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Original Article

Pharmacology Section

Estimation of Antimicrobial Properties of Aqueous and Alcoholic Extracts of *Salvadora Persica* (*Miswak*) on Oral Microbial Pathogens - An *Invitro* Study

SALMAN SIDDEEQH[1], AMRITA PARIDA[2], MAJI JOSE[3], VIDYA PAI[4]

ABSTRACT

Introduction: Twigs of *Salvadora persica* (Miswak) plant are being used as a means of oral hygiene since ages for brushing teeth. Though clinical research and trials have shown promising results on effectiveness of Miswak, but some reports are conflicting.

Aim: To evaluate the antimicrobial activity of crude aqueous and alcoholic extracts of *Salvadora persica* (Miswak) against the common microbial pathogens causing dental caries and periodontitis.

Materials and Methods: A prospective study of one year duration was conducted in Yenopoya dental and medical college, Mangalore. The twigs of *Salvadora persica* were collected and alcoholic and aqueous extracts were prepared using standard techniques. The antimicrobial properties of the extracts against common oral pathogens like *Streptococcus mutans, Streptococcus mitis, Candida albicans, Lactobacillus acidophilus, Prevotella intermedia, & Peptostreptococcus* were performed by agar well diffusion method and two fold broth dilution method.

Results: No significant results was obtained when water extracts of *Salvadora persica* was tested except for minimum inhibitory effect against *Streptococcus mutans, Prevotella intermedia & Peptostreptococcus* and *Candida albicans*. Relatively significant inhibitory effect was noted with respect to alcoholic extract of *Salvadora persica*.

Conclusion: Although comparatively less than chlorhexidine which is a known antimicrobial agent, the alcoholic extracts of *Salvadora persica* showed antimicrobial effect against the common microbial pathogens causing dental caries and periodontitis indicating a potential beneficial effect of this plant. However, further research with more standardized extraction procedure and advanced techniques is required to find out the exact chemicals responsible for the antimicrobial properties of the plant extract.

Keywords: Plant extracts, Phytotherapy, Oral hygiene

INTRODUCTION

Medicinal plants are being used since ages because of their considerable antibacterial activity against various microorganisms. Use of such plants for routine cleaning of teeth and treating various oral diseases has long been a part of Indian culture. *Salvadora persica*, commonly known as Miswak, is a twig that has been used as chewing sticks since centuries for tooth brushing as the only means of oral hygiene practice in some communities [1]. *Salvadora persica*, is a well branched evergreen shrub, usually found in the arid regions of India, often on saline soils. It is believed to have varied usefulness and has been traditionally used for epilepsy, scurvy, cough, rheumatism etc., Studies have reported that extracts of Miswak possess various biological properties, including significant antibacterial, antifungal, and anti-plasmodial effects [2]. Stems and roots of the plants are spongy and can easily be crushed between the teeth. Pieces of the root become soft when soaked in water. When used for cleaning teeth, the therapeutic and prophylactic effects of this chewing stick may be due to mechanical cleaning/ the potential release of biologically active chemicals, and/or a combination of both [3]. Pharmaceutical companies have now tried to incorporate certain properties of this twig into tooth pastes. Though clinical research and trials have shown promising results on effectiveness of Miswak [4], but some reports are incongruous [5]. Hence, the present study was undertaken as a step to assess and evaluate the antimicrobial activity of crude *Salvadora persica* (Miswak) extracts against the common microbial pathogens causing dental caries and periodontitis.

MATERIALS AND METHODS

I. Preparation of Alcoholic and Aqueous Extracts [4]

The study was carried out after obtaining clearance from Yenepoya University ethics committee, Mangaluru. Study duration was of one year conducted in 2014-15. Dry twigs of *Salvadora Persica* were purchased from authorized Ayurvedic medical shop in Mangaluru, Karnataka. The twigs were then powdered using household electric blender. Fifty grams of the plant powder was loaded in the thimble of Soxhlet apparatus. It was fitted with appropriate size round bottom flask with 250 ml absolute ethanol, and upper part was fitted with condenser for alcoholic extract and with water in round bottom flask for water extract. The filtrates were concentrated using a rotary evaporator. The extracts were transferred into clean vials and stored at 4^0C till further use. The yield from 50 grams of Miswak twigs was seven grams.

The aqueous and alcoholic extracts were dissolved in Dimethyl Sulfoxide (DMSO) to prepare different concentrations i.e., 200µg/ml, and 400µg/ml and used for microbial analysis.

