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Original Article

Dentistry Section

Anti-microbial Activity of Tulsi {*Ocimum Sanctum (Linn.)*} Extract on a Periodontal Pathogen in Human Dental Plaque: An Invitro Study

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ABSTRACT

Introduction: Tulsi is a popular healing herb in Ayurvedic medicine. It is widely used in the treatment of several systemic diseases because of its anti-microbial property. However, studies documenting the effect of Tulsi on oral disease causing organisms are rare. Hence, an attempt was made to determine the effect of Tulsi on a periodontal microorganism in human dental plaque.

Aim: To determine if *Ocimum sanctum* (*Linn.*) has an anti-microbial activity (Minimum Inhibitory Concentration and zone of inhibition) against *Actinobacillus actinomycetemcomitans* in human dental plaque and to compare the antimicrobial activity of *Ocimum sanctum*(*Linn.*) extract with 0.2% chlorhexidine as the positive control and dimethyl sulfoxide as the negative control.

Materials and Methods: A lab based invitro experimental study design was adopted. Ethanolic extract of *Ocimum sanctum* (*Linn.)* was prepared by the cold extraction method. The extract was diluted with an inert solvent, dimethyl sulfoxide, to obtain ten different concentrations (1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%) of extract.

Plaque sample was collected from 05 subjects diagnosed with periodontal disease. Isolation of *Actinobacillus actinomycetemcomitans* from plaque samples was done using Tryptic Soy Serum Bacitracin Vancomycin agar (TSBV) medium. Identification of *Actinobacillus actinomycetemcomitans* was done based on cultural, microscopic, biochemical characterization and multiple drug resistance patterns.

Anti-microbial activity of *Ocimum sanctum (Linn.)* extract was tested by agar well-diffusion method against 0.2% chlorhexidine as a positive control and dimethyl sulfoxide as a negative control. The zone of inhibition was measured in millimeters using Vernier callipers.

Results: At the 6% w/v concentration of *Ocimum sanctum (Linn.)* extract, a zone of inhibition of 22 mm was obtained. This was the widest zone of inhibition observed among all the 10 different concentrations tested. The zone of inhibition for positive control was 25mm and no zone of inhibition was observed around the negative control.

Conclusion: *Ocimum sanctum (Linn.)* extract demonstrated an antimicrobial activity against *Actinobacillus actinomycetemcomitans*. The maximum antimicrobial potential was observed at the 6% concentration level.

Keywords: *Actinobacillus actinomycetemcomitans*, Anti-microbial, Chlorhexidine, Dental plaque, Tulsi extract

INTRODUCTION

Periodontal disease is a chronic infectious disease of the oral cavity and one of the principal causes of tooth loss in humans. This chronic inflammatory disease that affects the supportive tissues of the teeth has a complex aetiology [1]. One of the major aetiological factors for periodontal disease is the dental plaque biofilm on the teeth surfaces [2]. The noxious products produced by the bacteria in dental plaque trigger the inflammatory process in the periodontal tissues.

Actinobacillus actinomycetemcomitans, in human dental plaque is one of the most commonly implicated micro-organisms in the causation of periodontal disease [3]. Hence, reducing their levels in the oral cavity is one of the rationales for the prevention and control of periodontal disease.

Currently, chlorhexidine is the most widely used chemotherapeutic agent against periodontal disease causing organisms. It has been studied extensively and is used as a gold standard against which other antimicrobial agents are compared [4]. Consequently, chlorhexidine is also often used as a positive control for assessing the antimicrobial potential of other agents used against periodontal pathogens. However, it's widespread and prolonged use is limited by its side effects in the form of teeth staining and taste disturbances [5]. Hence, there is a need to come up with an alternative and equally effective antimicrobial agent against periodontal disease causing organisms. One such alternative strategy would be to verify the antimicrobial properties of the medicinal plants.

