of chemical materials and the damage to vital macromolecules, including nucleic acids, proteins, carbohydrates and lipids. These harmful processes are directly linked to severe human disorders, including inflammatory conditions, cardiovascular diseases, diabetes and cancer. Moreover, essential antioxidants in the immune system are removed by excess free radical production, which leads to the disruption of gene expression and abnormal protein formation[1].

Reactive Oxygen Species (ROS) are the primary type of free radical produced during normal metabolism and from external sources such as X-rays, ozone and pollutants; they play a crucial role in oxidative damage[2]. The balance between free radicals and the antioxidative defense system must be maintained to ensure optimal physiological function[3]. The body's natural antioxidants often fail to sufficiently combat ROS-induced damage, making antioxidant supplements essential for health. Antioxidants, whether synthetic or natural, are indispensable for neutralizing free radicals by donating electrons[4]. Synthetic phenolic antioxidants have long been used to prevent oxidation, but growing concerns about their potential adverse health effects have necessitated a shift toward naturally occurring antioxidants derived from medicinal plants[5].

These plants are not only beneficial but also rich in potent compounds such as flavonoids and tannins, which are highly effective against serious diseases, including cancer, atherosclerosis and diabetes. A prime example is *Salvadora persica*, commonly known as Miswak, which is strongly endorsed by the World Health Organization (WHO) for oral hygiene[6]. It contains beneficial active chemical constituents that support its exceptional antibacterial and antifungal properties. The significance of medicinal plants in cancer treatment cannot be overstated, as many potent therapies stem from plant metabolites that exhibit substantial cytotoxic activity against cancer cells. Indeed, research consistently demonstrated that extracts from *S. persica* show remarkable effectiveness against various carcinoma cell lines, solidifying their critical role in cancer therapeutics[7]. Natural antioxidants are essential in the fight against oxidation, as they promote the neutralization of free radicals as well as ROS[5].

The medicinal and antioxidant properties of plants stem from potent compounds such as flavonoids, tannins and terpenoids. These compounds are highly concentrated in herbs and spices such as rosemary, thyme and cinnamon, as well as in extracts like tea and grape seed, making them crucial for health and well-being[8]. Folk medicine is prominently utilized in the Far East and GCC regions, where it is often the primary choice for treating serious illnesses, including cancers and inflammation. *Salvadora persica*, widely recognized as Miswak in Islamic culture, stands out as a traditional natural chewing stick. It is potentially the earliest form of toothbrush, dating back to the Babylonians around 3500 BC and its use underscores the significance of traditional practices in oral health[9].

The WHO endorses *S. persica*, known as Miswak, as a highly effective chewing stick for maintaining oral hygiene. Numerous studies have demonstrated its powerful ability to remove plaque and its robust antibacterial and antifungal properties[10]. Chemical analyses of *S. persica* revealed a diverse array of biologically active compounds, including alkaloids, chlorides, fluorides and sulfur-containing substances. Furthermore, it contains significant levels of silica, sulfur and vitamin C, underscoring its impressive nutritional profile[18]. Medicinal plants are pivotal in cancer treatment, with a wide range of plant secondary metabolites proven effective against cancer[11]. Substantial evidence showed that petroleum ether extracts from *S. persica* exhibit remarkable cytotoxic activity against lung and colon carcinoma cell lines[12]. Isolated ursolic acid outperforms oleanolic acid in effectiveness against HepG2 and MCF7 cell lines, while oleanolic acid demonstrates considerable potency against various cancer types[13,14].

This study was conducted to evaluate the antioxidant and anticancer potential of *Salvadora persica* (Miswak) root extracts prepared using different solvents water, ethyl acetate and ethanol. Specifically, the study sought to quantify the total phenolic and flavonoid contents of each extract, assess their antioxidant activity using the DPPH assay and evaluate the cytotoxic, anti-angiogenic and pro-apoptotic effects of the most potent extract (ethanolic) on human tongue carcinoma (HNO97) cells. Also, molecular docking analysis was conducted to explore the binding affinities of bioactive compounds from Miswak extract to apoptosis-related proteins, particularly Death-Associated Protein Kinase-1 (DAPK1), to support its potential as a natural therapeutic agent for oral squamous cell carcinoma.

MATERIALS AND METHODS

Study area and duration: The study was done between March, 2024 and August, 2025 at National Research Centre, Cairo, Egypt.

Materials: All solvents employed in this study were of analytical grade. The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA): Folin-Ciocalteu reagent, ascorbic acid, aluminum chloride, quercetin,

potassium acetate, ethanol, DPPH reagent, thiazolyl blue, gallic acid, sodium carbonate, methanol, tetrazolium bromide, ferric chloride and dimethyl sulfoxide. Millipore deionized water was consistently utilized throughout the entire research. Commercial kits (Elabscience, USA) were used, CAT No. E-EL-H0109 and CAT No. E-EL-H0111. The plate reader used was a BMG Labtech FLUOstar Omega from Germany, with a wavelength of 450 nm.

Methods

Preparation of the plant extract

Plant samples: Protected roots of *S. persica* were purchased from the local market and stored in labeled dry plastic zipper bags. Tap water was used to wash and clean the roots, which were then cut into small pieces and crushed with a grinder. Ten grams of the crushed roots were macerated in 150 mL of 70% (v/v) aqueous ethanol at 4°C for 48 hrs. The resulting mixture was filtered under vacuum and concentrated under low pressure using a rotary evaporator set at 40°C. A TELSTAR CRYODOS freeze dryer was then used to dry the extract further until no additional water could be distilled, after which it was stored at -20°C for further analysis. A 30 mg/mL solution of the extract was prepared in 50% ethanol for subsequent assessment. A stock solution was prepared by extracting freshly ground roots three times, each time with a different solvent: water, ethanol and ethyl acetate. For each solvent, 500 g of dried powder was percolated three times, with fresh solvent replaced every 24 hrs. The extracts were then dried under vacuum to ensure the complete removal of residual solvent[15].

Assessment of Miswak total phenolic content: The Folin-Ciocalteu method was used to evaluate the total phenolic content[16]. Specifically, 100 µL of the extract was placed in a test tube and the volume was adjusted to 3.5 mL with distilled water. Oxidation was initiated by adding 250 µL of Folin-Ciocalteu reagent. After a 5 min waiting period, the mixture was neutralized by incorporating 1.25 mL of a 20% aqueous Sodium Carbonate (Na_2CO_3) solution. The solution was then incubated for 40 min before the absorbance was measured at 725 nm, using the solvent blank as a reference.

Assessment of the entire content of Miswak flavonoids: We used the colorimetric assay of Aluminum Chloride ($AlCl_3$), as described by Zilic *et al.*[17] to determine the total flavonoid content of Miswak. In brief, 300 µL of 5% Sodium Nitrite ($NaNO_2$) was mixed with 100 µL of the extract. The mixture was incubated for 6 minutes, after which 300 µL of a 10% $AlCl_3$ solution was added and the volume was adjusted to 2.5 mL with distilled water. After 7 min, 1.5 mL of 1 M NaOH was introduced and the mixture was centrifuged at 5000 ×g for 10 min. The absorbance of the supernatant was measured at 510 nm against a solvent blank.