II. Assessment of Antimicrobial Properties

Collection & isolation of test organisms

Test organisms were collected from patients who visited the Yenopoya dental clinic, Mangalore for treatment. Informed consent was taken from the patients after explaining the study details. Cariogenic organisms were collected by scraping soft caries from carious cavities of affected teeth using excavator and periodontal pathogens from periodontal pockets using paper points. After

collection the paper points were dropped into 20 ml of Brain-Heart Infusion (BHI) broth which was used as transport media.

The collected samples were placed in an anaerobic chamber or incubator. After 48 hours of incubation, they were plated on variety of selective and nonselective media such as sheep blood agar, chocolate agar and SDA, manually under strict aseptic conditions in a laminar air flow chamber. Following a period of 24 hours of aerobic/anaerobic incubation, the culture plates were inspected for the colonies. Each colony was identified by colony morphology, gram staining, catalase test, pigment production and aerotolerance test, etc.

The isolates were preserved in BHI broth and RCM broth with serial subcultures every 72 hours for the entire study period. The purity of the broth cultures of each microorganism was confirmed by microscopic examination of gram-stained smears, cultivation of the organisms on sheep blood agar medium and used as inoculum for antibacterial assay. Cariogenic organisms such as *Streptococcus mutans*, *Streptococcus mitis* and Lactobacilli and periodontal pathogens such as *Peptostreptococcus* and *Prevotella intermedia* as well as Candida *albicans* were studied.

Preparation of Inoculum

The pure cultures of organisms were emulsified in BHI broth which was then incubated at 37°C for 24 hours. The suspensions were diluted by adding sterile BHI broth to match/obtain a turbidity equivalent to Mc Farland 0.5 standard $5X10^5$CFU/ml. These suspensions were used as inoculum for testing the effect of crude extracts by agar diffusion method and to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extract by broth dilution method [6].

Antimicrobial Analysis of the Extracts

The antibacterial screening was carried out using agar diffusion method described by Lino and Deogracious with slight modifications [7]. The freshly prepared inoculum was swabbed all over the surface of the Muller Hinton Agar plate using sterile cotton swab. Five wells of 6mm diameter were bored in the medium with the help of sterile cork-borer having 6mm diameter and were labelled properly and fifty micro-litres of different concentrations (200µg/ml and 400µg/ml) of plant extract and same volume of positive and negative control was filled in the wells with the help of micropipette. Chlorhexidine was used as positive control. Distilled water was used as control for aqueous extract and DMSO. Plates with lid closed were left for some time till the extract diffused in the medium. Plates were incubated at 37°C for 24 hour. Zone of inhibition was measured using scale [7].

Various dilutions of the extracts which had antibacterial activity in the previous assay were taken in sterile test tubes to determine MIC. Freshly prepared nutrient broth was used as diluents. Crude extract was diluted by two fold serial dilution method. Nutrient broth in a test tube was considered as control. Except the control tube, 50µl of the standard culture inoculums was added to each test tube. The tubes were incubated at 37°C for 24 hours and then examined for growth. Growth was confirmed by observing turbidity. One ml of bacterial culture was pipetted from the mixture obtained in the determination of MIC tubes which did not show any growth and subcultured onto Muller Hinton Agar and incubated at 37°C for 24 hour. The concentration at which there was no single colony of bacteria after incubation was taken as MIC [7].

STATISTICAL ANALYSIS

The results obtained were statistically analysed using Kruskal-Wallis test and Mann-Whitney U test. All statistical analysis was done using SPSS software version 15.0. The level of significance was set at $p < 0.05$.

RESULTS

With two different concentrations of water extract (200µg/ml and 400µg/ml), the tested cariogenic bacteria showed no zone of inhibition except for *Streptococcus mutans* at 400µg/ml which gave an inhibitory zone of 13mm. Periodontal pathogens, when subjected to the same concentrations of water extracts, both *Prevotella Intermedia* and *Peptostreptococcus*, gave a zone of inhibition of 6mm and 6.67mm and 12.67mm and 10.67mm at concentration of 200 and 400µg/ml respectively. Only the higher concentration showed an inhibitory zone of 6mm against *Candida albicans* while 200µg/ml did not show any effect.

