Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS Research Journal of Microbiology

ISSN 1816-4935
DOI: 10.3923/jm.2017.58.73

Research Article

In vitro Study of the Antimicrobial Effects of Phenolic Extract of the *Salvadora persica* (Miswak) on the Growth of Certain Microorganisms Responsible for Oral Infections

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Abstract

Background and Objective: *Salvadora persica*, commonly known as the miswak tree or the toothbrush tree is thought to contain a number of phenolic compounds. The objective of this study is to identify these phenolic compounds and to evaluate their antimicrobial effects on the growth of some germs implicated in certain oral infections. **Materials and Methods:** Phenolic ethanol extracts were obtained by vacuum evaporation of hydroalcoholic solutions after extraction from varying amounts of crushed root, bark and stem of the test plant. The resulting pure extracts were then diluted with sterile distilled water at different increasing ratio from 0-100%. The phenolic compounds were analyzed by the HPLC method. The antimicrobial effects of these extracts were tested on many reference germs. The antimicrobial activity was tested by monitoring the growth of the germs in specific media while using disk diffusion assays. The Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of the plant extracts were determined according to the micro broth dilution technique. Data were statistically analyzed by ANOVA and the Newman-Keuls test. **Results:** The qualitative analysis revealed that chlorogenic acid, catechin and epicatechin emerged as major phenolic compounds from root and stem of *Salvadora persica*, while bark extracts were rather rich in caffeine, theobromine and trigonelline. The MIC and MFC of *Candida albicans* were obtained with 40% phenolic extracts of the stem. The data seems to indicate that stem extracts caused a fungicidal action against *Candida albicans*. The growth of *Streptococcus mutans* was not affected by the different solutions of phenolic extracts. However, other bacteria belonging to *Streptococcus* genus such as *Streptococcus mitis* and *Streptococcus faecalis* and those belonging to *Staphylococcus* genus including *Staphylococcus aureus* and *Staphylococcus epidermidis* and the *Lactobacillus casei* were completely inhibited with the extracts prepared at 7.5 g of vegetal matter. **Conclusion:** The antimicrobial effects of phenolic extracts of miswak coming out of this study were close to those described in the study by most researchers. These extracts could be used as a medicament to prevent and to cure oral diseases in Algeria.

Key words: *Salvadora persica*, phenolic compounds, antimicrobial, oral, germs, infections

Received: August 11, 2016 Accepted: November 19, 2016 Published: December 15, 2016

Citation: Ouiza Ait Chabane, Djamel Ait Saada, Ahmed Mohamed Ali Bekada, Ghalem Selselet-Attou, Kaddour Bouderoua, Djamel Eddine Kati and Noel Durand, 2017. *In vitro* study of the antimicrobial effects of phenolic extract of the *Salvadora persica* (Miswak) on the growth of certain microorganisms responsible for oral infections. Res. J. Microbiol., 12: 58-73.

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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

Currently, using fluoride, consuming non-cariogenic foods and treatment with sanitary products whose composition is very diverse (alcohol, detergents, organic acids, dyes, etc.) have effectively contributed to the maintenance of good oral health as increasingly less people suffer from dental caries. This is especially the case in developed countries[1-3]. However, in developing countries preventive measures and practical strategies to reduce risks of oral diseases are still insufficient. This is due to the low standard of living of the population, lack of odontological medical structures in rural areas and ineffective awareness strategies[4,5]. Therefore, the extraction of decayed teeth remains the only accessible treatment for swathes of the population in these countries.

In addition to conventional treatments, some Asian, African, American and European populations also used traditional treatments for a long time. To these days, these populations consider that some plant sticks prepared from the roots and stems of some plants, particularly of *Salvadora persica* or other vegetal essences rich in many bioactive compounds (such as *Citrus mantafolia*, *Citrus sinensis*, *Azadirtachta indica* etc.) as an effective protective practice against oral diseases[6-9].

Commonly known as "Miswak or siwak" by Asian and African populations, it is well established that sticks from the *Salvadora persica* plant have interesting beneficial medicinal properties and can be used as a natural means to prevent dental caries and gum swelling[10-15]. In addition to their use as a means for cleaning teeth and mouth, these sticks have been used to treat some chronic diseases such as: Splenomegaly, rheumatism, tumors, gonorrhea, kidney stone, gastritis, diabetes etc.[16-25]. The World Health Organization (WHO)[26] also recommends and encourages the use of *Salvadora percica* as an effective way to achieve a good oral hygiene. A new treatment approach for oral health combining the use of vegetal sticks and prevention may thus be envisioned and implemented.

A special interest should be given to the present line of research in developing countries characterized by arid or semi-arid climates where in *Salvadora persica* belongs to these agro-forestry systems[27]. It is also an interesting bio-resource, which could be used, without financial burden, in favor of poor populations.

Knowledge of the principal compounds of *Salvadora persica* involved in the inhibition of microorganisms that cause oral infections (candidiasis, gingivitis, periodontitis and dental caries) in humans is very limited so far. However, numerous studies conducted by Farooqui and Srivastava[28], Ezmirly *et al.*[29], Malik *et al.*[30], Hardie and Ahmed[31], Al Sadhan and Almas[32], Al-Otaibi *et al.*[33], Sofrata *et al.*[34], Al-Ghonaiem *et al.*[35], Mariod *et al.*[36], Sofrata *et al.*[37], Halawany[38], Zafar and Ahmed[39] and Ngule *et al.*[40] have indeed confirmed that it contains several biologically active substances such as N-benzyl-2-phenylacetamide, benzyl isothiocyanate, chloride, sulfur, sulfate, nitrate, NaCl, KCl, sulfurized organic substances (salvadourea and salvadorine) and trimethylamine (alkaloid). It also contains an important number of phenolic compounds such as tannins and flavonoids, which exert antimicrobial activity against many germs causing multiple oral infections in humans, including: *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus salivarius*, *Streptococcus faecalis*, *Streptococcus mitis*, *Streptococcus pyogenis*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Haemophilus influenzae* and *Candida albicans*[9,12,14,40-46].

In this context, this study aims to identify the essential components of the phenolic compounds of *Salvadora persica*, which is found in abundance in the region of Aine Salah region located in the South of Algeria. The study proceeds by evaluating their potential effectiveness in maintaining oral hygiene through the study of their *in vitro* antimicrobial effects against certain bacteria (*Candida albicans*, *Lactobacillus casei*, *Streptococcus mitis*, *Streptococcus mutans*, *Staphylococcus epidermidis*, *Streptococcus faecalis* and *Staphylococcus aureus*) which are understood to be involved in various oral diseases.