Finding healing power in plants is an ancient idea. Tulsi or the Indian Holy Basil, is a time tested premiere medicinal herb. It is an annual herb belonging to the mint family. It grows extensively in India, Africa and Mediterranean regions. It belongs to the family of Labiatae and has a botanical name *Ocimum sanctum (Linn.)* [1]. The three commonly available varieties of Tulsi are Rama Tulsi (green leaves and white to purplish flowers), Vana Tulsi (green leaves, green stem and white flowers) and Krishna Tulsi (purple leaves, stem and flowers) [6].

Ocimum sanctum (*Linn.)* or Tulsi has been the pillar of Ayurvedic holistic health system in India. Since time immemorial, various parts of the plant have been used extensively in the treatment of several systemic diseases like upper respiratory infections, bronchitis, skin diseases, malaria etc. *Candida albicans*, *Staphylococcus aureus*, enteric pathogens, *Klebsiella*, *E.-coli* and *Proteus* are the microorganisms against which the antimicrobial property of Tulsi has been tested [7]. Tulsi has demonstrated anti-gonorrheal efficacy against multiresistant strains of *Neisseria gonorrhoea* and clinical isolates of beta-lactamase producing methicillin resistant *Staphylococcus aureus* [8].

However, its use in the treatment of oral diseases and its action against oral disease causing microorganisms has been poorly documented. Scientific literature search yielded no studies documenting the effect of *Ocimum sanctum (Linn.)* against periodontal disease causing microorganisms. Therefore, a study was conducted to determine the antimicrobial activity of the *Ocimum sanctum (Linn.)* extract against *Actinobacillus actinomycetemcomitans*, a periodontal pathogen in human dental plaque.

AIM

Hence, the aim of the present study was to determine the anti-microbial activity (Minimum Inhibitory Concentration and zone of inhibition) of *Ocimum sanctum (Linn.)* extract against *Actinobacillus actinomycetemcomitans* and compare it with 0.2% chlorhexidine (positive control) and dimethyl sulfoxide (negative control).

MATERIALS AND METHODS

Study Design and Selection of Study Subjects

The study was conducted at Mahatma Gandhi Dental College and Hospital in Jaipur over a period of one month in the year 2012. Before the start of the study clearance was obtained from the institutional ethical review board of the Dental College Hospital. A lab based invitro experimental study design was employed. Five male subjects aged between 50-52 years, diagnosed with periodontal disease based on clinical examination were selected from the Department of Periodontology and Implantology of the dental college and hospital. Voluntary written informed consent was obtained from all the selected subjects prior to the start of the study. Diagnosis of periodontal disease was made according to the criteria of Community Periodontal Index [9]. All the selected subjects had a score of 3 (pocket depth of 4-5mm) as determined using the Community Periodontal Index probe. All the patients selected were using tooth brush and toothpaste (without fluoride and anti-plaque agents) once daily in the morning as a primary oral self care. None of the patients were using mouthwash, dental floss or inter-dental cleaning aids. Those volunteers agree to refrain from oral hygiene procedures 24 hours prior to plaque collection and subjects having periodontal disease corresponding to score 3 on CPI index were included in the study. The selected subjects did not suffer from any cognitive deficiencies, chronic medical conditions, systemic diseases or infectious diseases. There was no history of drug, alcohol or tobacco addiction. Subjects did not have any other oral diseases other than periodontal disease. They did not have crowns, veneers, dentures or any other prosthesis or oral appliances. The selected subjects were not taking any medications/antibiotics in the recent past.

Collection of Plaque Samples

Buccal surfaces of maxillary and mandibular premolar and molar teeth were selected. The subjects were asked to swish their mouth with plain water to reduce the contamination of plaque with soft debris. The area of plaque collection was isolated with cotton gauze. Sterile stainless steel jaquette scaler was used to collect the supragingival plaque by inserting it slightly into the gingival sulcus and running it over the surface occlusally and a sterile Gracey curette was inserted into the pocket to collect the subgingival plaque sample. Plaque samples thus collected were transported to the laboratory in 200µl peptone water as the transport medium.

