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DETERMINING THE EFFICACY OF OCIMUM SANCTUM LEAVES EXTRACT ON CARIOGENIC PROPERTIES OF STREPTOCOCCUS MUTANS - AN IN VITRO STUDY

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ABSTRACT

Context: Ocimum sanctum has often been cited as one of the main pillars of herbal medicine as it possesses greater medicinal value. It has been proved to be effective against gram positive and gram negative bacteria. **Aim**: To assess the antimicrobial efficacy of Ocimum Sanctum extract against Streptococcus mutans and to analyze its mode of action on key virulence factors of Streptococcus mutans in vitro. **Methods and material:** Ocimum Sanctum extract using methanol, ethanol and aqueous solvents was prepared from freshly collected, air dried and powdered leaves. These extracts obtained at different concentrations were subjected to antimicrobial screening for cariogenic microorganism. Further its effect on cariogenic properties of streptococcus mutans such as acid production, cell-surface hydrophobicity, sucrose-dependent adherence to glass surface and growth were evaluated with assorted assays. **Statistical analysis:** One sample t-test was done. **Results:** Methanol extract of ocimum sanctum demonstrated good antimicrobial activity and inhibitory effect on key virulence factors of streptococcus mutans. **Conclusion:** From the results of the present study, ocimum sanctum could be considered as a potential phytotherapeutic anticariogenic agent. Thus with further research, it could be recommended as an anticaries mouthwash in children.

KEYWORDS: ocimum sanctum, antimicrobial activity, streptococcocus mutans, virulence factors, anticaries, plant extracts, herbs, tulsi.

INTRODUCTION

Plant and plant extracts have been used in traditional medicine since time immemorial.[1] In western and eastern societies, natural products were used to treat oral diseases, several 1000 years back. Even though, there is a mention about large number of recipes for mouthwashes composed of natural substances in Ebers papyrus[2] and therapeutic rinsing being practiced by Europeans (early 18th century).[3] Only, recently there has been a renewed interest in the use of herbs in dentistry. Herbs have been tried as an endodontic irrigant[4] and its antibacterial efficacy against various oral pathogens has been evaluated.[3,5-8]

Ocimum sanctum (OS), commonly known as tulsi is a small shrub belonging to the mint family lamiaceae. It has small leaves with a strong smell and purple flower.[9] Studies have proved its antibacterial effectiveness against various gram positive and gram negative bacteria.[9-18] In dentistry, its effect on E faecalis[19] has been assessed by one study. Till date there are only three studies[3,8,20] evaluating its effectiveness on streptococcus mutans (S. mutans). Thus, this study was planned to evaluate the antimicrobial efficacy of Ocimum Sanctum extract and to assess its mode of action on key virulence factors of S mutans in vitro.

METHODS

Preparation of Herbal extracts: Fresh leaves of OS were collected from Bangalore, which was identified by a botanist and with literature review. The OS leaves were washed with distilled water, air dried and ground to powder form. The leaf powder was extracted with different solvents (water, 100% methanol, and 100% ethanol) for 30 hrs. Then it was centrifuged at 12000 rpm for 10mins, supernatant was collected and the solvent was evaporated in hot air oven at 50°C. The dried extract was suspended in 75% of the above mentioned solvents and used as a stock and stored at 4°C.

Bacterial strains and culture: The bacterial strain streptococcus mutans MTCC 890 (Institute of Microbial Technology, Chandigarh, India) was grown in Brain Heart Infusion (BHI) broth (Himedia Labs, Mumbai, India) at 37°C for 48 hours. BHI broth was prepared by using BHI broth powder (37gm/lit) and BHI agar plates

were prepared by using 1.5% agar (15gm/lit). Media was sterilized by autoclaving at 120°C for 20 mins. S. mutans lyophilized powder was suspended in the media. A loop full of the above suspensions were streaked onto the media plates and incubated. Single colony of S. mutans was inoculated into broth and incubated at 37°C for 24-48 hrs.