MATERIALS AND METHODS

Sampling area and pretreatment of the studied plant: The plant species *Salvadora persica*, used in the experimental study was taken from the Arak region at 3°.70' East longitude and 25°.28' North latitude to about 1500 km North of the town of Tamanrasset going towards the town in Aine Salah in the South of Algeria. This plant is a protected species, well adapted to the soil and grows in the wild state in the study area. Branches of stem and root once picked up were cut into sticks of 10-12 cm in length, then spread over paper, dried in the shade for 15 days and stored in a dry place for analysis and subsequent use.

Extraction method of phenolic compounds: According to Almas and Al-Bagieh[47] and Almas[16], crude water extracts generally act on bacterial growth, particularly *Streptococcus mutans* and *Streptococcus faecalis* at extraction rates of 5 g

100 mL of plant material of kikar (*Acacia arabica*) found in Pakistan and arak (*Salvadora persica*) found in Saudi Arabia.

In a first experimental approach, the extraction of the main phenolic compounds of *Salvadora persica* was carried out on weight-differentiated samples, each with six repetitions for each part of the plant (root, stem and bark) at rates of (0, 2.5, 5, 7.5, 10, 12.5, 17.5 and 15 g) successively. The samples were crushed and subjected to a hydro-alcoholic extraction of polyphenols constituent by the method described by Sultana *et al.*[48]. The samples of plant material were each extracted with aqueous methanol (ethanol:water, 80:20 v/v) (100 mL) for 6 h at room temperature in an orbital shaker. Then, the extracts were filtered through a Whatman paper filter and freed of solvent under reduced pressure at 45°C, using a rotary evaporator. The obtained extracted solutions were stored in a refrigerator (-4°C), until they were used in the analyses.

In another experimental approach, the extraction was performed only on the stem of plant, which is thought to contain more polyphenols than the root and the bark. The extraction of phenolic compounds was repeated three times on samples of 10 g crushed plant material. Each powder sample was then mixed with (100 mL) of ethanol aqueous solution (80/20, ethanol/water, v/v) for 6 h at room temperature in an orbital shake. The duration of the extraction thus promotes depolymerization of the main components such as lignin, pectic substances and better solubilization of polyphenols. The extract solutions were filtered using Whatman paper filter and freed of solvent under low pressure at 45°C[48]. The aqueous extracts rich in polyphenols obtained were diluted with sterile distilled water at varying concentrations of 0 (water), 20, 40, 60, 80 and 100%.

Determination of Total Phenolic Content (TPC): The amount of total phenolic content were assessed using the Folin-Ciocalteu reagent[49]. Briefly after drying the phenolic extracts in a solution, the crude extracts (50 mg) were mixed with Folin-Ciocalteu reagent (0.5 mL) and deionized water (7.5 mL). The mixture was kept at room temperature for 10 min and then 20% sodium carbonate (w/v, 1.5 mL) was added. The mixture was heated in a water bath at 40°C for 20 min and then cooled in an ice bath. Absorbance was read at 755 nm using a spectrophotometer. Amounts of TPC were calculated using gallic acid calibration curve. All samples were analyzed thrice and the results were expressed as Gallic Acid Equivalents (GAE) mg/100 g of Dry Weight (DW) of plant matter [GAE mg/100 g DW] or as Gallic Acid Equivalents (GAE) mg/100 mL of hydralcolic extract (HAE) [GAE mg/100 mL HAE].

Activation of inoculums: The study involved a yeast species deemed to be responsible for oral candidiasis in immunocompromised men particularly affected by aids[50,51]. This is a reference strain of *Candida albicans* labeled with the code of (ATCC 10231) in the Pasteur Institute of Algiers databank (Algeria). The microbial species was first activated before its experimental use. A sample of the *Candida albicans* strain was set out on an agar medium for conservation by using a platinum loop. It was cultured in 10 mL of nutrient broth at 37°C for 3 h, then 0.2 mL of the resulting solution was taken and seeded on the surface of a solid medium of agar namely Sabouraud and the mixture was finally incubated at 37°C for 24 h.

The effects of the phenolic extracts of *Salvadora persica* were also tested on the growth of certain microbial species causing multiple oral infections (periodontitis, dental caries and gingivitis) including: *Lactobacillus casei*, *Streptococcus mitis*, *Streptococcus mutans*, *Staphylococcus epidermidis*, *Streptococcus faecalis* and *Staphylococcus aureus*. These microbial species, referenced were stored at 4°C in an agar medium for conservation and were each initially activated at 37°C for 3 h in 10 mL of a nutrient broth. Each activated species was subsequently cultured either on the surface of or inside a specific agar medium at incubation temperatures of 37°C for 24 h (Table 1).

Table 1: Microbial strains tested

Microbial species	Provider	Reference	Culture medium	Culture type
Lactobacillus casei	Lal'food probiotics and health ingredients in food-114th Terr., 66210 Overland park (KS)	ATCC334	MRS (Man, Rogosa et Sharpe)	On surface
Streptococcus mitis		ATCC6249	M 17	
Streptococcus mutans		ATCC700610		
Staphylococcus epidermidis	Lyo-San. Inc., CP 598, 500 Boulevard of LACHUTE airpark, Québec, J8H4G4	ID32 Staph., test	Chapman	
Staphylococcus aureus	Regional Laboratory Veterinary of Mostaganem-Algeria	ATCC33862	Baird Parker	
Streptococcus faecalis		Clinical strain	Slanetz	Inside
Candida albicans	Pasteur institute of Algiers-Algeria	ATCC 10231	Sabourauld	On surface

Direct contact method: A colony of each species of microorganisms activated on specific agars was collected using a sterile platinum loop. Each was then inoculated into a tube containing 10 mL of nutrient broth, followed by incubation at 37°C for 3 h. From these solutions, which constitute a stock solution of tested microorganisms, increasing isotopic decimal dilutions in physiological water were prepared: up to 10^{-11} for *Streptococcus mutans* and *Streptococcus mitis* from 10^{-2} to 10^{-5} for *Candida albicans* and other studied germs (*Lactobacillus casei*, *Staphylococcus epidermidis*, *Streptococcus faecalis* and *Staphylococcus aureus*). Samples of 1 mL of each final dilution fractions were then individually added to 9 mL of a sterile solution of distilled water (control) and to 9 mL of each phenolic extract (root, stem and bark) of *Salvadora persica*. The solutions mixtures were finally seeded, respectively on the surface at 0.2 mL or inside at a depth of 1 mL of three petri dishes containing the specific medium of growth for each microbial species. The reading of the developed colonies was performed after incubation of inoculated media for 72 h at 37°C for *Lactobacillus casei* as well as *Candida albicans* and after 24-48 h for other studied organism[52].