Determination of the scavenging activity of DPPH radical: The free radical scavenging capacity was effectively assessed using the stable compound 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\cdot}$)[18]. A final concentration of 50 µM $DPPH^{\cdot}$ was utilized and the total reaction volume was maintained at 4.0 mL. After 60 min, the absorbance at 517 nm (A) was measured against a blank of pure methanol. The inhibition percentage of the $DPPH^{\cdot}$ free radical was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

HPLC analysis: The HPLC analysis was conducted according to the method established by Kim *et al.*[19] using an Agilent Technologies 1100 series liquid chromatograph equipped with an autosampler and a diode-array detector. The analytical column was an Eclipse XDB-C18 (150×4.6 mm; 5 µm), supported by a C18 guard column from Phenomenex (Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was maintained at 0.8 mL/min over a total run time of 60 min. The gradient program was set to transition from 100% solvent B to 85% B within 30 min, then to 50% B in 20 min, to 0% B in 5 min and finally back to 100% B over another 5 min. The injection volume was 50 µL, with peaks monitored simultaneously at 280 nm for benzoic acid derivatives, 320 nm for cinnamic acid derivatives and 360 nm for flavonoids. All samples were filtered through a 0.45 µm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by comparing their retention times and UV spectra with those of standard compounds.

Assessment of anticarcinogenic effect of Miswak extract on the HNO97 cell line

Cell culture: The HNO-97 tongue carcinoma cells were acquired from Nawah Scientific Inc. in Mokattam, Cairo, Egypt. These cells were maintained in DMEM supplemented with 100 mg/mL streptomycin, 100 units/mL penicillin and 10% heat-inactivated fetal bovine serum. They were cultured in a humidified atmosphere with 5% (v/v) CO_2 at a constant temperature of 37°C[20].

Cytotoxicity assay: Cell viability was determined using the SRB assay[21]. Initially, 100 µL aliquots of cell suspension (5×10^3 cells) were placed in 96-well plates and incubated in complete media for 24 hrs. Following incubation, the cells were treated with an additional 100 µL of media containing drugs at various concentrations. After drug exposure, the media was replaced with 150 µL of 10% TCA to fix the cells and they were incubated at 4°C for 1 hr. The TCA solution was then removed and the cells were washed five times with distilled water. Subsequently, 70 µL of SRB solution (0.4% w/v) was added to each well and the plates were incubated in the dark at room temperature for 10 min. After incubation, the plates were washed three times with 1% acetic acid and air-dried overnight. Finally, 150 µL of TRIS (10 mM) was added to dissolve the protein-bound SRB stain and the absorbance was measured at 540 nm using an Infinite F50 microplate reader (TECAN, Switzerland). This method ensures accurate and reliable assessment of cell viability.

Total antioxidant capacity of Miswak extract: A commercial kit from Biodiagnostic, Egypt, was employed for the analysis. The process involved using a specific amount of substrate along with exogenously provided Hydrogen Peroxide (H_2O_2) to eliminate the total antioxidants present in the sample (IC_{50} of Miswak extract). The chromogen, 3,5-dichloro-2-hydroxybenzenesulfonate, underwent an enzymatic reaction that produced a colored product while retaining some residual H_2O_2. The resultant colored product was measured at 505 nm using a plate reader (FLUOstar Omega, BMG Labtech).

ELISA for TNF-α and VEGF

Assay principle: The micro-ELISA plate was pre-coated with a specific antibody targeting human TNF-α. After the addition of samples and standards, a biotinylated detection antibody specific to human TNF-α was introduced, followed by the addition of an avidin horseradish peroxidase (HRP) conjugate. These components were added to each well in succession and incubated. The optical density (OD) of the TNF-α complex formed with the biotinylated detection antibody was measured. This OD value directly correlated with the concentration of human TNF-α. The procedure was then repeated for VEGF to ensure consistent and accurate results.

Real time PCR for caspase-3/caspase-7: Total RNA was extracted from the samples using the RNeasy Mini Kit (Cat. No. 74104, Qiagen, Hilden, Germany) following the manufacturer's instructions. The concentration and quality of the RNA were assessed using a FLUOstar Omega Plate Reader (BMG LABTECH) spectrophotometer by measuring the A260/A280 ratio. The isolated RNA was reverse transcribed into complementary DNA (cDNA) using the GScript First-Strand Synthesis Kit (Cat. No. MB305-0050, GeneDireX, Taiwan) according to the manufacturer's guidelines. Gene expression levels were measured using SYBR Green qPCR master mix (Xpert Fast SYBR (uni), Cat. No. GE20.100, Porto, Portugal) on the Bio-Rad CFX OPUS 96 system. Each reaction was conducted in a total volume of 20 µL, including 2 µL of cDNA, 2 µL of forward and reverse primers (0.3-0.5 µM each), 10 µL of master mix and 6 µL of nuclease-free water.

Molecular docking analysis: To explore the potential antimicrobial and anticancer activities of the extracted compounds, molecular modeling was conducted. This study evaluated the binding modes and estimated the binding affinities of the compounds toward a selected protein. The targets were selected based on the molecular similarity between the extracted compounds and apigenin, the co-crystallized ligand and the crystal structure was obtained from the Protein Data Bank (www.rcsb.org). DAPK1 (PDB ID: 5AUV)[22].

At first, the target protein was prepared by removing water molecules and unnecessary residues from the protein complex. then, protons were added and unfilled valence atoms were corrected. The protein structure's energy was minimized and saved as PDBQT files[23]. The target compounds 2D structures were drawn using Chem-Bio Draw Ultra16.0, saved as an SDF file[24]. Protonation and energy minimization were carried out and saved as a PDBQT file. The prepared ligands were docked against DAPK1. The processes were conducted using Autodock Vina 1.5.7 software[25]. then the best-scoring candidates were selected. The docking scores of the best-fitted poses with the active sites were recorded and figures were generated using Discovery Studio 2024 visualizer[23].

RESULTS

Assessment of Miswak total phenolic content: The calibration curve built with gallic acid standards (5-50 mg) to ascertain the total phenolic content of Miswak extracts is illustrated in Fig. 1. The absorbance values at 725 nm ranged from 0.18 to 1.25, indicating a substantial linear relationship between absorbance and concentration, which supports the accuracy of the quantification method. This curve was used for estimating the phenolic content of the tested extracts, which was then expressed as milligrams of gallic acid equivalent per gram of sample (mg GAE/g). Ethanolic extract showed the highest phenolic concentration (39.06 mg GAE/g) of the

