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Uses of Salvadora Persica L. (Chewing Sticks) As A Therapeutic to Inflammation Periodontal

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ABSTRACT

Salvadora persica is a type of chewing sticks which is widely used as an oral hygiene tool in many parts of the world. Moreover, the use of miswak has been a very long tradition which is more economical, ecological and it can be used without dentifrices. Nowadays, miswak is used as the only tool for tooth cleaning in many communities in the developing world. This study was carried out to highlight the antioxidant activity, fractionation of phenolic and flavonoid compounds, and the effect of miswak sticks' extracts on microbes isolated from saliva inflammation iperiodontal, and know their beneficial effects on oral health promotion.
The results showed that the total antioxidant capacity, total phenolic acids, total condensed tannin, total flavonoids compounds and reducing powder were higher in methanol extract than in aqueous acetone and ethyl acetate extracts. The results of the identification of phenolics content and flavonoids compounds by HPLC showed that the methanol extract from Salvadora persica had contained 18 compounds from phenolic content and 14 compounds from flavonoids compounds.
The zone inhibition of measurements was conducted by the disc diffusion assay, where bacterial strains of Staphylococcus aureus, Streptococcus mutans and Candida albicans isolated from saliva inflammation periodontal showed susceptibility to Salvadora persica discs aqueous acetone, ethyl acetate, and methanol extract paper discs. The results showed that methanol extract had the greatest effect on all of the tested bacteria in all the three concentrations used.
Therefore, it could be recommended that the extract from Salvadora persica as an antimicrobe isolated from saliva inflammation periodontal caused the Salvadora persica extract to have high contained phenolic and flavonoids compounds as a natural antioxidant.

Key words: *Salvadora Persica, Chewing Sticks, Periodontal, Microbial Isolated, Phenolic Acid, Flavonoids Compounds.*

INTRODUCTION

In the past, Islamic people used miswak which became a part of their mode of life and as a prominent feature of Islamic hygienic jurisprudence. Today, cross-cultural knowledge can help the motivated public health dentists and dental hygienists to recognize culturally accepted behaviors for the purpose of strengthening patient-provider relationships and optimizing public health outcomes [1].
The sticks of miswak have been used for centuries as oral hygiene tools in many countries of the world. Many studies reported that the fresh and dried leaves, dried fruits and stems were used to treat swellings, ulcers and blisters, scorpion stings, regulating menstruation, gases and worms. Also, in the UAE, the sticks of the miswak were used as toothbrushes, and the leaves' powder was used with an oil to treat knee pains. The miswak has been incorporated into commercially available toothpastes [2-4].

Among 182 types of plants which are used as chewing sticks, the toothbrush tree, Salvadora persica L, also known as miswak from Salvadoraceae family, is one of the most prominent. It has been extensively used in many Asian, African, and Middle Eastern countries. People in these countries use the roots, twigs, and stems of this plant for oral hygiene, and they use small miswak sticks as toothpicks for keeping oral hygiene [5,6]. The aqueous and methanol extracts existing in miswak possess biological characteristics which act against plaque and periodontitis developing organisms.

According to some previous in vitro studies [1,7,8], the cariogenic bacteria and periodontal pathogens including Staphylococcus aureus, Streptococcus mutans, Streptococcus faecalis, Streptococcus pyogenes, Lactobacillus acidophilus, Pseudomonas aeruginosa, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Haemophilus influenzae, and Candida albicans are affected by the antibacterial and antifungal qualities of miswak. Moreover, the data collected from controlled clinical studies indicated that Salvadora persica extract is also an effective antimicrobial tool and used clinically in the endodontic treatment of teeth [9-11].

Alili et al [12] and Fallah et al [13] assessed the in vitro antibacterial activities of miswak extracts against 10 isolated bacteria from clinical oral pathogens including methicillin-resistant Staphylococcus aureus (MRSA), methicillin-resistant Staphylococcus epidermidis (MRSE), penicillin-resistant Streptococcus pyogenes, Enterococcus faecalis, and 6 carbapenem-resistant Gram negative bacilli: Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia marcescens, Acinetobacter baumannii, and Stenotrophomonas maltophilia.