- **Antibacterial activity:** The antibacterial activity of the OS extracts was evaluated using Agar well diffusion method. Grown S. mutans inoculum (100µl) was swabbed onto BHI agar plates. Wells of 5mm diameter were punched into the agar plates. Using a micropipette, 20 µl of different concentrations of plant extracts (20%, 10%, 5% and 2.5%) were added to the wells made in the plate and incubated. Antibacterial activity was evaluated by measuring the zone of inhibition (mm) against the S. mutans strains. The test was performed with positive (chlorhexidine 0.2%) and negative (75% of suitable solvent) controls.

- **Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC):** The OS extracts that were found effective, as antimicrobial agent, were later tested to determine the MIC and MBC values. To the 5ml of BHI broth in each tube, 50 µl of grown S. mutans was added and then increasing concentrations of OS extracts ranging from 0.1 to 6.4mg/ml were added in a series of two fold dilutions and the tubes were incubated. Two control tubes were maintained (inoculum control and extract control).The lowest concentration of plant extract that produced no visible growth after incubation when compared with the control tubes was considered as MIC.
- MBC value was determined by sub culturing the test dilution (which showed no visible growth) onto freshly prepared BHI agar plates and incubated. The lowest concentration of the extract that yielded no single bacterial colony on the BHI agar plates was taken as MBC.

- **Cell adhesion:** S. mutans culture was grown in a test tube containing 5ml of BHI broth with 5% (w/v) sucrose and with different concentrations (ranging from 0.1 to 6.4 mg/ml) of OS extracts at 37°C for 48 hrs. After incubation, the glass tubes were slightly rotated and the planktonic cells were decanted. The adhered cells were then removed by adding 2ml of 0.5M NaOH followed by agitation. The cells were washed and suspended in sterile 1x PBS. The adherence was quantified spectrophotometrically at 600nm. Similarly, the test was performed with controls (Broth control and solvent control, 1% methanol). Adherence was calculated using the following formula-

Percentage of adherence = (Optical density(OD) of adhered cells/ O.D. of total cells) x 100

- **Cell surface Hydrophobicity:** S. mutans culture was grown in a test tube containing 5ml of BHI broth with 5% (w/v) sucrose and with different concentrations (ranging from 0.1 to 6.4 mg/ml) of OS extracts at 37°C for 48 hrs. After incubation, the culture was centrifuged and cells were washed twice and suspended in sterile 1x PBS, giving an absorbance at 600 nm equivalent to 0.3, the cell suspension (3 ml) was placed in tubes and 0.3ml of benzene was added. The tubes were agitated uniformly in a vortex mixer for 2 min and allowed to stand for 10 min at room temperature. After the benzene phase got separated from the aqueous phase, the optical density of aqueous phase was determined spectrophotometrically at 600 nm. The test was performed with controls (Broth control, solvent control 1% methanol) also. The formula used to calculate cell surface hydrophobicity : Percentage of Hydrophobicity = O.D of initial – O.D. of final x 100 ÷ O.D. of initial.

- **Acid production:** Five ml of BHI broth containing 5% (w/v) of sucrose and different concentrations of OS extracts were inoculated with 100 µl of grown cultures of S.mutans and incubated. The pH of the bacterial broth was assessed at the onset and after incubation using pH meter. The test was performed with growth controls (Broth and Broth with 1% v/v methanol).

- **Effect of ocimum sanctum leaves extract on Glucan Synthesis**
- **Preparation of crude GTFase**

The crude Glucosyltransferases (GTFase) were prepared and assayed to evaluate the effect of plant extracts on glucan synthesis. 30ml of BHI broth was inoculated with S.mutans and incubated at 37°C for 48 hrs. Grown culture was centrifuged at 10,000rpm for 15 mins and supernatant was collected. The GTFase were precipitated from culture supernatant by adding solid ammonium sulphate to 70% cut. The mixture was stirred at 4°C for 1hr and allowed to stand for another 1hr at 4°C. The mixture was centrifuged at 12,000rpm at 4°C for 20 min and precipitate was dissolved in 3ml of 20mM Phosphate buffer (pH 6.8) and then dialysed against 2mM Phosphate buffer (pH 6.8) at 4°C for 24hrs. The dialysed crude GTFase were stored at -20°C for further assay.