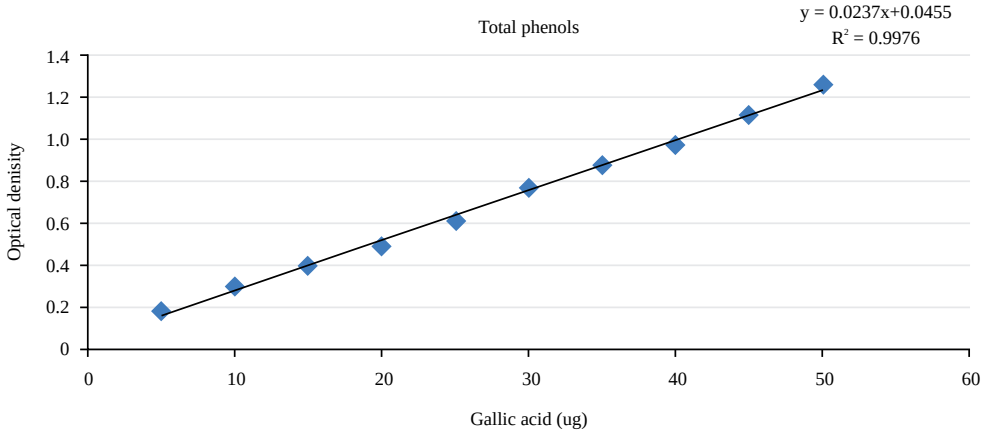

Fig. 1: Total phenolic concentrations in Miswak extract, highlighting its remarkable health-promoting properties

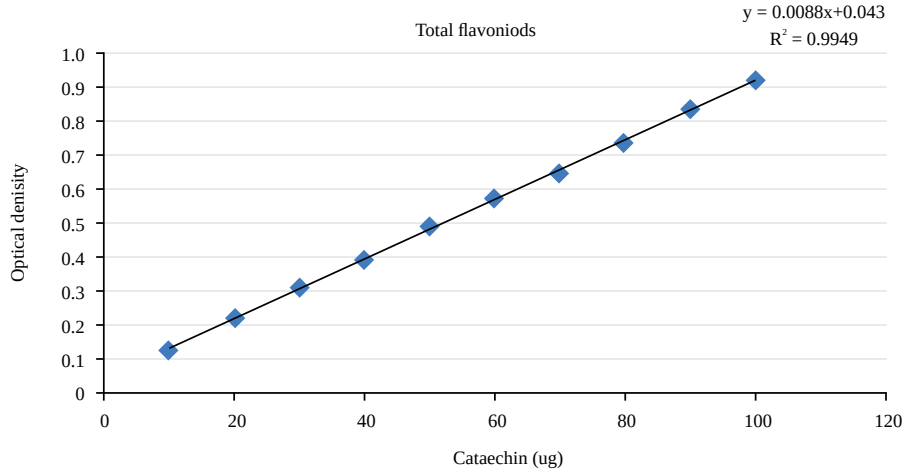

Fig. 2: Concentrations of total flavonoids present in Miswak extract

three solvents utilized, followed by ethyl acetate (25.64 mg GAE/g) and water extract (13.11 mg GAE/g). The results obtained indicate that ethanol is the most effective solvent for extracting phenolic compounds from Miswak roots, which may help elucidate its better antioxidant and anticancer properties observed in subsequent tests.

Assessment of the entire content of Miswak flavonoids: The calibration curve formed using catechin standards (10-100 mg) for calculating the overall flavonoid content of Miswak extracts is provided in Fig. 2. The absorbance values ranged from 0.12 to 0.92 at 510 nm, demonstrating a strong linear correlation between catechin concentration and absorbance, which validates the reliability of the assay. The flavonoid concentration is expressed as milligrams of catechin equivalent per gram of sample (mg CE/g) according to this curve. The highest concentration of flavonoids among all of the examined extracts was found in the ethanolic fraction (14.58 mg CE/g), which was followed by ethyl acetate (7.19 mg CE/g) and water extract (0.21 mg CE/g). Owing to these results, ethanol is the most efficient solvent to extract flavonoids from Miswak roots, which may help explain why further analyses found that it boosted antioxidant and anti-cancer properties.

Determination of the scavenging activity of DPPH radical: Figure 3 illustrates the calibration curve produced using Trolox standards (2-20 mg) to estimate the antioxidant activity of Miswak extracts through the DPPH radical scavenging assay. The absorbance values ranged from approximately 5.39 to 63.14, signifying a strong linear relationship between Trolox concentration and antioxidant capacity. Based on this curve, the antioxidant activity of the extracts was expressed as milligrams of Trolox equivalent per gram of sample (mg TE/g). Among the tested extracts, the ethanolic extract revealed the highest antioxidant activity (23.47 mg TE/g), followed by ethyl acetate (18.50 mg TE/g) and water extract (11.57 mg TE/g).These results prove that ethanol is the most effective solvent for extracting antioxidant compounds from Miswak roots, which aligns with its phenolic and flavonoid content identified in Fig. 1 and 2.

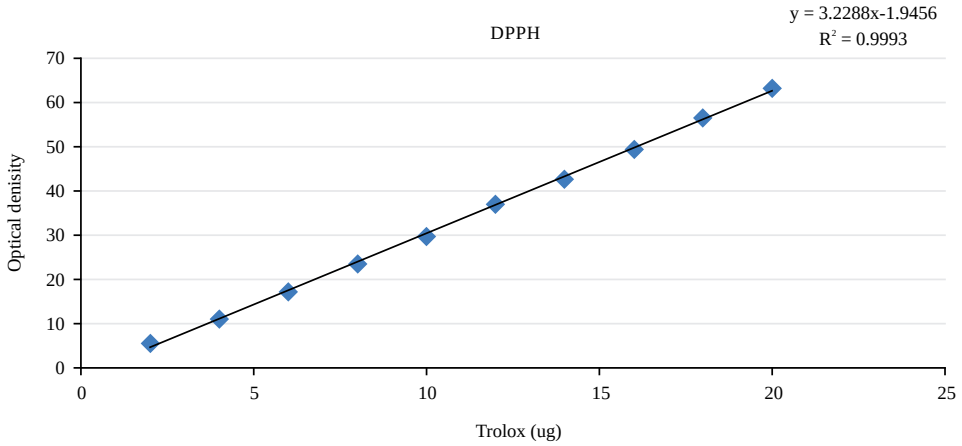

Fig. 3: Remarkable antioxidant capability of Miswak extract, highlighting its potent health-promoting benefits

Table 1: Comparison between water, ethanolic and ethyl acetate Miswak extract

Sample	Whole phenols content (mg GAE/g)	Whole flavonoids content (mg CE/g)	Antioxidant activity (mg TE/g)
Water	13.11	0.21	11.57
Ethanol	39.06	14.58	23.47
Ethyl acetate	25.64	7.19	18.50

Table 2: Phenolic compounds content (µg/g) in different solvent extracts

Compound	Water	Ethanol	Ethyl acetate
Gallic acid	98.39	461.79	302.04
Protocatechuic acid	57.54	75.85	386.96
Gentisic	0.00	0.00	0.00
p-hydroxybenzoic acid	29.40	39.78	7.36
Catechin	253.44	208.90	0.00
Chlorogenic acid	348.33	70.12	16.97
Caffeic acid	226.59	88.99	11.81
Syringic acid	103.50	227.96	27.83
Vanillic acid	55.25	181.36	130.99
Ferulic acid	58.58	477.76	193.47
Sinapic acid	7.72	37.57	45.65
Rutin	148.23	538.94	176.25
p-coumaric acid	104.79	723.95	35.13
Apigenin-7-glucoside	18.40	125.88	141.56
Rosmarinic acid	36.11	668.31	155.61
Cinnamic acid	4.24	254.30	179.85
Quercetin	0.80	150.85	130.78
Apigenin	0.00	100.89	67.16
Kaempferol	0.00	65.50	98.46
Chrysin	7.66	23.63	15.52

Analysis of Miswak extraction results using different solvents clearly demonstrated that the alcoholic extract of Miswak possessed the highest antioxidant power. Moreover, it contained a significantly greater concentration of phenols and flavonoids than both the water and acetyl acetate extracts (Table 1).