The aim of the present work was to evaluate the antioxidant properties of different extracts of the Salvadora persica, and identify the phenolics content and flavonoids compounds using the HPLC technique. Moreover, the effect of Salvadora persica extracts on the microbial isolated from the saliva inflammation periodontal was examined.

MATERIALS AND METHODS

Materials :

A dried plant sample was used in this study. Dried stems of Salvadora persica imported from Saudi Arabia were purchased from a local market. The sample was grounded by a household grinding machine.

Methods :

Antioxidants extracts from Salvadora persica

Fresh plant samples were used. Extraction was made with three different solvents : a mixture of acetone : water (80 :20 ; v/v), ethyl acetate and methanol. The extracts of the Salvadora persica were prepared by adding 4 g of small particles of the fresh plant powdered by a commercially available food blender to 40 ml of a solvent, and allowing the mixtures to stand overnight at the room temperature. After which the supernatants were filtered and then purified by a fine mish, then sterilized by Millipore filter papers 0.45μm, and dried/evaporated under a controlled temperature (40°C), and finally their biological properties were evaluated.

Evaluation of total antioxidant capacity (TAC)

The assay was based on the reduction of the extracts according to the method described by [14]. The antioxidant capacity was expressed as mg gallic acid equivalent per gram dry weight (mg GAE/g DW). The calibration curve range was 0 to 500 μg/ml. All the samples were analyzed in triplicate.

Total phenolic content

Phenolic content was assayed using the Folin-Ciocalteu reagent, following Singleton's method slightly modified by [15]. The absorbance was measured at 760 nm, after incubation for 90 min at 23°C in dark. Total phenolic content of leaves was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE.g-1 DW) through the calibration curve with gallic acid. Triplicate measurements were taken for all the samples.

Total condensed tannins

Condensed tannins were measured using the modified vanillin assay described by [16]. The absorption was measured at 500 nm against the solvent as a blank. The amount of total condensed tannins is expressed as mg (+) - catechin g-1 DW.

Estimation of total flavonoids contents (TFC)

The total flavonoids contents were determined by Aluminum chloride method [17]. The reaction mixture (3.0 ml) comprised of 1.0 ml of extract (1 :10 dilution), 0.5 ml of aluminum chloride (1.2%) and 0.5 ml of potassium acetate (120 mM) was incubated at the room temperature for 30 min, and the absorbance was measured at 415 nm. The total flavonoids contents were expressed in terms of ascorbic acid equivalent (mg/g) [18].

Determination of reducing powder (RP)

The ability of the extracts to reduce Fe3+ was assayed by the method of Oyaizu [19]. The absorbance was measured at 700 nm. The mean of absorbance values were plotted against concentration, and a linear regression analysis was carried out. The increase in the absorbance of the reaction mixture indicated the increase in the reducing powder. The effective concentration is EC50 value (mg.ml-1) at which the absorbance was 0.5 for reducing powder. Ascorbic acid was used as the positive control.

Preparation of methanol extract

The sticks were broken into small pieces, ground in a grinding machine to a fine powder, mixed with methaol, and extracted for 24 h at 150 rpm at 25°C in a shaker. The mixture was then centrifuged at 3000 rpm for 20 min. The supernatants were subsequently filtered through Whatman No. 1 filter paper, and in a rotary evaporator (Buchi Rota vapor R-200) the filtrate was concentrated at 70°C and then lyophilized. The obtained powder was packed in a glass bottle and stored at 4°C until needed.