Microbiological Procedures

Isolation, identification and characterization of *Actinobacillus actinomycetemcomitans* from dental plaque samples was done using Tryptic Soy Serum Bacitracin Vancomycin agar (TSBV agar). TSBV agar is an enriched selective medium for the isolation and presumptive identification of *Actinobacillus actinomycetemcomitans*. TSBV agar media was poured in sterile petriplates and inoculated with 05 different dental plaque clinical samples. The plates were incubated at 37°C for 24 hours.

Actinobacillus actinomycetemcomitans was identified from the incubated plaque samples based on cultural, microscopic, biochemical characterization and multiple drug resistance patterns. Based on the above screening tests, isolate 1 was identified as *Actinobacillus actinomycetemcomitans* [Table/Fig-1].

Preparation of Cold Extract of Ocimum Sanctum (*Linn.*)

Fresh leaves of the green variety of *Ocimum sanctum* (*Linn.*) were plucked from courtyards and were dried in sunlight. The dried leaves were then powdered finely. Three hundred grams of finely powdered *Ocimum sanctum* (*Linn.*) was then macerated with 100% ethanol. It was then subjected to filtration with Whatman filter paper to obtain a clear filtrate. The filtrate so obtained was reduced at a low temperature of less than 60°C to obtain a solid residue of *Ocimum sanctum* (*Linn.*) extract. From 300 grams of *Ocimum sanctum* (*Linn.*) powder dissolved in 1 liter of ethanol, 18 gram of extract (residue) was obtained and thus the yield was 6% w/v.

Characterization	Test	*Actinobacillus actinomycetemcomitans*	Isolate 1	Isolate 2
Cultural	TSBV agar	Yellowish	Yellowish , Round configuration, Drop like elevation	White mucoid with irregular configuration, Raised elevation
Microscopic	Mobility	Non-motile	Non-motile	Sluggishly motile
	Gram satin	Gram negative short rods	Gram negative short rods	Gram negative rods
Biochemical	Catalase	P†	P	P
	Oxidase	N‡	N	N
	Indole	N	N	N
	Urease	N	N	N
	Glucose	P	P	P
	Fructose	P	P	P
	Lactose	N	N	P
	Sucrose	N	N	N
Multiple drug resistance pattern	Methicillin	S§	S	R¶
	Chloramphinicol	S	S	S
	Tetracycline	S	S	R

[Table/Fig-1]: Isolation, identification and characterization of *Actinobacillus actinomycetemcomitans* from dental plaque samples using TSBV agar.
† positive; ‡ negative; § susceptible; ¶resistant

Preparation of 10 Different Concentrations of *Ocimum Sanctum (Linn.)* Extract

One gram of cold extract of *Ocimum sanctum* (*Linn.*) was dissolved in 10 milliliter (ml) of dimethyl sulfoxide to obtain a 10% concentration of extract. 1ml of the extract was transferred to a sterilized test tube and labelled as 10%. The remaining 9 ml of the extract was then diluted further with dimethylsulfoxide to obtain 09 different concentrations (1%,2%,3%,4%,5%,6%,7%,8%,9%) of *Ocimum sanctum* (*Linn.*) extract. A total of 10 concentrations ranging from 1% to 10% were prepared.

Testing for antimicrobial activity of *Ocimum sanctum* (*Linn.*) extract:

The antimicrobial activity of *Ocimum sanctum* (*Linn.*) extract against *Actinobacillus actinomycetemcomitans* was performed by agar well diffusion method on Muller Hinton agar media using 50 micro liter (µl) of 0.2% chlorhexidine as the positive control and 50µl dimethyl sulfoxide as the negative control.