- **Glucan Assay setup**

A reaction mixture consisting of 0.5ml of crude enzyme and varying concentrations of the ocimum sanctum leaf extract in 20mM Phosphate buffer pH 6.8 (1ml) and 0.5ml of 0.4M Sucrose was incubated at 37°C for 18hrs. After incubation centrifuge the reaction mixture at 12,000rpm for 30mins. Discard the supernatant and wash the pellet with distilled water and suspend the pellet in 0.3ml of 0.1N NaOH. The total amount of glucans were measured by Phenol-Sulphuric acid method. A control reaction was maintained by mixing 1ml of 20mM Phosphate buffer pH 6.8, 0.5ml of crude GTFase and 0.5ml of 0.4M sucrose.

- **Glucans estimation by Phenol-Sulphuric acid method**

0.1mg/ml sugar solution (sucrose) was used as a standard. Pipette out 0.2, 0.4, 0.6, 0.8 and 1ml of

standard sucrose solution into a series of tubes and make the volume to 1ml in each tube with distilled water. Test samples were prepared by pipetting 0.3ml of glucan solution (prepared as above) into the tubes and make the volume to 1ml with distilled water. To all tubes (Blank+Standards+Test) add 1ml of 5% phenol and 5ml of Conc. Sulphuric acid and the contents are mixed and incubated in a water bath (30-35°C) for 20-30 mins. Measure the absorbance at 490 nm

- **Growth:** Its effect on the growth of S.mutans was examined at 37°C in the broth containing OS extract. The growth rate was monitored spectrophotometrically by measuring the optical density of the culture periodically. The test was performed with inoculum control containing broth and bacteria without extract.

RESULTS

One sample t-test was done to correlate the efficacy of various concentrations of ocimum sanctum/tulsi extract and chlorhexidine on S. mutans. The statistical analysis showed a significant correlation (p-value = 0.003) between effect of various antimicrobial agents on S. mutans. S. mutans had shown sensitivity to OS methanol extract, whereas it showed no sensitivity to ethanol and aqueous extract. It was sensitive to antibiotic 0.2% Chlorhexidine containing mouthwash. The diameter of Inhibition zone increased as the extract concentration of extract increased **(Fig 1)**.

OS methanol extract showed MIC value at 3.2mg/ml and MBC value at 6.4 mg/ml against S. mutans. The inhibitory effects of different concentrations of OS methanol extract on adherence of S.mutans to glass tubes are shown in figure 2.

The statistical analysis was done using one sample t-test to assess the correlation of cell adherence of S.mutans with various concentrations of ocimum sanctum/tulsi extract. A statistically significant correlation (p-value = 0.004) was found with cell adhesion and various tulsi extract concentrations. As the concentration of the OS methanol extract increased the adherence percentage of S.mutans to the glass tubes decreased. The adherence without extract was 52.1%, while at MIC 12.66% adherence was observed against S mutans.