Disc diffusion assays: Discs of 5 mm in diameter were made from filter paper (Whatman No. 3). To avoid any risk of contamination with exogenous bacteria during testing, the discs were thermised at 65°C for 30 min in an oven. A *Candida albicans* colony taken from the agar medium of Sabouraud after activation was seeded in 10 mL of nutrient broth; this mixture constitutes the stock solution. A number of samples with volumes of 1 mL of the latter solution were separately spread out on the surface of several petri dishes containing the Sabouraud medium. Three disks soaked for 5 min in each test solution of phenolic extracts, as well as in a solution containing a powerful fungicide whose fungizone[53] were then successively deposited on the surface of each petri dish containing the agar medium of Sabouraud seeded with *Candida albicans*. The reading of the inhibition diameters was performed after incubation of the boxes at 37°C for 24 h[54].

Minimal inhibitory concentration: The minimal inhibitory concentration is the lowest concentration of antibiotic, antifungal and/or other bio-active compounds required to inhibit the growth of a microorganism[55]. In the case of this study, the phenolic extracts of the stem of miswak were used to determine the minimum inhibitory concentration of yeast species responsible for oral thrush. Thus, a young colony of *Candida albicans* taken using a platinum loop was deposited into 10 mL of nutrient broth and then incubated for 3 h at 37°C to obtain the inoculum. Samples of 0.2 mL of inoculum were introduced respectively into 2 mL of each stem extract of miswak diluted with the Mueller Hinton broth. The tubes containing the mixture of phenolic extracts of *Salvadora persica* prepared at different concentrations (0, 20, 40, 60, 80 and 100%) and the inoculum of *Candida albicans* were then incubated at 37°C during 18-24 h[56]. Determination of the Minimum Inhibitory Concentration (MIC) was performed by measurements induced by growth of the studied microorganism. Thus, the MIC corresponds to the smallest concentration for which there is no turbidity. Therefore this is the first tube where the di value is equal to d_f ($d_f = d_i$). The survival rate of the microorganism was measured by a spectrophotometer adjusted at 560 nm as follows:

$$S = \frac{d_f - d_i}{D_f - D_i} \times 100$$

where, S is survival rates of microorganism (%), d_i-d_f is optical density difference in the phenolic solution seeded with the studied germ before and after incubation at 37°C for 18 h, D_f- D_i is optical density difference in a water solution distilled without extracts of *Salvadora persica* seeded with the studied germ before and after incubation at 37°C during 18 h[57,58].

Minimal Fungicidal Concentration (MFC): The minimal fungicidal concentration of the species *Candida albicans* represented the smallest concentration of phenolic extract of miswak leaving at least 0.01% of the initial inoculum surviving after incubation[56]. For its determination, the control tube (inoculum) was diluted with physiological water to 10^{-4}. This dilution constitutes 0.01% of survival of the microorganism. It was inoculated by streaking of 5 cm on Mueller Hinton agar and incubated at 37°C during 24 h. The number of the yeast colonies obtained on streak of the dilution 10^{-4} was compared to that of each test tube containing the inoculum, also inoculated on the same culture medium in 5 cm streak and incubated at 37°C for 18-24 h. Thus, the first experimental tube of which the number of colonies present on its streaks is lower or equal to that 10^{-4} dilution corresponds to the MFC.

Physicochemical measures: The dry matter was measured after drying a sample from the plant of *Salvadora persica* to a constant mass in an isothermal oven at a temperature of (105±1°C) during 24 h[59]. The ash content is conventionally the residue of the sample after the destruction of organic matter by incineration for 3-4 h at 550°C in a muffle furnace[60]. Organic matter was determined by the difference between the dry matter and mineral matter.

In the presence of sodium salicylate, nitrates contained in an extraction solution of water give sodium paranitrosonylate

colored yellow and likely to be determined by colorimetry at 415 nm[61]. The principle of determining of sulfate ions levels in an aqueous solution is to precipitate these ions in the presence of barium chloride in hydrochloric acid medium in the form of barium sulfate and then stabilize the precipitated material using a stabilizer. Nephelometric measurement of the test solution was made at a wavelength of 650 nm while determining the concentration of sulfate contained in the specimen is performed by comparison to a standard curve[62]. The method for determining chloride ion concentration in aqueous medium was based on the combined determination of chlorine in the form of chloride with silver nitrate in the presence of potassium chromate as indicator[63]. The method used to determine the fluoride ions concentration in water extracts of *Salvadora persica* was based on the analysis of the potential difference between a selective fluoride ion electrode and a reference electrode, at a pH between 5 and 8 and temperature and total ionic force of the constant medium. The determination of the fluoride ion concentration was performed by direct reading on a calibration curve[64].

Profile of polyphenols and alkaloids compounds in root, stem and bark were determined by HPLC SHIMADZU model on phenolic extracts obtained, respectively on plant materials of *Salvadora persica* species studied by the method of Jerez *et al.*[65]. Samples of 2 g of powder (bark, stem or root) of the plant after grinding was dissolved in 100 mL of methanol at 98°C (Carlo Erba, Milano, Italy). The mixture prepared in bottles of 100 mL while stirring at 140 rpm was left at room temperature for an extraction time of 20 h because of the woody character of the vegetable material, the long extraction time promotes depolymerization of the main components such (lignin and pectin substances) and generates a gradual solubilization of polyphenols. Phenolic extracts were then filtered through a filter (cellulose nitrate-sartorius) with a porosity of 0.2 µm.

Statistical methods: Data was analyzed using the Stat box 6.4 software and is expressed as Mean and Standard Deviation (SD). Parametric values were compared with one way ANOVA and Newman-Keuls test. The level ($p<0.05$) was considered as the cut-off value for significance.