PROCEDURE

The hot media was poured into petriplates and allowed to cool for 20 minutes. Three wells were punched in the plate at a distance of 25 millimeter (mm) from each other, one well for the *Ocimum sanctum (Linn.)* extract, one for the positive control and one for the negative control. 50µl of the particular concentration of the *Ocimum sanctum* (*Linn.*) extract was taken in a micropipette and loaded into the well marked for test sample. Ten such plates were loaded corresponding to ten different concentrations of *Ocimum sanctum* (*Linn.*) extract. Similarly 50µl of positive control (0.2% chlorhexidine mouth wash) and 50µl of negative control (dimethyl sulfoxide) were loaded into wells marked for positive and negative control respectively. Ten such plates were prepared for ten different concentrations of *Ocimum sanctum* (*Linn.*) extract, each plate having a well for one particular concentration of *Ocimum sanctum* (*Linn.*) extract, one for the positive control and one for the negative control. The plates were incubated at 37 degree Celsius for 24 hours. Following incubation, the zone of inhibition was measured with unaided eye using a Vernier caliper measuring the zone sizes to the nearest millimeter, including the diameter of the disk in the measurement.

RESULTS

At the 6% w/v concentration of *Ocimum sanctum* (*Linn.*) extract, a zone of inhibition of 22mm was obtained. This was the widest zone of inhibition observed among all the 10 different concentrations tested. Hence, the Minimum Inhibitory Concentration of cold ethanolic extract of *Ocimum sanctum* (*Linn.*) against clinically isolated *Actinobacillus actinomycetemcomitans* was 6% w/v [Table/Fig-2]. The zone of inhibition for positive control was 25mm and no zone of inhibition was observed around the negative control [Table/Fig-3].

Microorganism	Concentration of cold extract of *Ocimum sanctum* (linn.)	50µl† of *Ocimum sanctum* (linn.) extract Zone of inhibition (mm)‡
Actinobacillus actinomycetemcomitans	1%	09
	2%	10
	3%	10
	4%	12
	5%	16
	6%	22
	7%	20
	8%	20
	9%	21
	10%	21

[Table/Fig-2]: Minimum inhibitory concentration and zone of inhibition for *Ocimum sanctum* (linn.) extract.
† Microlitre; ‡ Millimeters

Microorganism	Control	Zone of inhibition (mm) ‡
Actinobacillus actinomycetemcomitans	Positive control (50µl† of 0.2% chlorhexidine solution)	25
	Negative control (50µl† of Dimethyl sulfoxide solution)	No zone of inhibition

[Table/Fig-3]: Zones of inhibition for positive control and negative control.
† Microlitre; ‡ Millimeters

DISCUSSION

Plants are one of the important sources of medicine [10]. It is estimated that 25% of modern medicines of the 21st century have plant derivatives as their active ingredients or principles [11]. Among a wide array of medicinal herbs, *Ocimum sanctum* (*Linn.)* or the Indian Holy Basil stands out as a time tested premiere medicinal herb.

The chemical composition of *Ocimum sanctum* (*Linn.*) is highly complex containing many biologically active compounds and nutrients. *Ocimum sanctum* (*Linn.*) leaves have antibacterial agents mainly in the form of essential oils. The five major constituents found in the essential oils of *Ocimum sanctum (Linn.)* leaves are eugenol, caryophyllene, germarene-A, clemene and caryophylline oxide. [11]. They also contain many biologically active compounds in the form of phytochemicals like ursolic acid, rosmarinic acid and oleanolic acid [6]. The essential oils and biologically active compounds have antibacterial properties and are effective against gram positive and gram negative bacteria [10-13].

Studies have shown that the essential oils and biologically active compounds in fresh leaves of *Ocimum sanctum* (*Linn.*) are effective against bacteria like E *coli*, *Shigella* species, *Salmonella typhi*, *B.anthracis* and *P.aeruginosa* [10,13]. The oils are known to inhibit the invitro growth of M. *tuberculosis* bacteria [1,4]. A study conducted in Lahore, Pakistan found that essential oils from fresh *Ocimum sanctum* (*Linn.*) leaves displayed marked antibacterial efficacy against *Pseudomonas aeuroginosa*, *E.-coli*, *Klebsiella*, *Proteus mirabulus* and *Staphylococcus aureus* [14].

The anti microbial action of essential oils in *Ocimum sanctum* (*Linn.*) is attributed to monotrepene components which are mostly phenolic in nature. They exert membrane damaging effects to microbial strains and stimulate leakage of cellular potassium which is responsible for a lethal action related to cytoplasmic membrane damage [14].