Quantitative determination of flavonoids by HPLC

The Dionex Ultimate 3000 liquid chromatography (Germany) with four solvent delivery system quaternary pumps (LPG 3400 SD) containing a diode array detector (DAD 3000) with 5 cm flow cell, a manual sample injection valve equipped with a 20 µl loop, was used for HPLC analyses and Chromeleon 6.8 system manager was utilized as a data processor. A reversed-phase Acclaim TM 120 C18 column (5 µm particle size, 4.6 x 250 mm) [20] was applied for separation. Solvent A containing 1% aq. Acetic acid solution and solvent B containing acetonitrile comprise The mobile phase, the flow rate was attuned to 0.7 ml/min, the column was controlled at 28°C thermostatically and the injection volume was kept at 20 µl. By changing the proportion of solvent B to solvent A, a gradient elution was achieved. The gradient elution was shifted from 10% to 40% B in a linear fashion in a period of 28 min, from 40 to 60% B in 39 min, from 60 to 90% B in 50 min. Before the injection of another sample, the mobile phase composition was returned back to the initial condition (solvent B: solvent A: 10: 90) in 55 min, and allowed to run for another 10 min. The total time for analyzing each sample was 65 min. Using a photo diode array UV detector at three different wavelengths (272, 280 and 310 nm) based on the absorption maxima of analyzed compounds, HPLC chromatograms were discerned. Each compound was detected by its retention time and impaling with standards under the same conditions. The integrated peak was measured for the quantification of the sample, and the content was determined using the calibration curve by plotting peak area against concentration of the respective standard sample. The obtained data was reported with convergence limit in triplicate.

Quantitative determination of phenolic compounds by HPLC

According to the method of [21], Phenolic compounds were determined by HPLC as follows : 5 gm of samples were mixed with methanol and centrifuged at 10000 rpm for 10 min and the supernatants were filtered through a 0.2 µm Millipore membrane filter, then 1-3 ml was collected in a vial for injection in to HPLC Hew let Packared (series 1050) equipped with autosamplling injection, solvent degasser, ultraviolet (UV) detector set at 280 nm and quaternary HP pump (series 1050). Hewlett Packard using a column Altman C18, 5mm (150mm x 4.6mm Alltech) the column temperature was maintained at 35°C. Gradient separation was carried out with methanol and acetonitrile as a mobile phase at flow rate of 1 ml/min. Phenolic acid standard from sigma Co. were dissolved in a mobile phase and injected into HPLC. Retention time and peak area were used for calculation of Phenolic compounds concentration by the data of Hewllet Packared software.

Microbial isolated

Oral inflammation periodontal saliva was collected from fifty patients of private dental clinics in Saudi Arabia in addition to ten normal samples. Oral saliva samples were collected to test the effect of Salvadora persica extracts against oral microbes.

The oral inflammation periodontal saliva was cultured directly by streaking method on nutrient agar plates (10 mm), and then was incubated at 37°C. The growing microbes were isolated, purified and identified following standard methods by [22].

The yeast pure cultures were prepared on nutrient agar (NA) with chloramphenicol (250 mg/l) to avoid bacterial growth. Germ tube test was followed to identify Candida albicans [23].

Two species of the bacteria and one of the yeasts were isolated from oral inflammation periodontal saliva and they were identified. They were Staphylococcus aureus, Streptococcus mutans and Candida albicans. The percentage of the three microbes is common in the samples shown in Table (1).

Table 1. The percentage of microbial occurrence in the samples

Microorganisms	Occurrence %*
Staphylococcus aureus	82
Streptococcus mutans	60
Candida albicans	94

*Occurrence% = (no. of samples with microorganism/50) x100

Microbial assay for inhibitory concentrations using Salvadora persica extracts

The powder of Salvadora persica was used to prepare 2.5%, 5% and 10% (w/v) of three different solvent : a mixture of acetone : water (80: 20; v/v), ethyl acetate and methanol. The plant extracts were purified by a fine mish then sterilized by Millipore filter papers 0.45µm. The agar media were inoculated by full loop of pure culture of S. aureus, S. mutants and C. albicans according to [24].