When the same organisms were subjected to alcoholic extracts of similar concentrations, significant zone of inhibition was noted particularly in 400µg/ml concentration. The cariogenic bacteria *Lactobacillus acidophillus, Streptococcus mutans* and *Streptococcus mitis* gave an inhibitory zone of 10.67 mm, 17.33 mm and 8.67 mm, respectively at a concentration of 400µg/ml. The periodontal pathogens namely *Prevotella* and *Peptostreptococcus* showed an inhibitory zone of 11.33 mm and 10 mm, respectively at concentration of 200µg/ml and 16.67 mm and 16.63 mm at concentration 400µg/ml. *Candida* also showed an inhibitory zone of 10.67 mm at 400µg/ml and 6.33 mm at 200µg/ml. Statistical analysis of the results showed a significant increase in effect from 200µg/ml to 400µg/ml in both water and alcoholic extracts (p-value < 0.05). Similarly alcoholic extract showed a significantly higher effect compared to water extract (p-value < 0.05) [Table/Fig-1].

When the effect on different pathogens was compared, it was noted that both extracts had significant effect on periodontal pathogens compared to others [Table/Fig-1]. The MIC and MBC recorded is shown in [Table/Fig-2].

DISCUSSION

Plants are important sources of therapeutic medicines and the tribal and ethnic communities rely a lot on these natural products for their healthcare needs. According to WHO, herbal medicines serve the health needs of about 80% of the world population, especially for millions of people in the rural areas of developing countries [8]. The beneficial medicinal effects of plant materials including the antibacterial activity typically result from the secondary products

Microorganism	AE- 400 With WE- 400	AE - 400 with WE - 200	AE- 400 with AE -200	AE-400 with control	WE- 400 with AE– 200	WE- 400 with WE- 200	WE- 400 with control	AE-200 with WE- 200	AE - 200 with control	WE - 200 with control
Lactobacilli	0.034*	0.034*	0.034*	0.043*	1.000	1.000	0.034*	1.000	0.034*	0.034*
Prevotella intermedia	0.068	0.034*	0.043*	0.043*	0.197	0.034*	0.197	0.034*	1.000	0.034*
Streptococcus mutans	0.046*	0.034*	0.034*	0.043*	0.037*	0.037*	0.046*	1.000	0.034*	0.034*
Candida albicans	0.034*	0.034*	0.034*	0.043*	0.025*	0.025*	0.034*	1.000	0.034	0.034*
Peptostreptococcus	0.046*	0.046*	0.037*	0.037*	0.317	0.043*	0.317	0.034*	1.000	0.034*
Streptococcus mitis	0.034*	0.034*	0.034*	0.043*	1.000	1.000	0.034*	1.000	0.034*	0.034*

[Table/Fig-1]: Mann-Whitney test for alcoholic and water extracts of *Salvadoraa persica*.
**p-value significant (<0.05)*
AE- Alcoholic Extract, WE – Water Extract, Control – Positive Control Chlorhexidine.

Microorganism	MIC	MBC
S. mutans	12.5	25
S.mitis	200	400
Lactobacillus	200	200
Candida	000	000
Peptostreptococcus	12.5	25
Prevotella	200	400

[Table/Fig-2]: The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentrations (MBC) of the *Salvadoraa Persica* extracts against different test bacteria.

present in the plant although, it is usually not attributed to a single compound but a combination of the metabolites [9]. Various chemicals in Miswak extracts, such as sodium chloride, potassium chloride, salvadourea, Salvadoraine, saponins, tannins, vitamin C, silica, and resin, cyanogenic or lignan glycosides, alkaloids, terpenoids, oleic, linoleic, and stearic acids may be responsible for the antimicrobial and cleaning effects of Miswak [4].

Though the exact mechanism by which the active components of the plant materials contribute to antibacterial activity is not known, the antimicrobial effect may be through one of the mechanisms such as inhibition of cell wall synthesis, damage to cell membrane, inhibition of nucleic acid synthesis, inhibition of protein synthesis etc. Useful antimicrobial phytochemicals can be divided into several categories like phenolics, polyphenols, flavones, flavonoids, flavonols, quinones, tannins, coumarins, terpenoids, essential oils, alkaloids, lectins and polypeptides etc., [10]. Phenolic compounds exert antimicrobial property by causing enzyme inhibition, probably through interaction with sulfhydryl groups [11]. Quinones probably act on adhesins, cell wall polypeptides and membrane-bound enzymes. They also deprive microorganisms of substrates [11]. Flavones, flavonoids, and flavonols complex with bacterial proteins and cell walls and exhibit antimicrobial activity. Tannins form a layer over enamel by their astringent effect, thus providing protection against dental caries. Coumarins act by stimulating macrophages. Terpenoids and essential oils act against bacteria, fungi, viruses and protozoa by causing membrane disruption [10]. Alkaloids also exhibit antimicrobial properties [12]. Lectins and polypeptides act possibly through formation of ion channels in the microbial membrane [13,14] or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors [10]. Other compounds that have antimicrobial properties are polyamines [15], isothiocyanates [16,17] thiosulfinates [18], and glucosides [19,20].