Legends for illustration

The statistical analysis was done using one sample t-test to assess the correlation of cell surface hydrophobicity of S.mutans with various concentrations of ocimum sanctum/ tulsi extract. A statistically highly significant correlation (p-value = 0.001) was found with cell surface hydrophobicity and various ocimum sanctum/ tulsi extract concentrations.The cell surface hydrophobicity of S. mutans was found to drastically reduce in a concentration dependent manner figure 3. The hydrophobicity without the extract was 81.5% and at MIC concentration 22.80 % of hydrophobicity was noticed. One sample t-test was done to assess the effect of various concentrations of ocimum sanctum/tulsi extract on pH onset immediately and after 48hrs. A statistically highly significant correlation (p-value = 0.000) was found with various ocimum sanctum/tulsi extract concentrations on acid production (pH changes) by S. mutans. Results regarding its effect on acid production showed an expected effect on the acid production of S. mutans at MIC. There was a significant change from acidic to neutral pH with gradual increase in the concentration of the extracts. At MIC concentration there was a decrease in the pH from onset i.e. 7.42 to 7.27 (after 48 hrs) table 1.

The effect of ocimum sanctum /Tulsi leaf extract was assessed for the synthesis of Glucans by GTFase of S.mutans.The synthesis of Glucans was efficiently inhibited in a concentration dependent manner. GTFase in the absence of extract showed 98.18 µg/ml glucan synthesis, whereas, GTFase in the presence of extract concentration 0.8 and 1.6 mg/ml showed 60.88 and 49.22 µg/ml glucan synthesis. statistical analysis was done to estimate effect of change in concentration of ocimum sanctum extract tulsi extract on O.D & Glucan synthesis Insignificant correlation was found between various ocimum sanctum/ tulsi extract concentrations with O.D & Glucan synthesis. (table 2) There was no significant change in the growth pattern of the control and OS methanol extract treated cells.

Figure 1: Antibacterial activity of Ocimum Sanctum on S Mutans determined by using Agar Well Diffusion Method.

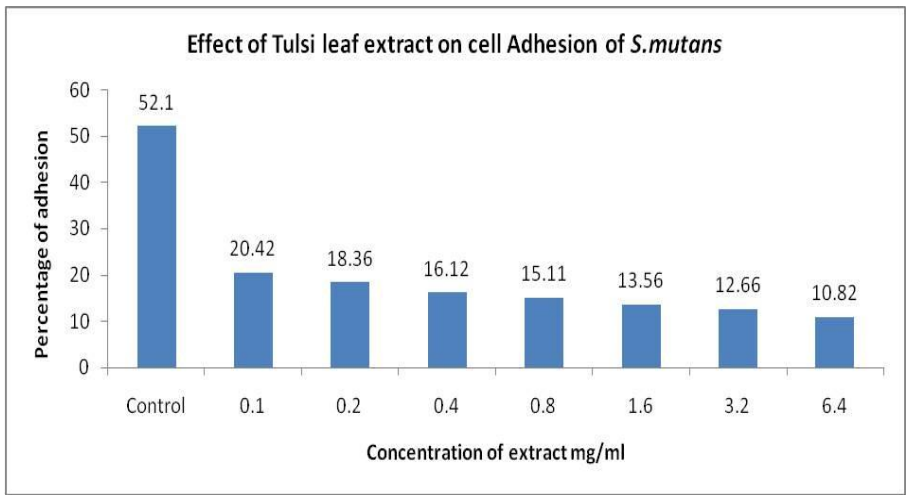

Figure 2: Figure 2: Effect of ocimum sanctum /tulsi methanol extract on Cell adhesion of S.mutans.