Disc diffusion assays

To make 30 mg extract impregnated disks, 1 ml of different concentration extracts from methanol or ethyl acetate or aqueous acetone was applied onto the sterile disks in 10 µl increments with sufficient time in between, to allow to dry. Plates were then incubated at 37oC for 48 h aerobically, the zone of diffusion from the well into the agar was measured in millimeters. The shortest distance (mm) from the outer margin of the well to the initial point of microbial growth was considered as the inhibitory zone according to [25]. The results were recorded as the average of the two measurements.

RESULTS AND DISCUSSION

Phytochemical in different extracts from Salvadora persica

Total antioxidant capacity, total phenolic acids, total condensed tannin, total flavonoids compounds and reducing powder were determined in ethyl acetate methanol and diluted acetone extracts from Salvadora persica or miswak, and the results are reported in Table (2). The study reveals that the antioxidant capacity of methanol extract was 5.23 and 1.63 folds higher than that of ethyl acetate and the diluted acetone extracts from miswak (225, 52.8 and 125 mgGAE.g-1 dry weight). The highest results in methanol extract might be attributed to the high presence of phytochemical as phenolics acid content and flavonoid compounds. Total phenolic acids content from miswak extract was increased in the methanol extract to 32.83 mg GAE.g-1 dry weight, while only12.4 and 4.43 mg GAE.g-1 dry weight was found in the diluted acetone and ethyl acetate extracts of miswak. Similarly, the total condensed tannin contents in methanol extract, ethyl acetate and diluted acetone extract were 13.16, 3.9 and 5.23 mg EC. g-1 dry weight, respectively. Moreover, total flavonoids compounds were higher in methanol extract (8.15 mg AAE.g-1 dry weight) than in ethyl acetate and diluted acetone which were 3.94 and 4.68 mg AAE.g-1 dry weight, respectively. Meanwhile, the obtained reducing powder indicated that in ethyl acetate and diluted acetone, the methanol extract from Salvadora persica or miswak was the highest, that is may be because of rich amounts of natural antioxidants existing in the methanol extract.

The chemical materials like flavonoids, salvadorine, cyanogenic glycosides, lignans, saponins, alkaloids, tannins, linoleic acid, steraric acid, vitamin C, silica and different salts which exist in various extracts from Salvadora persica or miswak are also recognized to have significant antimicrobial activity [26,27].

Table 2. Phytochemical components and antioxidant activities in diluted acetone, ethyl acetate and methanol from Salvadora persica extracts.

Antioxidant activities	Ethyl acetate	Methanol	Diluted acetone
TAC: (mg GAE.g-1 DW)	52.8	225	125
Total phenolic acid: (mg GAE.g-1 DW)	4.43	32.83	12.4
Tannins (mg EC.g-1 DW)	3.9	13.16	5.23
Total flavonoids (mgAAE.g-1 DW)	3.94	8.15	4.68
RP: EC50 (µg.ml-1)	176	940	299

Phenolic compounds in Salvadora persica extract

The results from Table (3) showed that the profile of phenolic content by HPLC apparatus in methanol extract from Salvadora persica had contained 18 compounds. The major compounds were Pyrogallol, Salycilic, e-vanillic, Ceumarin, Benzoic, Ellagic, Protocatchuic, Ferulic, Caffeine, Chlorogenic, Catechein, P-OH-benzoic and Gallic had contained 886.5, 764.31, 729.24, 651.26, 428.94, 301.25, 213.43, 175.56, 152.18, 129.56, 120.1, 116.27 and 103.86 mg/100g^{-1}, respectively. Moreover, the other compounds from S. persica methanol extract were Vanillic, P-coumaric, Epicatachin, Caffeic, 3,4,5-methoxy-cinnami, Catechol, Cinnamic, Alpha-coumaric and 4-Amino-benzoic had contained from 95.75 to 21.63 mg/100g^{-1}, respectively. [28] observed that Salvadora persica or miswak is rich in phenolic compounds often known to have antimicrobial activity against other various bacteria involved in numerous oral diseases such as periodontitis and dental caries.