RESULTS

Chemical composition: The phenolics quantified in stem were remarkably ($p<0.01$) higher than in root and bark of *Salvadora persica*, 411.91 vs 195.23 vs 207.12 (GAE) mg/100 g DW (Fig. 1).

The bark showed a significantly higher amount ($p<0.01$) of dry matter (93.367% RW) compared to the two other parts of the plant, namely the stem (91.37% RW) and the root (88 and 68% RW). As for mineral material, high levels ($p<0.01$) were observed in bark (13.06% DW), while low levels ($p<0.01$) were detected in the root (9.78% DW). Furthermore, stem and bark have comparable values in ash ($p>0.05$): 12.24% vs 13.06% of DW, respectively. The organic matter level in stem (87.76% DW) was statistically ($p>0.05$) similar to what was found in the bark (86.94% DW), whereas, these rates were significantly ($p<0.01$) lower than the root (90.22% DW). The nitrate concentrations in water extracts of the root (00.34 mg/100 mL) and the stem (00.29 mg/100 mL) are comparable ($p>0.05$) and significantly ($p<0.01$) lower than those in the bark (02.65 mg/100 mL) of *Salvadora persica*. In addition, the crude extracts of the root and the stem recorded similar levels of sulphate with values varying ($p>0.05$) from 08.38-10.51 mg/100 mL. These values are significantly lower ($p<0.01$) than those measured in the bark solution (19.57 mg/100 mL). The water extracts of the stem are highly loaded ($p<0.05$) in chlorine ions, more than the root and the bark (37.27 vs 19.52 vs 12.75 mg/100 mL). Finally, there was no detection of fluoride in the different aqueous extracts of the studied plant (Table 2).

Profile of phenolics and alkaloids: 5-O-caffeoylquinic acid and 4,5-O-Dcaffeoylquinic acid are major phenolic compounds found in the root. The stem is very rich in

Table 2: Variation in chemical composition of the root, stem and bark of *Salvadora persica*

Chemical composition	Root	Stem	Bark	Effect of plant parts
Dry matter (RW%)	88.68±00.91^c	91.37±00.84^b	93.367±01.06^a	**
Mineral matter (DW%)	09.78±00.55^b	12.24±00.44^a	13.06±00.42^a	**
Organic matter (DW%)	90.22±00.55^a	87.76±00.44^b	86.94±00.42^b	**
Nitrates (mg/100 mL)	00.34±00.3^b	00.29±00.22^b	02.65±01.97^a	**
Sulfates (mg/100 mL)	10.51±06.53^b	08.38±08.27^b	19.57±01.92^a	**
Chlorine ions (mg/100 mL)	19.52±00.19^b	37.27±00.29^a	12.75±00.43^c	**
Fluorides (mg/100 mL)	00.00	00.00	00.00	NS

For each group the number of repetitions n is equal to 5. Results expressed as mean value followed by the corresponding standard deviation, NS: Not significant effect ($p>0.05$), **Highly significant effect ($p<0.01$). Means of each category followed by different letters are significantly different at 5% level of probabilities, DW: Dry weight, RW: Raw weight

Table 3: Variations in certain phenolic compounds and alkaloids in the stem, root and bark of *Salvadora persica* (PCA%)

Profile of phenolic compounds and alkaloids			Root	Stem	Bark	Influence of plant parts
Chlorogenic acids	C3Q	3-O-caffeoylquinic acid	00.08±00.04	00.45±00.02	01.70±00.95	NS
	C5Q	5-O-caffeoylquinic acid	02.65±01.37	04.88±02.48	02.63±00.41	NS
	C4Q	4-O-caffeoylquinic acid	00.00±00.00^{c}	01.57±00.04^{a}	00.55±00.07^{b}	**
	F5Q	5-O-feruloylquinic acid	00.20±00.06	01.22±0.45	02.53±01.85	NS
	Di3,4CQ	3,4-O-Dcaffeoylquinic acid	00.37±00.10	00.65±00.20	00.63±00.13	NS
	Di3,5CQ	3,5-O-Dcaffeoylquinic acid	00.32±00.05^{b}	06.76±03.12^{a}	00.79±00.20^{b}	*
	Di4,5CQ	4,5-O-Dcaffeoylquinic acid	02.01±01.00	00.75±00.25	02.07±01.53	NS
Kaempherol	Flavonol		00.00±100.00^{c}	00.45±00.02^{a}	00.09±00.01^{b}	**
Narengenine	Flavanones		00.00±00.00^{b}	00.00±00.00^{b}	00.16±00.03^{a}	**
Quercetin	Flavonol		00.00±00.00^{c}	00.24±00.02^{a}	00.07±00.03^{b}	**
Catechin	Flavonol		00.11±00.04^{c}	07.20±00.51^{a}	02.42±01.15^{b}	**
Epicatechin	Flavonol		00.65±00.03^{b}	06.35±00.53^{a}	00.00±00.00^{b}	**
Trigonelline	Alkaloid		01.00±00.59^{b}	00.54±00.29^{b}	04.31±00.11^{a}	**
Theobromine	Alkaloid		00.04±00.01^{b}	00.36±00.14^{b}	01.24±00.15^{a}	**
Cafeine	Alkaloid		00.08±00.01^{b}	00.00±00.00^{c}	00.88±00.02^{a}	**

For each group the number of repetitions n is equal to 3 (n = 3). Results expressed as mean value followed by corresponding standard deviation, NS: Not significant effect (p>0.05), *Significant effect (p<0.05), **Highly significant effect (p<0.01). Means of each category followed by different letters are significantly different at 5% level of probabilities, PCA%: In percent of phenolic compounds and alkaloids

Table 4: Influence of the polyphenol extracts of *Salvadora persica* on the proliferation of *Candida albicans*

		Concentrations of ethanol extracts of the stem of *Salvadora persica*						
Measures	Fungizone	100% (62.73 mg GA)	80% (50.19 mg GA)	60% (37.64 mg GA)	40% (25.09 mg GA)	20% (12.55 mg GA)	0% control test	Effects of extracts solution
Diameter of inhibition (mm)	18.67±02.08^{a}	12.00±00.00^{b}	09.33±01.16^{bc}	09.33±01.16^{bc}	07.00±01.00^{c}	06.67±01.15^{c}	-	**
Inhibition rate (%)	100^{a}	64.17^{b}	49.89^{bc}	49.89^{bc}	37.43^{c}	35.67^{c}	-	*
Growth level (CFU mL^{-1})	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}	84 10^{4b}	115 10^{4a}	**