Consequently, the alcoholic extract was chosen for the assessment of its anticarcinogenic profile in subsequent tests. Results of HPLC analysis of Miswak extract using different solvents are presented in Table 2. The table shows the concentration of various phenolic compounds in water, ethanol and ethyl acetate extracts. Ethanol extracts generally exhibited higher levels of most phenolics, while gentisic acid was not detected in any extract. Some compounds, such as catechin and chlorogenic acid, were predominant in specific solvents.

Cytotoxicity assay: Alcoholic Miswak extract showed a concentration-dependent inhibition of HNO-97 cell viability with an IC_{50} of 260.03 µg/mL (Fig. 4 and 5).

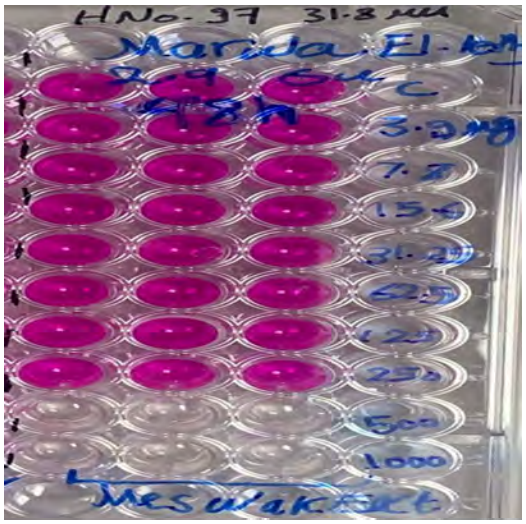

Fig. 4: Cytotoxicity assay using alcoholic Miswak extract on cultured HNO97 cells

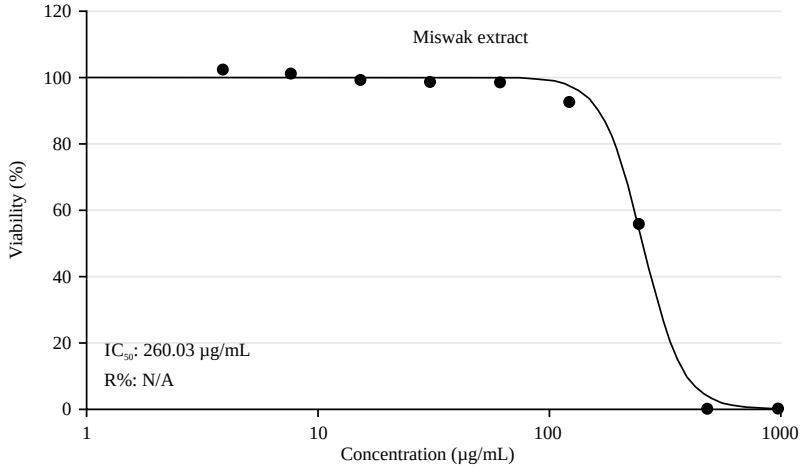

Fig. 5: Viability of HNO97 cells is robustly influenced by varying concentrations of Miswak alcoholic extract with an IC_{50} of 260.03 µg/mL

Table 3: Mean and standard deviation of TNF-α (pg/mL) and VEGF-A (pg/mL) levels observed in both the Miswak-treated group and the control group

	TNF-α (pg/mL)		VEGF-A (pg/mL)		Independent t test				
							95% Confidence interval of the difference		
	Mean	Standard deviation	Mean	Standard deviation	Mean difference	Std. error difference	Lower	Upper	p-value
Miswak treated	11.40	1.43	1.47	0.67	9.93	0.48	8.94	10.92	0.0001*
Control	16.74	1.13	3.26	1.16	13.48	0.49	12.47	14.50	0.0001*
p-value	0.0001*	0.0001*							

*Significant difference as $p \leq 0.05$

Total antioxidant capacity of the Miswak alcoholic extract: The total antioxidant concentration of the Miswak alcoholic extract was found to be 22 mM compared to 11 mM for the control group (routine culture media).

Measurement of TNF-α (pg/mL) and VEGF-A (pg/mL) using ELISA: Statistical analysis was performed using SPSS 16® (Statistical Package for the Social Sciences), GraphPad Prism and Microsoft Excel. The findings are presented in two tables and four graphs. Normality of the data was assessed using both the Shapiro-Wilk test and the Kolmogorov-Smirnov test, confirming that the data followed a normal distribution. Therefore, levels of TNF-α (pg/mL) and VEGF-A (pg/mL) were compared

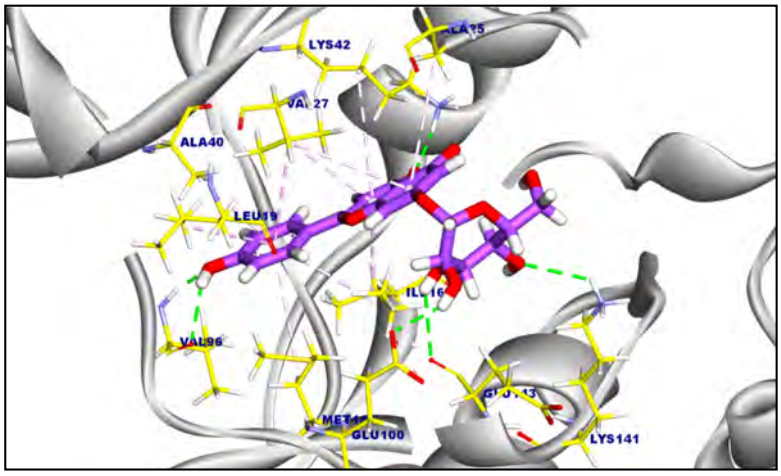

Fig. 6: 3D figure of the proposed binding mode of apigenin-7-glucoside against (DAPK1), amino acids colored in yellow, and the ligand colored in purple

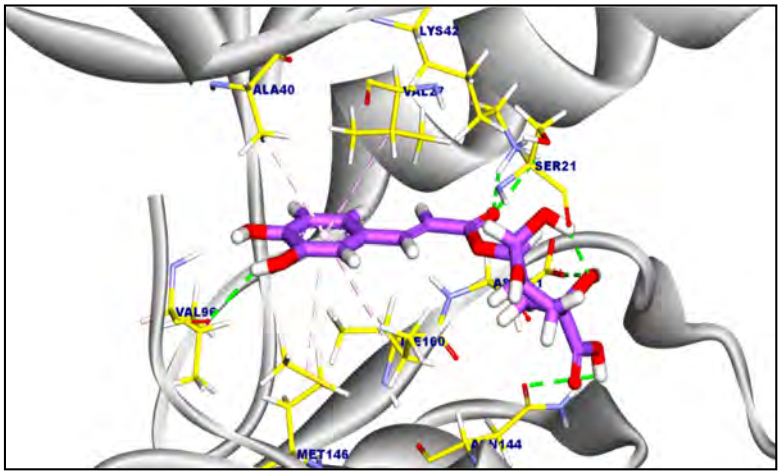

Fig. 7: 3D figure of the proposed binding mode of chlorogenic acid against (DAPK1), amino acids colored by yellow, and the ligand colored by purple

between the Miswak-treated group and the control group using an independent t-test. A p-value of ≤0.05 was considered significant (Table 3).