Phenolic acids play an important role in combating oxidative stress in the human body by maintaining a balance between oxidants and antioxidants. Polyphenolic compounds are secondary metabolites present in many plant species. Their content depends on various factors, such as cultivar, climatic and cultivation conditions [29].

Table 3. Profile of phenolic compounds in Salvadora persica extract

Phenolic compounds	(mg/100g^{-1}) phenolic	Phenolic compounds	(mg/100g^{-1}) phenolic
Pyrogallol	886.5	P-OH-benzoic	116.27
Salycilic	764.31	Gallic	103.86
e-vanillic	729.24	Vanillic	95.75
Ceumarin	651.26	P-coumaric	80.98
Benzoic	428.94	Epicatachin	69.31
Ellagic	301.25	Caffeic	58.71
Protocatchuic	213.43	3,4,5-methoxy-cinnami	49.71
Ferulic	175.65	Catechol	42.18
Caffeine	152.18	Cinnamic	31.68
Chlorogenic	129.65	Alpha-coumaric	25.33
Catechein	120.1	4-Amino-benzoic	21.63

Flavonoids compounds in Salvadora persica extract

Total flavonoids compounds were identified in methanol extract from Salvadora persica and the results are reported in Table (4). From the resultants, it could be noticed that the major compounds in the methanol extract were Acacetin, Hespirdin, Luteolin, Rosmarinic, Narengin, Quercetrin and Apignin 7-glucose which contained amounts were 584.38, 108.96, 102.92, 86.54, 62.13, 51.9 and 44.48 mg/100g, respectively. The other flavonoids compounds less than 40 mg/100g-1 were Hespirin, Quercetrin 3-o-glucoside, Naringenin, Kampferol, Rutin and Apegnin. The obvious results were confirmed by the study of [30] who found the presence of several flavonoids compound in the miswak extracts such as (Kaempferol, quercetin, quercetin rutin and quercetin glucose). Moreover, [31] reported that two flavanols which are belonging to the class of flavonoids have been identified in the miswak extracts including ; catechin and epicatechin. Catechins and their derivatives are very strong antioxidants.

Salvadora persica also possess the flavonoids and flavonoid glycosides like Kaempferol, Quercetin, Kaempferol 3-α-L-rhamnosyl 7-β-xylopyranoside, iso rhamnetin-3-O-robinobioside, kaempferol3-O-robinobioside, narcissin, kaempferol-3-O-rutinoside,isorhamnetin-3-O-β-galactoside,astragalin,isorhamnetin-3-O-β Dglucoside,isorhamnetin-3-(2,6-dirhamnopyranosylgalactopyranoside), Mauritanian, isorhamnetin-3-O-(2-Glcrhamnosylrutinoside) and kaempferol 3-O-(2-Glcrhamnosylrutinoside) [32].

The zone inhibition of measurements were conducted by the disc diffusion assay, where bacterial strains of Staphylococcus aureus, Streptococcus mutans and Candida albicans isolated from oral inflammation periodontal

saliva showed susceptibility to Salvadora persica discs aqueous acetone, ethyl acetate and methanol extract paper discs, the results are shown in Table (5).

The results obtained in this study, indicated that among all the three concentrations used, methanol extract had the greatest effect on all of the tested bacteria, whereas the aqueous acetone extract demonstrated less inhibition, especially on Salvadora persica and C. albicans. The least zone of inhibition among the three solvents was observed in the ethyl acetate extract. C.albicans showed less susceptibility to ethyl acetate extract than the aqueous acetone or methanol extracts. In the alcoholic extract, the highest value of the measured inhibition zones was observed, and it indicated the effect of alcohol extract on pathogenic bacteria [33].These findings show that there is a mixture of antimicrobial agents with different polarities in Salvadora persica chewing sticks which act against gram positive and negative bacteria [34]. Also, these results have induced that the different extracts of S. persica possess various biological properties, including significant antimicrobial [35] and anti-inflammatory [36] properties, and lack of toxicity [37]. The antimicrobial and cleaning effects of Salvadora persica may be attributed to various chemicals contained in its extracts such as trimethyamin, salvadorine, chloride, fluoride in large amounts, silica, sulfur, mustard, vitamin C, saponins, tannins, cyanogenic glycoside, and benzylisothiocyanate [26,38]. Salvadora persica has demonstrated cleansing efficacy, ability to remove the plaque, and decrease ginigival bleeding [39] when used as a chewing stick. As a mouth wash, Salvadora persica has improved periodontal health, reduced microbial plaque accumulation and lowered carriage rate of cariogenic bacteria [40,41].