For each group (n = 5), results expressed as mean value followed by corresponding standard deviation, NS: Not significant effect (p>0.05), *Significant effect (p<0.05), **Highly significant effect (p<0.01), GA: Gallic acid, CFU: Colony forming unit

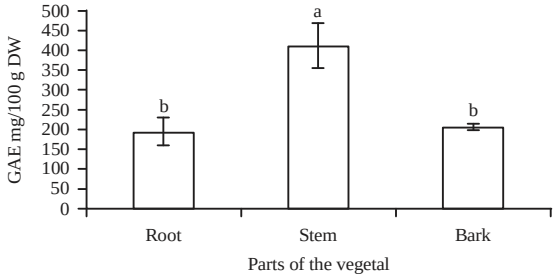

Fig. 1: Variations of phenolic contents in the root, stem and bark of *Salvadora persica*

5-O-caffeoylquinic acid, 3,5-O-Dcaffeoylquinic acid, catechin and epicatechin. The bark showed a high content in 5-O-caffeoylquinic acid, in narengenine and in some alkaloids such as: Caffeine, theobromine and trigonelline (Table 3).

Antimicrobial effects of ethanol extracts of the stem of *Salvadora persica* on *Candida albicans*: Fungizone showed better inhibitory effect on the growth of *Candida albicans* than the hydroalcoolic extract of stem of *Salvadora persica* (p<0.01) with diameters of inhibition of 18.6 vs 12 mm. The rate of inhibition of this yeast species was proportional to the variable concentrations of 20, 40, 60, 80 and 100% of stem extracts of miswak (p<0.05): 35.67, 37.43, 49.89, 49.89 and 64.17%, respectively. The direct contact method, also confirmed complete inhibition of the proliferation of *Candida albicans* in the solutions prepared at 40, 60, 80 and 100% of stem extracts of miswak rich of polyphenols (Table 4).

Minimal Inhibitory Concentration (MIC) of *Candida albicans*: The solution of phenolic extract of the stem prepared at 20% recorded a survival rate of 5.45% for the fungal species of *Candida albicans*, whereas at higher levels of phenolic extracts this microorganism has been unable to survive after 18 h of incubation at 37°C. It is especially noticed that in extracts solution prepared from 40-100%, the growth of *Candida albicans* is stymied completely. The solution at 40% of phenolic extract of stem of *Salvadora persica* was therefore selected as the minimum inhibitory concentration of this yeast species (Table 5).

Minimal Fungicidal Concentration (MFC) of *Candida albicans*: Through Fig. 2 it appears that the solution

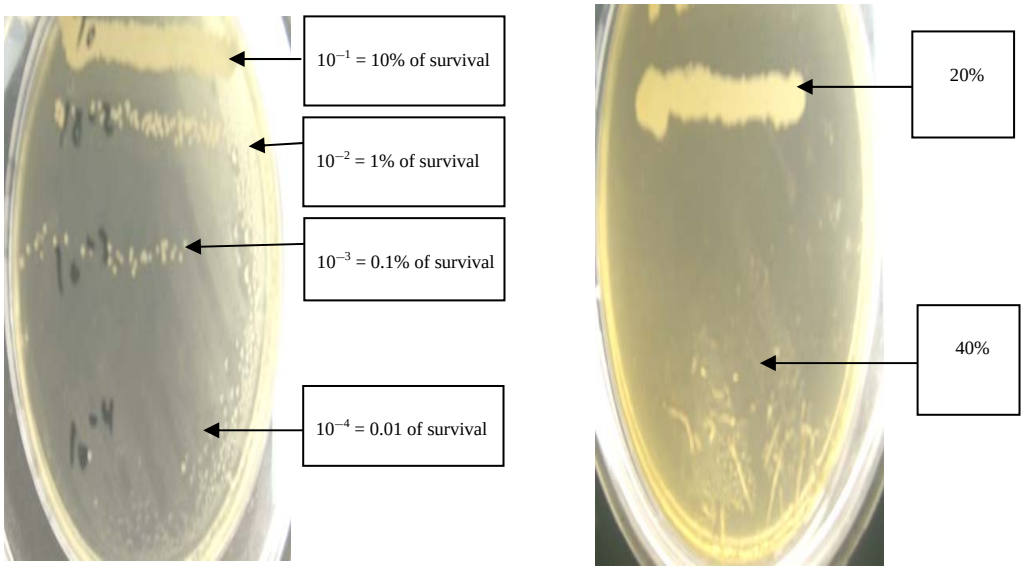

Fig. 2: Determination of Minimum Fungicidal Concentration (MFC) of phenolic extracts of *Salvadora persica* in *Candida albicans*

Table 5: Evaluation of Minimum Inhibitory Concentration (MIC) of phenolic extracts of *Salvadora persica* on the growth of *Candida albicans*

		Concentrations of ethanol extracts of the stem of *Salvadora persica*				
Parameters	Control test (0 mg AG)	20% (12.55 mg AG)	40% (25.09 mg AG)	60% (37.64 mg AG)	80% (50.19 mg AG)	100% (62.73 mg AG)
d_i	1.25	0.22	0.42	0.81	2.7	2.38
d_f	1.8	0.25	0.42	0.81	0.81	1.17
d_f-d_i	0.55	0.03	0	0	-1.89	-1.21
S (%)	100.0	5.45	0	0	0	0
MIC		40%				

d_f: Optical density after incubation, d_i: Optical density before incubation, S: Percentage of survival, MIC: Minimum inhibitory concentration, GA: Gallic acid

Table 6: Inhibitory action of *Salvadora persica* extracts on the growth of *Candida albicans*

Parameters	MFC	MIC	MFC/MIC	Inhibitory action
Candida albicans	40%	40%	1	Fungicide
Norms	According to Denis *et al*.[55]: • MFC/MIC≤2 (fungicide effect) • MFC/MIC>2 (fungistatical effect) According to Marmonier[66]: • MFC/MIC≤4 (fungicide effect) • MFC/MIC>4 (fungistatic effect)			

prepared at 20% of stem extract of *Salvadora persica* did not inhibit completely the growth of *Candida albicans*. However, the solution prepared at 40% resulted in a 0.01% survival rate among the studied yeast. This solution containing 40% of extract of miswak is therefore the Minimal Fungicidal Concentration (MFC) of *Candida albicans*.