Comparison between the Miswak and control groups: Comparison between these groups was conducted using an independent t-test. Significant differences were as follows:

- **TNF-α (pg/mL):** The Miswak group exhibited a significantly lower level (11.4±1.43) compared to the control group (16.74±1.13), with a p-value of 0.0001 (Table 3)
- **VEGF-A (pg/mL):** Similarly, the Miswak group had a significantly lower level (1.47±0.67) than the control group (3.26±1.16), with a p-value of 0.0001 (Table 3)

Comparison between TNF-α (pg/mL) and VEGF-A (pg/mL):

- **Miswak-treated group:** A significant difference was detected, with TNF-α levels (11.40±1.43) being significantly higher than VEGF-A levels (1.47±0.67), indicated by a p-value of 0.0001 (Table 3)
- **Control group:** A similar significant difference was observed, with TNF-α levels (16.74±1.13) being significantly higher than VEGF-A levels (3.26±1.16), as indicated by a p-value of 0.0001 (Table 3 and Fig. 6)

Real-time PCR for caspase-3/caspase-7 gene expression: Comparison between the Miswak and control groups:

An independent t-test definitively showed the following results:

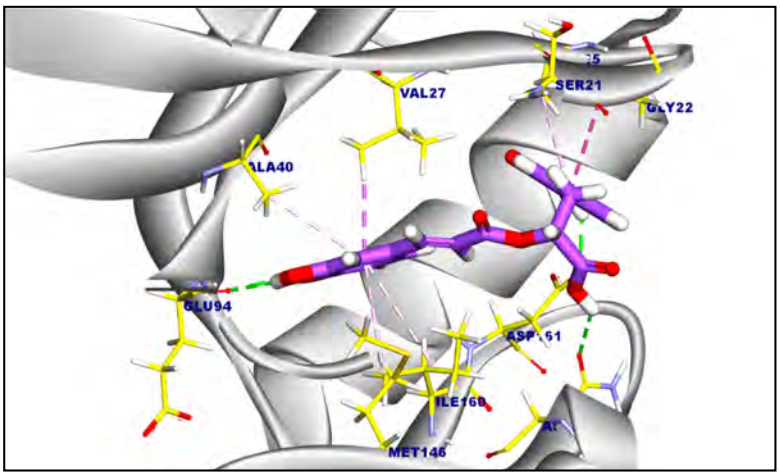

Fig. 8: 3D figure of the proposed binding mode of rosmarinic acid against (DAPK1), amino acids colored by yellow, and the ligand colored by purple

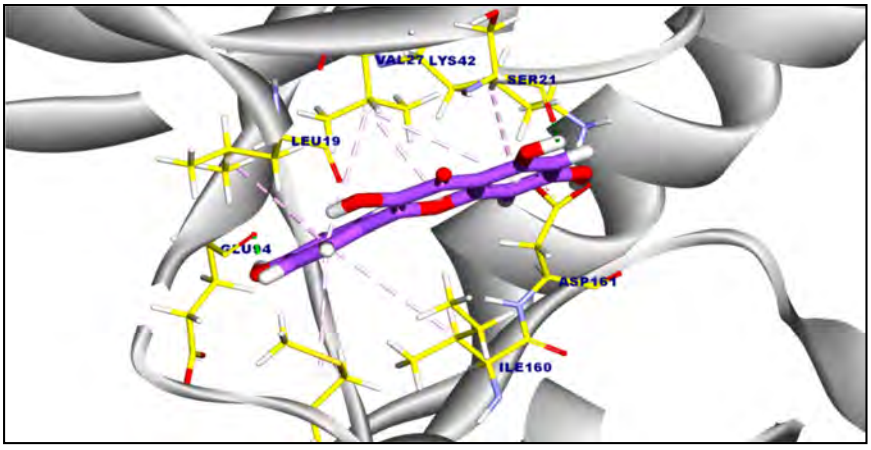

Fig. 9: 3D figure of the proposed binding mode of kaempferol against (DAPK1), amino acids colored by yellow, and the ligand colored by purple

- **Caspase-3 (ng/µL):** The Miswak group exhibited a statistically significant elevation (192.03±41.07) compared to the control group (92.28±4.17), with a p-value of 0.0001 (Table 4)
- **Caspase-7 (ng/µL):** Similarly, the Miswak group displayed a significant increase (188.95±32.20) relative to the control group (88.48±11.21), with a p-value of 0.0001 (Table 4).

Comparison between caspase-3 (ng/µL) and caspase-7 (ng/µL):

- **Miswak-treated group:** No significant difference was observed between caspase-3 (192.03±41.07) and caspase-7 (188.95±32.20), evidenced by a p-value of 0.14, indicating comparable levels
- **Control group:** No significant difference was observed, as caspase-3 (92.28±4.17) was marginally higher than caspase-7 (88.48±11.21), with a p-value of 0.78

Molecular docking study: Miswak extract is recognized as one of the most valuable sources of phytochemicals, containing a wide range of bioactive compounds with potential anticancer properties. In the present study, the active constituents of Miswak extract were subjected to molecular docking analysis to explore their potential binding affinities as anticancer agents. Death-Associated Protein Kinase 1 (DAPK1) was chosen as the representative target due to the notable structural similarity between the extracted compounds and apigenin, the co-crystallized ligand bound to this protein.The docking results, including the predicted interactions and binding scores of the most promising compounds, are presented in Table 5.

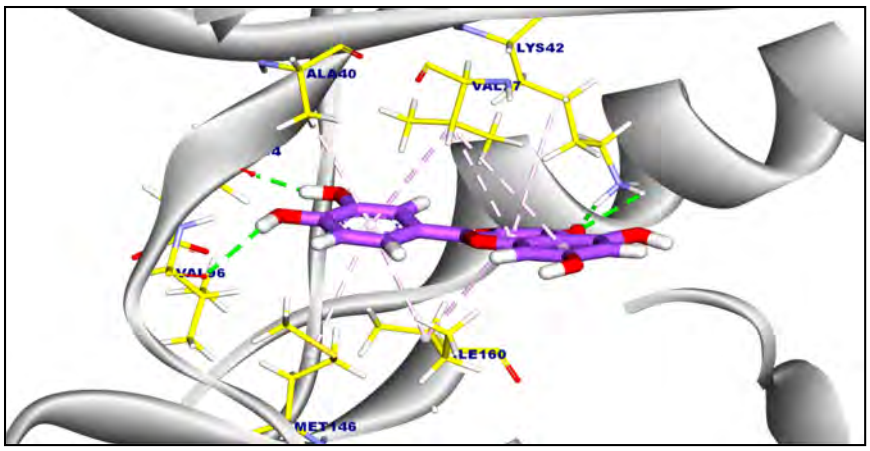

Fig. 10: 3D figure of the proposed binding mode of quercetin against (DAPK1), amino acids colored by yellow, and the ligand colored by purple