Table 4. Profile of flavonoids compounds in Salvadora persica extract

Flavonoids compounds	Flavonoids (mg/100g)	Flavonoids compounds	Flavonoids (mg/100g)
Acacetin	584.38	Hespirin	38.92
Hespirdin	108.96	Quercetrin 3-o-glucoside	25.84
Luteolin	102.92	Naringenin	19.51
Rosmarinic	86.54	Kampferol	15.69
Narengin	62.13	Rhamnetin	10.95
Quercetrin	51.9	Rutin	5.89
Apignin 7-glucose	44.48	Apegnin	2.27

Antimicrobial effects using different extracts from Salvadora persica

To compare ethanol and hot water extracts on growth of isolated microbes, plant extracts were used to prepare the culture media. The results showed that the ethanolic extract has a higher antimicrobial activity than aqueous extracts against bacterial and yeast isolates. Such effects may be related to several chemical compounds found in miswak sticks including trimethylamine, salvadorine (alkaloids), chlorides, fluorides, sulfur, tannins, flavonoids and sterols [42]. Moreover, Tannic acid may have caused such effect on C. albicans [43].

All collected samples showed microbial growth on nutrient agar plates. The patients' swabs and mouth washing samples showed more intensive microbial growth than normal swabs.

The solvents may give different results, so do the age of plant parts used in preparing the extracts. Alcoholic extracts showed considerable effects, which were increased by the extract concentrations of shoots, and these results agreed with [44] and [45].

In all tests followed here, the inhibition effects of extracts against microbial growth were increased from 2.5%, 5% and 10%, respectively (Table 2). These method and inhibition zones give the same results which agree with [46] who found using water extract reported (20mm and 24mm) as an inhibition zones for S. aureus and S. mutans, respectively. The differences of the present study may be due to the degree of miswak extract concentration, or/and are related to different bacterial isolates.

Salvadora persica exhibited significant antimicrobial activity against aerobic and anaerobic bacteria which were collected from teeth by many researchers in the various parts of world. Moreover, many studies have reported that Salvadora persica extracts were effective against Streptococcus mutans [47].

Table 5. The inhibition zone (mm.) of stems extracts according to different solvents and concentration

Salvadora persica extracts	Staphylococcus aureus			Streptococcus mutans			Candida albicans		
	2.5	5.0	10.0	2.5	5.0	10.0	2.5	5.0	10.0
Aqueous acetone	4	3	1	6	4	1	4	2	1
Ethyl acetate	10	8	3	11	9	3	7	3	1
Methanol	13	10	3	18	14	4	11	8	4

CONCLUSION

From the results, the use of miswak as an inexpensive, simple and also effective oral hygiene tool in many zones could be recommended. The uses of miswak have been widely examined in many areas around the world where miswak can play a major role in the development of oral hygiene. As a result, the dental profession should become acquainted with the application of miswak within its traditional customs.

The World Health Organization [48] has suggested that the use of these sticks, known as miswak, as a tool for oral hygiene in regions where their use is effective to obtain optimum oral health and hygiene. Miswak (Salvadora persica) can be used alone or as an adjunct to a traditional toothbrush. Hence, miswak use should be encouraged and promoted based on scientific knowledge of its numerous therapeutic effects on oral health and its easy availability. Therefore, achieving the optimum effects of miswak (Salvadora persica) depends on its regular use with effective techniques.

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