When used as chewing sticks, in addition to these biologically active chemicals, the beneficial effect of Miswak could be because of mechanical cleansing effect of fibrous component. The substantial amount of silica detected in *S. persica* ashes contribute to Miswak*'s* mechanical action in plaque removal [21]. However, there is no sufficient literature regarding the mechanical cleansing efficacy of Miswak, as compared to the manual or electric tooth brushes. Current studies available frequently lack specific details concerning the time, duration, and frequency of their use which prevents meaningful assessment of the mechanical cleaning effect of Miswak upon oral health. The increase in saliva production when the chewing stick is left in the mouth for some time could also be responsible for promoting better cleansing and thus maintenance of oral health [3].

While comparing the efficacy of different extraction methods, the alcoholic extract of *Salvadora Persica*, showed better inhibitory zones compared with aqueous extracts. This observation is in accordance with the findings of Maria et al., and Doughari et al., who reported a strong antibacterial effect of alcoholic extract of tested plants in comparison to aqueous extract [7,9]. The differences in the activities of alcoholic and water extracts may be due to varying degrees of solubility of the active constituents in these two solvents. From this observation we can infer that alcohol is relatively a more efficient solvent than water for the extraction of bioactive compounds of *Salvadora Persica* than water. Difference in potency and diversity of compounds extracted by using organic solvents and water has also been reported previously [9]. However, in contrast to our results, one study done in Iraq has shown that the aqueous extract was more potent than the alcoholic form in inhibiting microorganisms [4]. In a study conducted by Almas K it was seen that even the alcoholic extracts of Salvadora persica (obtained from Pakistan) had no inhibitory effect on *Streptococcus mutans*, Staphylococcus aureus and Candia *albicans* [5]. He has attributed this to the fact that it took him almost one month to test the antimicrobial activity of extracts, so the extract was not fresh, at the time of experiment which could have caused the loss of antimicrobial activity.

In the present study, the results showed that the alcoholic extracts of *Salvadoraa persica* at a concentration of 400µg/ml was more effective against the anaerobic bacteria (*lactobacilli*, *Peptostreptococcus*) than the facultative aerobes *(streptococci)*. Anaerobic bacteria are inherently more sensitive to antimicrobial substances than the aerobes; this could be the reason behind above observation.

Numerous extrinsic and intrinsic parameters influence the inhibition produced by the plant extracts against particular organism. Variable diffusability of plant extract in agar medium may also affect zone of inhibition. The antibacterial property may not demonstrate as zone of inhibition in proportion to its efficacy if there is decreased diffusability [9]. Therefore, MIC and MBC values, the lowest concentration of antibacterial substance required to produce a sterile culture has also been computed here. Demonstration of antibacterial activity against the test bacteria is an indication that alternative antibiotic substances in these plants could be used for the development of newer antibacterial agents in future. The observed low MIC and MBC values against these bacteria suggest that the plant extracts has the potential to treat ailments arising from the bacterial pathogens effectively.

It is possible that these plant materials can also be contributing to oral health through efficient free radical scavenging activity and antioxidant capacity [7].

LIMITATION

With respect to plant materials, the beneficial antimicrobial effects could be related to the active chemical constituents It was beyond the scope of our study to isolate the active principle. Further studies are needed to bring forth the details of the active components in *Salvadora persica*, mechanism of action of these chemicals as well as their potentials in combination with other plant extracts.

CONCLUSION

Our study has confirmed the antimicrobial beneficial effects of alcoholic extracts of *Salvadora persica*. However, with respect to this plant, an alternative mechanism of synergistic effects of various natural substances when used in combination or when used along with other antibiotics should be considered as an area of future research.

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PARTICULARS OF CONTRIBUTORS:
1. Assistant Professor, Department Oral Pathology and Microbiology, Century Dental College, Kasargod, Kerala, India.
2. Assistant Professor, Department of Pharmacology, Kasturba Medical College, Mangaluru, Manipal University, Manipal, Karnataka, India.
3. Professor, Department of Oral Pathology and Microbiology, Yenepoya Dental College, Mangaluru, Karnataka, India.
4. Professor and Head, Department of Microbiology, Yenepoya Medical College, Mangaluru, Karnataka, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Amrita Parida,
Assistant Professor, Department of Pharmacology, Kasturba Medical College, Manipal University,
Light House Hill Road, Mangalore, Karnataka-575001, India.
E-mail: amrita_parida@yahoo.com

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