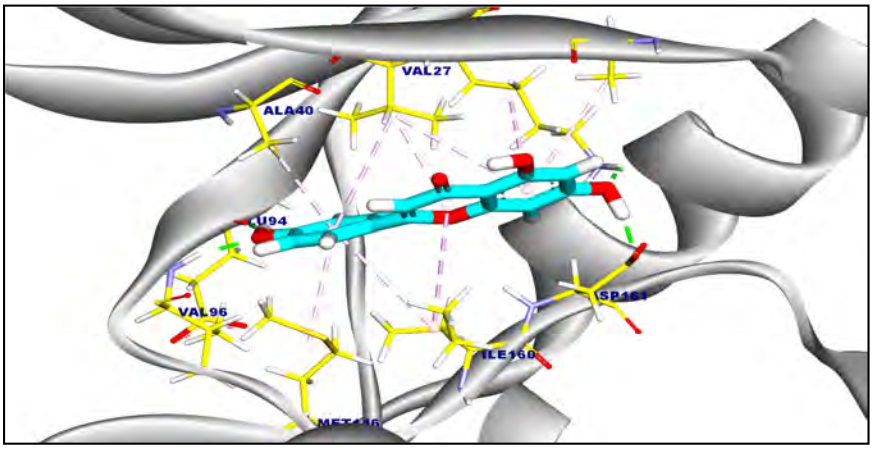

Fig. 11: 3D figure of the proposed binding mode of the co-crystalized ligand (apigenin) complexed with (DAPK1), amino acids colored yellow, and erlotinib colored by turquoise

Table 4: Mean and standard deviation of caspase-3 (ng/µL) and caspase-7 (ng/µL) in both Miswak treated group and control group:

					Independent t test				
	Caspase-3 (ng/µL)		Caspase-7 (ng/µL)				95% Confidence interval of the difference		
Group	Mean	Standard deviation	Mean	Standard deviation	Mean difference	Std. error difference	Lower	Upper	p-value
Miswak treated	192.03	41.07	188.95	32.20	3.07	0.88	-44.39	50.54	0.14
Control	92.28	4.17	88.48	11.21	3.79	0.45	-7.09	14.67	0.78
p-value	0.0001*	0.0001*							

*Significant difference as p≤0.05

Table 5: Molecular docking analysis of the extracted compounds against (DAPK1)

Target proteins	Tested compounds	RMSD value (Å)	Docking (Affinity) score (kcal/mol)
(DAPK1)	Apigenin-7-glucoside	1.15	-8.86
	Chlorogenic acid	0.77	-7.95
	Rosmarinic acid	1.42	-8.35
	Kaempferol	1.29	-7.07
	Quercetin	0.76	-7.43
	Co-crystalized ligand (apigenin)	0.45	-7.25

Molecular docking of target compounds against (DAPK1): The molecular docking analysis revealed that Apigenin-7-glucoside exhibited an affinity score of -8.86 kcal/ mol against (DAPK1) . Apigenin-7-glucoside interacted with Met146, Ala40, Leu19, Ile160, Val27 and Ala25 by ten hydrophobic π-alkyl interactions. Meanwhile, Apigenin-7-glucoside is attached to Lys141, Glu143, Glu100, Val96 and Lys42 by six hydrogen bonds with distances of 2.91, 2.96, 2.31, 2.40, 2.41 and 1.96 Å, respectively (Fig. 6). Chlorogenic acid interacts by four hydrophobic π-alkyl interactions with Val27, Ile160, Ala40 and Met146. Additionally, showed four classical hydrogen bonds with Asn144 (2.82 Å), Asp161 (2.64 Å), Lys42 (2.95 Å) and Val96 (2.45 Å) with an affinity score equal to -7.95 kcal/ mol (Fig. 7). Similarly, Rosmarinic acid showed an affinity score of -8.35 and exhibited six hydrophobic π-π, π-sigma and π-alkyl interactions with Ser21, Val27, Met146, Ala25, Ala40, Ile160 and was supported by four hydrogen bonds with Asp161, Asn144 and Glu94, with distances of 2.12, 2.88, 2.56 and 2.15 Å (Fig. 8).

On the other hand, the proposed binding mode of Kaempferol showed a binding affinity of -7.07 kcal/mol against (DAPK1). Kaempferol formed seven hydrophobic π-alkyl interactions with Lys42, Val27, Ile160, Leu19 and Met146. Moreover, it interacted with Asp161, Ser21 and Glu94 by three hydrogen bonds with distances of 1.63, 2.48 and 2.12 Å (Fig. 9). The proposed binding mode of Quercetin exhibited an affinity score of -7.43 kcal/mol, Many hydrophobic π-alkyl interactions were noted with Lys42, Val27, Met146, Ile160 and Ala40. Moreover, Quercetin interacted with Lys42, Glu94 and Val96 by three hydrogen bonds with distances of 2.71, 2.08 and Val96 Å (Fig. 10). Furthermore, The co-crystalized ligand (Apigenin) complexed with (DAPK1) exhibited an affinity score of -7.25 kcal/mol. Apigenin formed eight hydrophobic π-Alkyl interactions with Lys42, Ala25, Val27, Ile160, Ala40 and Met146. Furthermore, it interacted with Asp161, Lys42, Glu94 and Val96 by four hydrogen bond with distances of 195, 2.47, 1.70 and 1.94 Å (Fig. 11).

DISCUSSION

Cancer is the second leading cause of death globally. Numerous natural bioactive compounds derived from plants provide significant health benefits, particularly in cancer prevention and they often outperform synthetic alternatives. There is a critical need to discover effective cancer-fighting agents that minimize adverse effects and herbal extracts have emerged as a promising focus of research over the past decade. The most common type of Miswak is sourced from the Arak tree, which predominantly thrives in Saudi Arabia and other areas of the Middle East[26].

Research unequivocally demonstrated that extracts from *Salvadora persica* possess remarkable biological properties, including potent antimicrobial and anti-inflammatory effects, while exhibiting minimal toxicity[27]. Several methods have been used to obtain *S. persica* extract, primarily through aqueous and alcohol extraction. Some studies have utilized pieces of *S. persica* without any extraction[28]. In the present study, the roots of Miswak were ground and the extract was obtained using three different solvents: water, ethanol and acetic acid. The extracts were analyzed for their flavonoid and phenolic content, as well as their antioxidant effects. The alcoholic extract of Miswak demonstrated the highest antioxidant power and contained significantly greater concentrations of phenols and flavonoids compared to both the water and acetyl acetate extracts.