Consequently, it follows from the equality of the ratio between MFC and MIC to 1 that the phenolic extracts of *Salvadora persica* exert a fungicidal effect on the tested yeast of *Candida albicans* (Table 6).

Other antimicrobial effects of *Salvadora persica* extracts: The solutions of phenolic extracts (either root, stem or bark) of *Salvadora persica* did not affect the growth of *Streptococcus mutans*. In contrast, extracts prepared from 2.5-17.5 g of crushed root and containing a polyphenols rate equal or higher than (07.21 Gallic Acid Equivalents (GAE) mg/100 mL of hydralcolic extract (HAE)) inhibited all the other cariogenic bacteria studied, found responsible of certain oral infections including *Streptococcus mitis*, *Streptococcus faecalis*, *Lactobacillus casei*, *Staphylococcus aureus* and *Stahylococcus epidermidis*. The complete inhibition of these germs was also achieved with the extract prepared at 5 g of crushed stem containing (31.36 GAE mg/100 mL HAE) and at 7.5 g of crushed bark containing (24.17 GAE mg/100 mL HAE) (Table 7).

DISCUSSION

The quantities of organic matter recorded in the root of *Salvadora persica* was remarkable and significantly higher

Table 7: Effect of the phenolic extracts of root, stem and bark of *Salvadora persica* on the growth levels of certain germs responsible for oral infections

		Quantity of vegetable matter used during the extraction of the phenolic compounds of *Salvadora persica*										
		00.00 g (Control)	02.50 g	05.00 g	07.50 g	10.00 g	12.50 g	15.00 g	17.50 g	F1	F2	Int. F1XF2
Lactobacillus casei (CFU mL^{-1})	Root	222 10^{5a}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	**	NS	**
	Stem	222 10^{5a}	106 10^{4b}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}			
	Bark	222 10^{5a}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}			
Streptococcus mutans (CFU mL^{-1})	Root	142 10^{11}	630 10^{11}	43 10^{12}	49 10^{12}	UNC	UNC	UNC	UNC	-	-	-
	Stem	142 10^{11}	62 10^{12}	98 10^{12}	71 10^{12}	UNC	UNC	UNC	UNC			
	Bark	142 10^{11}	67 10^{12}	65 10^{12}	105 10^{12}	UNC	UNC	UNC	UNC			
Streptococcus mitis (CFU mL^{-1})	Root	580 10^{11a}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	**	**	**
	Stem	580 10^{11a}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}			
	Bark	580 10^{11a}	296 10^{10b}	30 10^{10b}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}			
Streptocoques faecalis (CFU mL^{-1})	Root	243 10^{5a}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	**	NS	**
	Stem	243 10^{5a}	133 10^{3b}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}			
	Bark	243 10^{5a}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}			
Staphylococcus aureus (CFU mL^{-1})	Root	110 10^{5a}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	**	NS	**
	Stem	110 10^{5a}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}			
	Bark	110 10^{5a}	33 10^{3b}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}			
Staphylococcus epidermidis (CFU mL^{-1})	Root	123 10^{5a}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	**	NS	**
	Stem	123 10^{5a}	100 10^{3b}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}			
	Bark	123 10^{5a}	43 10^{5a}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}	00^{c}			

For each group (n = 5), results expressed as mean value, F1: First studied factor (quantity of vegetable matter used in the extraction of the phenolic compounds of *Salvadora persica*), F2: Second studied factor (parts of plant of *Salvadora persica* used during the extraction). Int. F1XF2: Interaction of the two studied factors (F1XF2), CFU : Colony Forming Unit, Unc: Uncountable number of germs, NS: Not significant effect (p>0.05), **Highly significant effect (p<0.01), means of each category followed by different letters are significantly different at 5% level of probabilities

(p<0.01) than the stem and the bark. This can reflect the multiple biologically active compounds that may be contained in different parts of the plant, which are not fully explored until now. In fact, this plant species is rich in phenolic compounds particularly in the stem where the measured contents appears twice higher (p<0.01) compared to those measured in the root and the bark. This was confirmed by study of Abdel-Wahab *et al.*[67] who have stated the existence of several flavanoides in the miswak such as (Kaempferol, quercetin, quercetin rutin and quercetin glucose). Two flavanols belonging to the class of flavonoids[68,69] have been identified in the stem including:catechin and epicatechin. Catechins and their derivatives are very potent antioxidants[70-73].

These elements play a very important anti-cancer role[74,75] and also help prevent inflammatory diseases[76,77] as well as coronary and cardiovascular diseases[78-83] by inducing a significant decrease in the plasma levels of lipoproteins, cholesterol and triglycerides[84,85]. These elements cause atherosclerosis[86,87]. According to Macheix *et al.*[68,69], about a dozen other of these compounds were often found in plants, in particular these include: (+) gallocatechin and (-) epigallocatechin.

The principal chlorogenic acids recorded in the stem are 5-O-caffeoylquinic acid and 3,5-O-Dcaffeoylquinic acid. These compounds are among the major subgroups representing 98% of chlorogenic acids ehich are most frequently encountered in plants and consisting mainly of esters of quinic acid with caffeic acid such as caffeoylquinic acids and dicaffeoylquinic acids or with ferulic acid as feruloylquinic acids. For lack of appropriate standards, the rest of these minor compounds whose feruloyl-feruloylquinic acids, caffeoyl-feruloilquinic acid and coumaric acid were not identified in *Salvadora persica* species[88,89].

According to Clifford[90,91], 5-O-caffeoylquinic acid and chlorogenic acid are generally the most predominant in the majority of plants especially in coffee, apples, pear and tubers of potato. The chlorogenic acid and 4,5-O-Dcaffeoylquinic acid were also found but at low levels in the root of the studied plant. These substances possess strong antioxidant properties involving the free radical scavenging, metal ion chelation and the inhibitory effect of specific enzymes responsible for the formation of free radicals and hydro peroxide[92,93]. As for the bark and in comparison to other parts of the studied plant it seems to contain a high proportion of naringenin, which is a flavanones compound[94,95]. Also, it is relatively rich in caffein (1.3.7 trimethylxanthin) and theobromin (3.7 dimethylxanthin) which are the most important methylxanthin alkaloids found in plants[96].