Based on this mechanism it may be supposed that the essential oils and phytochemicals in *Ocimum sanctum* (*Linn.*) effective against systemic disease causing bacteria might have also acted against the periodontal pathogen *Actinobacillus actinomycetemcomitans* in human dental plaque.

In this study, leaves of green variety of *Ocimum sanctum* (*Linn.*) were used (green leaves and white to purplish flowers) over other varieties for no specific reason other than convenience and ease of availability. Studies have shown that the green and black varieties of *Ocimum sanctum* (*Linn.*) have similar chemical constituents and common medicinal properties [12].

Ethanol was used as a solvent because the essential oils in *Ocimum sanctum* (*Linn.*) are more soluble in alcohol when compared to distilled water [15]. Dimethyl sulfoxide, an inert solvent, was used to dilute the *Ocimum sanctum* (*Linn.*) extract to neutralize the effect of alcohol, which itself is an antiseptic, attributing the result solely to *Ocimum sanctum* (*Linn.*).

In the present study, 0.2% chlorhexidine was found to be more effective against *Actinobacillus actinomyctemcomitans* when compared to Tulsi extract. However, the well known side effects of chlorhexidine, i.e., staining of teeth and restorations, alteration of taste sensation and development of resistant organisms, may

limit its long term use. In comparison, *Ocimum sanctum* (*Linn.*) has a high safety margin with exceptionally low toxicity and no human drug interaction data is currently available [6,16]. Hence, it may be recommended for long term use. Moreover, herbal medicine like *Ocimum sanctum* (*Linn.*) is abundantly available, easily accessible, economically feasible and culturally acceptable. Hence, using herbs like *Ocimum sanctum* (*Linn.*) in the management of oral diseases can overcome many barriers that exist for the utilization of dental services like affordability, accessibility, availability and acceptability.

Literature search revealed very few studies that have tested the antimicrobial action of *Ocimum sanctum* (*Linn.*). on oral disease causing organisms. A triple blind randomised clinical trial which tested 4% w/v of *Ocimum sanctum* (*Linn.*) mouthrinse against 0.12% chlorhexidine mouthwash and saline water as placebo found that *Ocimum sanctum* mouthrinse was equally effective in reducing plaque and gingivitis as chlorhexidine [17]. An in vitro study found that the methanolic extracts of *A. indica* (neem), *O.sanctum* (Tulsi), *M.elengi* (bakul), *T.cardifolia* (Giloy) and chlorhexidine gluconate had antimicrobial activity against common endodontic pathogens like *S. mutans*, *E.faecalis* and *S. aureus*. At 3mg concentration, *O.sanctum* was the most effective against *S. mutans* and chlorhexidine was the most effective against *S.aureus* [18].

Further, the laboratory results obtained in this study need reconfirmation in real life situations. If confirmed so, the active ingredients of *Ocimum sanctum* (*Linn.*) could be incorporated into oral hygiene products such as toothpastes and mouthwashes as an agent to fight the periodontal disease causing organisms.

One of the limitations of the present study was that it employed an invitro lab based study design. Hence, the laboratory results need reconfirmation in real life situation. Further, the study tested the anti-microbial effect of *Ocimum sanctum* (*Linn.*) against only one periodontal pathogen i.e. *Actinobacillus actinomyctemcomitans*. However, many other microorganisms have been implicated in the causation of periodontal disease including P.*gingivalis* and *Prevotella intermedia*. Hence, it is also recommended to determine the anti-microbial effect of *Ocimum sanctum* (*Linn.*) extract against other common periodontal pathogens like P.*gingivalis* and *Prevotella intermedia* to find out the spectrum of its activity.

CONCLUSION

Given the high prevalence of periodontal disease in developing countries like India, the undesirable effects due to prolonged use of currently used antibacterial agents and financial considerations there is a need for alternate preventive and treatment strategies that are safe, effective and economical when compared to existing treatment methods. In this direction, natural phytochemicals isolated from traditional medicinal plants like Tulsi serve as a good alternative.

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