Haque and Alsareii[29] extracted Miswak using different solvents, specifically ethanol and hexane, to test their antimicrobial activity against *Streptococcus mutans*, *Streptococcus sanguis* and *Streptococcus salivarius*. This study definitively demonstrated, for the first time, the impact of the ethanolic extract of Miswak on tongue squamous cell carcinoma (HNO97 cell line), a type of solid malignant tumor. The results were exceptionally promising. The ethanolic extract exhibited strong cytotoxic effects on malignant keratinocytes, achieving an IC_{50} of 260.03 µg/mL. Furthermore, its anti-angiogenic properties were evident, as it effectively inhibited VEGF in cultured cells. Notably, this extract emerged as a novel pro-apoptotic agent, successfully inducing the intrinsic apoptotic pathway and significantly upregulating caspases 3 and 7.

In vitro studies have demonstrated that TNF-α activates the NF-κB pathway, resulting in increased protein expression of IKKβ and p65. This activation enhances the invasive capabilities of oral squamous cell carcinoma cells, thereby amplifying their metastatic potential. In this context, the ethanolic extract of Miswak has been proven to significantly downregulate TNF-α, underscoring its therapeutic potential.

In a pivotal study conducted by Balto *et al.*[30], researchers prepared a stock solution by extracting fresh, ground Miswak roots using three distinct solvents: Hexane, ethyl acetate and 10% ethanol. They rigorously evaluated the cytotoxic effects of each extract on human gingival fibroblasts to determine their viability as canal irrigants in endodontic treatment. The findings are unequivocal: The ethanol extract of *Salvadora persica* at concentrations of 0.5 and 1 mg/mL, along with the hexane extract at 0.5 mg/mL, exhibited no cytotoxic activity. This evidence confirms the promising potential of these extracts for use in dental procedures. However, the hexane extract at 1 mg/mL showed some cytotoxicity, with a cell survival rate of 88% compared to the controls.

In their 2018 study, Al-Dabbagh *et al.*[31] clearly demonstrated that Miswak extract possesses significant antioxidant activity, high polyphenolic content and notable anticancer properties. Their findings revealed that Miswak extract is not only rich in antioxidants and polyphenols but also effectively exhibits anti-angiogenic and antiproliferative effects on the HCC cell line.

In another study, Hammad *et al.*[32] conclusively proved that the aqueous root extract of Miswak exhibited significant cytotoxic effects on oral epithelial dysplasia, oral cancer and normal human periodontal ligament fibroblast cell lines. The results indicated that Miswak has promising potential in cancer prevention.

Al Bratty *et al.*[14] conducted a comprehensive study on the cytotoxic effects of ethanol fruit extract from *S. persica*, utilizing the MTT assay over 72 hrs. Their findings clearly indicated that the extract is effective against breast, ovarian and colon cancer cells. Furthermore, the *S. persica* fruit extract demonstrated significant protective effects on normal lung and oral cells, strongly affirming its robust safety profile as a widely used plant.

Mohany *et al.*[33] demonstrated that persicaline, an alkaloid derived from Miswak, plays a significant role in inhibiting the growth of MCF-7 cells. Their findings revealed that persicaline effectively induces G1 phase arrest and triggers apoptosis. The study showed that ROS and the upregulation of pro-apoptotic genes by persicaline are essential mediators in activating the intrinsic apoptotic pathway.

Furthermore, molecular docking analysis provided strong computational support for the in vitro findings. Key bioactive compounds identified in the ethanolic Miswak extract such as apigenin-7-glucoside, rosmarinic acid and chlorogenic acid displayed high binding affinities toward Death-Associated Protein Kinase 1 (DAPK1), a critical regulator of apoptosis. These compounds formed multiple hydrogen bonds and hydrophobic interactions with active site residues, suggesting a direct role in triggering apoptotic pathways. According to Farag and Roh[34], the DAPK family plays a vital role in regulating several biological functions, including autophagy, apoptosis, tumor suppression and inhibition of metastasis. The strong interaction between Miswak-derived compounds and DAPK1 detected in this study aligns with the noteworthy upregulation of caspase-3 and caspase-7 in treated HNO97 cells, reinforcing the hypothesis that Miswak extract induces apoptosis via DAPK1-mediated signaling. These findings not only validate the therapeutic potential of Miswak at the molecular level but also highlight its promise as a natural anticancer agent for oral squamous cell carcinoma.

CONCLUSION

The present study highlights the promising therapeutic potential of *Salvadora persica* (Miswak) extract, particularly the ethanolic fraction, which demonstrated the highest antioxidant activity and high concentrations of phenolic and flavonoid compounds. The extract revealed potent cytotoxic effects against human tongue carcinoma (HNO97) cells, along with significant anti-angiogenic and pro-apoptotic activities. These biological effects were further supported by molecular docking analysis, which revealed strong binding affinities between key Miswak-derived compounds and DAPK1, a critical apoptosis-regulating protein. Collectively, the ethanolic Miswak extract is considered a promising natural candidate for the development of anticancer therapies targeting oral squamous cell carcinoma. Future research should focus on isolating the active constituents, validating their mechanisms *in vivo* and advancing toward preclinical and clinical applications.

SIGNIFICANCE STATEMENT

This study discovered the potent antioxidant, anti-inflammatory, anti-angiogenic and pro-apoptotic properties of *Salvadora persica* ethanolic extract that can be beneficial for developing natural therapeutic strategies against oral squamous cell carcinoma. By demonstrating its cytotoxic effects on tongue carcinoma cells and molecular interactions with apoptosis-regulating proteins, this study will help the researchers to uncover the critical areas of plant-derived anticancer mechanisms that many researchers were not able to explore. Thus, a new theory on natural phytochemical-based cancer therapy may be arrived at.

FUNDING

This research was conducted without financial support from any funding agencies in the public, commercial, or non-profit sectors, underscoring its independence and integrity.

REFERENCES

1. Chandimali, N., S.G. Bak, E.H. Park, H.J. Lim and Y.S. Won *et al.*, 2025. Free radicals and their impact on health and antioxidant defenses: A review. Cell Death Discovery, Vol. 11. 10.1038/s41420-024-02278-8.
2. Manful, C.F., E. Fordjour, D. Subramaniam, A.A. Sey, L. Abbey and R. Thomas, 2025. Antioxidants and reactive oxygen species: Shaping human health and disease outcomes. Int. J. Mol. Sci., Vol. 26. 10.3390/ijms26157520.