These substances have several interesting effects on different body systems including: Central nervous, cardiovascular, gastrointestinal, respiratory and kidney[97,98]. The

therapeutic dose of 500 mg kg^{-1} of theobromin has long been used in the treatment of the cardiac oedema and the angina pectoris and its analogs of pentoxifyllin, suramin-theobromin and lisophyllin are currently exploited in chemotherapy of certain cases of cancers[99,100].

Another alkaloids more prevalent in the bark than the stem and the root of *Salvadora persica* is trigonellin (N-mthylnicotinic acid) which is derived from a pyridin compound[101-103]. The study reports several beneficial effects induced by trigonellin on human health: Hypocholesterolemia[104,105], hypoglycemic[106,107], antibacterial[108], antiviral[109], anti-inflammatory[110], antioxidant[111], stimulating appetite[112] etc.

The contents of mineral matter which were recorded are remarkably high and significantly ($p<0.01$) different concentrations were found in the root, 9.8% DW to 12.65% DW found in the stem and the bark. According to Ezmirly *et al.*[29], Abdel-Wahab *et al.*[67], Porteres[113], Ray *et al.*[114] and Massassati *et al.*[115], high levels of minerals are found in the stem of *Salvadora persica* which include mainly a particular form of chlorides and gypsum. Furthermore, Dorner[116] and Almas and Al-Lafi[117] reported the presence of NaCl as well as KCl and substantial quantities of silica in this plant. According to Le Houerou[118], the ash content of the leaves of *Salvadora persica* is found to be considerable (36% RW) with significant amounts of calcium (07.20% RW), silica (02.40% RW), magnesium (09.40% RW) and potassium (01.70% RW). Moreover, the silica content in the shewing sticks of miswak was often implied by Almas and Al-Lafi[117] in the physical removal of dental plaque during its use.

Moreover, several potential anionic compounds having some antimicrobial activity[119] have been detected in *Salvadora persica*, this may also partly explain the high levels of mineral observed in this plant species. Indeed, the bark is highly concentrated in sulphate ions and nitrate, more than the content of the root and the stem. Concerning the ions chlorinates, the bark showed lower levels ($p<0.01$) compared to the root and the stem. Concerning fluorides, the analysis showed an absence in the different parts of the studied plant. This confirms the results of Hattab[11] who has suggested the existence of few fluorides in the root and the stem of arak (*Salvadora persica*).

The phenolic extracts of *Salvadora persica* exerted a significant inhibitory effect ($p<0.01$) on the growth of *Candida albicans* deemed to be responsible for oral candidiasis in immunocompromised men particularly those affected by aids[50,51]. Regression of the proliferation of microbial species on the appropriate agar medium, namely the Sabouraud has already begun to appear in the solutions at 20 and 40% of ethanolic extract of miswak which is rich in polyphenols. Moreover, no yeast growth was observed in the presence of extracts prepared at 60, 80 and 100%. These responses are certainly due to the antimicrobial effect exerted by polyphenols on the fungal species. Indeed, many authors reported the presence of many phenolic compounds in miswak (tannins and flavonoids) which have an inhibitory effect against many oral microorganisms associated with caries and periodontal diseases such as: *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus salivarius*, *Streptococcus faecalis*, *Streptococcus mitis*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Staphylococcus epidermidis*, *Streptococcus pyogenis*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Haemophilus influenzae* and *Candida albicans*[9,28, 29,34,41-43].

The analysis of extracts of the stem of this plant species to date around the world has shown that it contains glycosides and sulfur compounds which have an approved antimicrobial activity[120]. Studies have also contributed to highlight the presence of four benzylamide exerting an inhibitory effect on platelet aggregation in humans and a moderate antibacterial action[121]. In addition, beta sitosterol, octacosanol, triacantanol, glucopyranoside and trimethylamine are antimicrobial substances reported a while back in this plant[114,122-125]. The stem extracts of *Salvadora persica* collected in the region of Aine Salah in South Algeria seem especially rich in 3,5-O-Dcaffeolylquinic acid, catechin and in epicatechin.

These main substances appear to exert a remarkable inhibitory effect on the growth of *Candida albicans*. In this regard, several researchers[7,9,42-44,126-128] have found *in vitro* inhibitory effect of phenolic extracts of miswak (*Salvadora persica*) on the growth of *Candida albicans*. The antifungal effect of *Salvadora persica* was also confirmed in other fungal species such *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus xylinium*[129] and *Aspergillus fumigatus*[128]. The analysis of MIC and MFC shows that *Candida albicans* was sensitive at 40% phenol extract of miswak. Consequently, it follows from the ratio between MFC and MIC being 1, that the phenolic extracts of *Salvadora persica* exert a fungicidal effect on the studied yeast. According to Denis *et al.*[55] this would be the right conclusion given that this ratio is less than or equal to two.

On the other hand, Marmonier[66] reports that the fungicidal effect of the substance is rather confirmed when the ratio (CMI/CMF) is less than or equal to four, whereas, if the ratio is greater than four the antimicrobial effect is called fungistatic. It is clear that ethanol extracts of the stem of

miswak rich in phenolic compounds are a powerful fungicide towards *Candida albicans*. It is well established that the inhibitory effect of polyphenols may be manifested by a multiplicity of individual influences affecting DNA, some specific proteins and the activity of certain cellular enzymes of the microorganisms[130,131]. The inhibitory effects of the phenolic compounds are further explained by their ability to be adsorbed in cell membrane and deprive the microorganisms of certain metal ions[132]. According to Luck and Jager[130] and Ikigai *et al*.[133] phenols can also attack the microorganisms by puncturing the cell membrane, which may cause an increase in the proton flux to the interior of the cell and an increase in energy requirements of germs. Some phenolic compounds such as catechin and epicatechin found in the miswak also demonstrate an antimicrobial effect by preventing the replication of DNA, as well as the activity of some enzymes like glucosyl transferase (GTase) and by denaturing the cell membrane of certain bacteria[134-135].