3. Tumilaar, S.G., A. Hardianto, H. Dohi and D. Kurnia, 2024. A comprehensive review of free radicals, oxidative stress, and antioxidants: Overview, clinical applications, global perspectives, future directions, and mechanisms of antioxidant activity of flavonoid compounds. J. Chem., Vol. 2024. 10.1155/2024/5594386.
4. Alsulami, F.J. and Sadr ul Shaheed, 2024. Role of Natural Antioxidants in Cancer. In: Nutrition and Dietary Interventions in Cancer, Saeed, R.F. and Sadr ul Shaheed (Eds.), Springer Nature, Switzerland, ISBN: 978-3-031-55622-7, pp: 95-117.
5. Alara, O.R., C.I. Ukaegbu, N.H. Abdurahman, J.A. Alara and H.A. Ali, 2023. Plant-sourced antioxidants in human health: A State-of-Art review. Curr. Nutr. Food Sci., 19: 817-830.
6. Soobrattee, M.A., V.S. Neergheen, A. Luximon-Ramma, O.I. Aruoma and T. Bahorun, 2005. Phenolics as potential antioxidant therapeutic agents: Mechanism and actions. Mutat. Res. Fundam. Mol. Mech. Mutagen., 579: 200-213.
7. Liu, R.H., 2003. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am. J. Clin. Nutr., 78: 517S-520S.
8. Arzani, V., M. Soleimani, T. Fritsch, U.M. Jacob, V. Calabrese and A. Arzani, 2025. Plant polyphenols, terpenes, and terpenoids in oral health. Open Med., Vol. 20. 10.1515/med-2025-1183.
9. Wu, C.D., I.A. Darout and N. Skaug, 2001. Chewing sticks: Timeless natural toothbrushes for oral cleansing. J. Periodontal Res., 36: 275-284.
10. Al-Otaibi, M., M. Al-Harthy, B. Soder, A. Gustafsson and B. Angmar-Mansson, 2003. Comparative effect of chewing sticks and toothbrushing on plaque removal and gingival health. Oral Health Prev. Dent., 1: 301-307.
11. Abhary, M. and A.A. Al-Hazmi, 2016. Antibacterial activity of Miswak (*Salvadora persica* L.) extracts on oral hygiene. J. Taibah Univ. Sci., 10: 513-520.
12. Alili, N., J.C. Turp, E.M. Kulik and T. Waltimo, 2014. Volatile compounds of *Salvadora persica* inhibit the growth of oral *Candida* species. Arch. Oral Biol., 59: 441-447.
13. Halawany, H.S., 2012. A review on miswak (*Salvadora persica*) and its effect on various aspects of oral health. Saudi Dental J., 24: 63-69.
14. Al Bratty, M., H.A. Makeen, H.A. Alhazmi, S.M. Syame and A.N. Abdalla *et al*., 2020. Phytochemical, cytotoxic, and antimicrobial evaluation of the fruits of miswak plant, *Salvadora persica* L. J. Chem., Vol. 2020. 10.1155/2020/4521951.
15. Ellithy, M. and A. Saleh, 2025. *Salvadora persica* (Miswak) extract inhibits invasion of oral malignant cells: *In-vitro* and *in-silico* assessment. Egypt. J. Chem., 68: 577-592.
16. Ainsworth, E.A. and K.M. Gillespie, 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nat. Protocols, 2: 875-877.
17. Zilic, S., A. Serpen, G. Akıllıoglu, M. Jankovic and V. Gokmen, 2012. Distributions of phenolic compounds, yellow pigments and oxidative enzymes in wheat grains and their relation to antioxidant capacity of bran and debranned flour. J. Cereal Sci., 56: 652-658.
18. Mensor, L.L., F.S. Menezes, G.G. Leitao, A.S. Reis, T.C. dos Santos, C.S. Coube and S.G. Leitao, 2001. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother. Res., 15: 127-130.
19. Kim, K.H., R. Tsao, R. Yang and S.W. Cui, 2006. Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. Food Chem., 95: 466-473.
20. Al-Ashmawy, G.M., D.A. Labah, O.M. Wahba, M.T. Abdel Ghafar and O.A. El-Feky, 2023. Cancer chemo-preventive role of grape seed oil and cisplatin as a combination adjuvant therapy in the treatment of tongue squamous cell carcinoma: A biological *in-vitro* study. Arch. Oral Biol., Vol. 151. 10.1016/j.archoralbio.2023.105698.
21. Orellana, E. and A. Kasinski, 2016. Sulforhodamine B (SRB) assay in cell culture to investigate cell proliferation. Bio-protocol, Vol. 6. 10.10.21769/BioProtoc.1984.
22. Yokoyama, T., Y. Kosaka and M. Mizuguchi, 2015. Structural insight into the interactions between death-associated protein kinase 1 and natural flavonoids. J. Med. Chem., 58: 7400-7408.
23. Ebaid, M.S., H. Farag, M. Abdelraof, A.M. Saleh and M.G. Thabit *et al*., 2025. Design, synthesis, and biological assessment of a novel series of coumarin-tethered thiazole derivatives as potential antibacterial agents. Front. Chem., Vol. 13. 10.3389/fchem.2025.1627186.
24. Nasser, A.A., I.H. Eissa, M.R. Oun, M.A. El-Zahabi and M.S. Taghour *et al*., 2020. Discovery of new pyrimidine-5-carbonitrile derivatives as anticancer agents targeting $EGFR^{WT}$ and $EGFR^{T790M}$. Org. Biomol. Chem., 18: 7608-7634.
25. El-Demerdash, A.S., R. Alfaraj, F.A. Farid, M.H. Yassin, A.M. Saleh and G.E. Dawwam, 2024. Essential oils as capsule disruptors: Enhancing antibiotic efficacy against multidrug-resistant *Klebsiella pneumoniae*. Front. Microbiol., Vol. 15. 10.3389/fmicb.2024.1467460.
26. Ahmad, H. and N. Ahamed, 2012. Therapeutic properties of meswak chewing sticks: A review. Afr. J. Biotechnol., 11: 14850-14857.
27. Nordin, A., Aminuddin Bin Saim, R. Ramli, A. Abdul Hamid, N.W.M. Nasri and R.B.H. Idrus, 2020. Miswak and oral health: An evidence-based review. Saudi J. Biol. Sci., 27: 1801-1810.
28. Balhaddad, A.A., L. Mokeem, M.A.S. Melo and R.L. Gregory, 2021. Antibacterial activities of methanol and aqueous extracts of *Salvadora persica* against *Streptococcus mutans* biofilms: An *in vitro* study. Dent. J., Vol. 9. 10.3390/dj9120143.
29. Haque, M.M. and A.S. Alsareii, 2015. A review of the therapeutic effects of using miswak (*Salvadora persica*) on oral health. Saudi Med. J., 36: 530-543.

30. Balto, H.A., K.K. Al-Manei, T.M. Bin-Mohareb, Z.A. Shakoor and S.M. Al-Hadlaq, 2014. Cytotoxic effect of *Salvadora persica* extracts on human gingival fibroblast cells. Saudi Med. J., 35: 810-815.
31. Al-Dabbagh, B., I.A. Elhaty, C. Murali, A. Al Madhoon and A. A min, 2018. *Salvadora persica* (Miswak): Antioxidant and promising antiangiogenic insights. Am. J. Plant Sci., 9: 1228-1244.
32. Hammad, H.M., K.M. Al-Qaoud, M.M. Hammad and M.A. Mansi, 2021. Effect of *Salvadora persica* Linn root aqueous extract on oral epithelial dysplasia and oral cancer cell lines. Trop. J. Pharm. Res., 18: 2591-2596.
33. Mohany, M., M. Al-Zharani, F.A. Nasr, M.S. El-Wetidy and M. Farag *et al*., 2023. Persicaline, an alkaloid from *Salvadora persica*, inhibits proliferation and induces apoptosis and cell-cycle arrest in MCF-7 cells. Open Chem., Vol. 21. 10.1515/chem-2022-0302.
34. Farag, A.K. and E.J. Roh, 2019. Death-associated protein kinase (DAPK) family modulators: Current and future therapeutic outcomes. Med. Res. Rev., 39: 349-385.