Many studies conducted on plants which are rich in phenolic compounds often indicate that they have some antimicrobial activity against various other bacteria involved in numerous oral diseases such as (gingivitis, periodontitis and dental caries)[9,12,15,44,136-141]. However, this results have not found evidence that polyphenols of *Salvadora persica* collected in South of Algeria exerted inhibitory effect on the growth of *Streptococcus mutans*. This is true even at high concentrations of ethanolic extract of the stem. These findings are in contrast to those reported by several researchers[12,15,34,41,44,142,143]. They observed a remarkable antimmicrobial effect of both alcoholic and aqueous extracts of miswak on *Streptococcus mutans* reputed to be the most cariogenic microbial species. It is very well established that polyphenols can affect the growth of germs especially by inhibiting the activity of certain enzymes such as glucosyl transferase (GTFs) which is caused by reducing the phenomenon of adsorption of GTFs on the bacterial cell[144]. It also appears that some catechins may even damage the membrane structures of the bacteria[133]. It appears that, most of the major antimicrobial compounds analyzed such as chlorogenic acid, flavanols (kaempherol, quercetin and catechin) and flavanones (narengénin) were only found at low levels in the different extraction solutions of *Salvadora persica*. These bioactive substances did not affect the growth of *Streptococcus mutans*.

However, the other bacteria of *Streptococcus* genus were remarkably inhibited by polyphenols of *Salvadora persica*. Thus, the bactericidal effect on *Streptococcus mitis* which is also a very cariogenic bacterial species has been noted with ethanol extracts prepared at 2.5 g of crushed root or stem and containing, respectively 7.21 and 15.68 mg of gallic acid equivalents/100 mL of hydralcolic extract (GAE mg/100 mL HAE). The antimicrobial effect on *Streptococcus mitis* was also observed with extracts prepared at 7.50 g of plant material of the bark and containing a higher rate of polyphenols, estimated at 24.17 GAE mg/100 mL HAE. Many researchers have confirmed this results and suggest that the extracts of *Salvadora persica* exert remarkable inhibitory effects against *Streptococcus mitis*[140,141,145,146]. Depending on the nature of phenolic compounds analyzed in *Salvadora persica*, it is very well known that some chlorogenic acids and alkaloids such as trigonellin exert a significant antimicrobial activity against several bacteria[108].

These extracts are very likely to contain other unidentified compounds whose action mechanisms on bacteria are currently less known. *Streptococcus faecalis* was completely inhibited with ethanol extracts prepared at 2.5 g of the root and/or the bark and containing from 7.21-8.05 GAE mg/100 mL HAE. Furthermore, the bactericidal effect of these enterobacteria was also found with the extraction solution prepared at 5 g of the stem and containing 31.36 GAE mg/100 mL HAE. Our observations confirm those advanced by Darout *et al*.[147] who claim that miswak extracts exhibit an antimicrobial activity against the *Streptococcus faecalis*. Al-Bayati and Sulaiman[41], Al-Ayed *et al*.[45] and Ngule *et al*.[40] also show the antibacterial effectiveness of aqueous extracts and methanol extracts of *Salvadora persica* on this pathogen of the oral cavity.

The extracts prepared at 2.5 g of raw matter of the root or the bark of *Salvadora persica* and containing 7.21 and 8.06 GAE mg/100 mL HAE exercised a significant bactericidal effect on *Lactobacillus casei*. In addition, the ethanol extracts prepared at 5 g of the stem of *Salvadora persica* and containing a quantity of 31.36 GAE mg/100 mL HAE has completely inhibited the proliferation of this germ. These findings do not corroborate those reported by Almas and Al-Zeid[148] which confirm the ineffectiveness of *Salvadora persica* extracts to inhibit all the microorganisms of the genus *Lactobacillus* such as *Lactobacillus casei*. However, recent studies report conflicting evidence and show a drastic effect of methanol extracts of *Salvadora persica* against *Lactobacillus casei*[9,149].The various polyphenols identified in the different parts of the plant (root, stem and bark) of *Salvadora persica* are probably at the origin of the various antimicrobial effects observed in the studied bacteria. This does not exclude the existence of other biologically active substances in the plant, which have not been identified in the different extraction solutions.

The most pathogenic bacteria species is *Staphylococcus aureus* which is not cariogenic and which can colonize mainly the nasophyrynx was completely inhibited with the

phenolic extracts prepared from 5-17.5 g of plant material of the stem, the bark or the root. These extract solutions presented the same type of bactericidal effect on cariogenic species of *Staphylococcus epidermidis*. These results do not confirm those reported by Almas[16] who have not noticed any inhibition of the growth of both bacteria (*Staphylococcus aureus* and *Staphylococcus epidermidis*) with aqueous extracts and solvent extracts of *Salvadora pesica* (miswak). However, these findings are in agreement with those advanced by Kumar *et al.*[9], El-Desoukey[14], Abhary and Al-Hazmi[15], Ngule *et al.*[40], Naseem *et al.*[44], Al-Zubaidy[46] and Yarbrough *et al.*[150] who have confirmed the existence in the miswak plant many antimicrobial constituents such as nitrates and sulfates which are capable of preventing the activity of phospho-oxidative transport as well as the consumption of oxygen in *Staphylococcus aureus*. The antimicrobial effect of the phenolic compounds such as glycosylflavones was also reported by Afifi *et al.*[151] in *Staphylococcus aureus*, but no detailed studies have been performed to our knowledge to date in *Staphylococcus epidermidis*.

CONCLUSION

The results significantly showed that *Salvadora persica* stems, root and bark extracts have a broad-spectrum activity against a panel of bacteria and fungi responsible for most oral diseases. The experiments show that a quantity of 7.5 g of plant matter of miswak collected in the arak region located in South of Algeria contains essential phenolics compounds, able to completely inhibit the growth of the following germs: *Candida albicans*, *Lactobacillus casei*, *Streptococcus mitis*, *Staphylococcus epidermidis*, *Streptococcus faecalis* and *Staphylococcus aureus*. However, *Streptococcus mutans* considered as the most cariogenic microbial species was not affected by phenol extracts of the studied plant. Further study is needed to identify the many bioactive compounds of miswak and to characterize their acting mechanisms on various microorganisms.

ACKNOWLEDGMENTS

This study was supported by the Laboratory of Food Tecnology and Nutrition located at Mostaganem University in Algeria. We are grateful to Dr. NOEL DURAND and Dr. KATI. D exercising at CIRAD-France and in the Laboratory of Applied Biochemistry, University of Bejaia-Algeria, for their help in identification by HPLC of major phenol compounds of the experimental plant of *Salvadora persica* and their cooperation throughout the study.

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