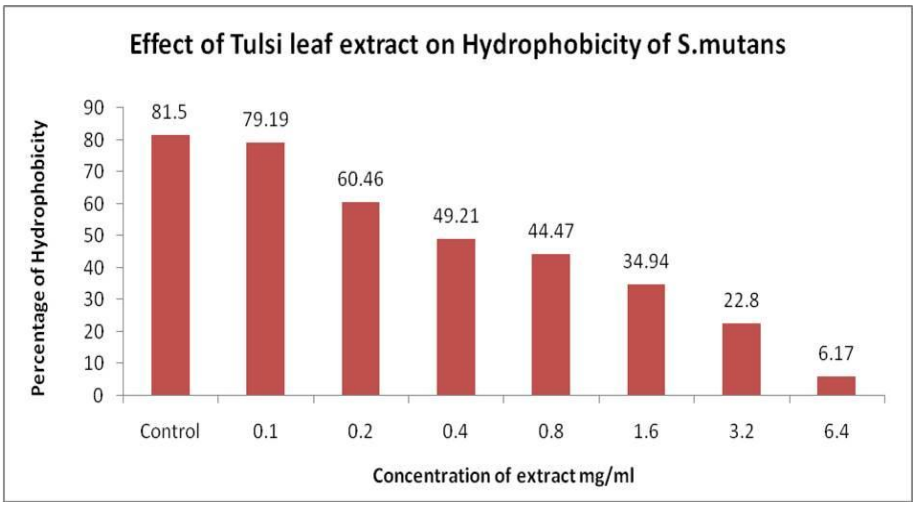

Figure 3: Effect of ocimum sanctum/tulsi methanol leaf extract on Cell surface Hydrophobicity of S.mutans

Table 2: Effect of ocimum sanctum methanol Extract on Glucan Synthesis

Sample	Conc mg/ml	O.D. at 490nm	Glucan synthesized µg/ml
Blank	0.00	0.000	-
Std 1	0.02	0.369	-
Std 2	0.04	0.703	-
Std 3	0.06	1.001	-
Std 4	0.08	1.137	-
Std 5	0.1	1.620	-
Inoculum control S.m	-	1.553	98.18

Extract	0.8	0.974	60.88
Extract	1.6	0.793	49.22

Table 1: Effect of ocimum sanctum methanol extract on Acid production of S.mutans

Extract Conc (mg/ml)	pH onset		pH after 48 hrs	
0.1	7.51		5.71	
0.2	7.50		5.72	
0.4	7.53		5.73	
0.8	7.50		5.75	
1.6	7.47		6.60	
3.2	7.42		7.27	
6.4	7.42		7.11	
Inoculum control	7.5		4.25	
Control (BHI broth)	7.58		7.56	
Control (BHI + methanol 1%)	7.55		7.51	
Statistical analysis				
t-test	513.48		17.58	
p-value	0.000*		0.000*	
95% Confidence Interval of the Difference	**Upper**	**Lower**	**Upper**	**Lower**
	7.52	7.45	6.83	5.21

DISCUSSION

India is a land filled with nature's bounty comprising abundant medicinal plants.[9] Plant extracts are known to possess antimicrobial compounds especially against bacterial pathogens.[15] But still the reported data so far available on medicinal use of plants are comparatively meager while taking into consideration the vast number of plant population.[13] In dentistry, Literature search has revealed handful of studies assessing the antimicrobial activity of natural products against oral pathogens.[21]

Streptococcus mutans is considered the main culprit among cariogenic microorganisms. A brief outline of S mutans role in the pathogenesis of dental caries determines that initially there is adhesion mediated attachment of S mutans, which adhere by hydrophobic bonds to the enamel surface and ferment sucrose. This sucrose metabolism is known to further promote firm adherence and aggregation of the bacteria to the tooth surface resulting in acid production and cavity formation.[2,22]

The use of an antimicrobial agent in the oral cavity might inhibit the growth of microorganisms, thus preventing the development of dental caries. Commonly used anticarigenic oral rinses are fluoride and chlorhexidine. Although chlorhexidine is a bacteriostatic and bactericidal chemical plaque control agent. It causes staining of teeth, mucous membrane and restoration, mucosal desquamation in children, develops resistant microorganisms, hypersensitivity reactions, and carcinogenicity. A well proven anticariogenic Fluoride exhibits fluorosis and toxicity. These adverse effects of chemotherapeutic rinses has led researchers to search for naturally safer anticariogenic alternative agents especially for children.[8,23]

Studies have proved that among ocimum species, OS has displayed higher antibacterial activity. It has been used in ayurvedic medicine, since ancient times. It is known as the mother medicine of nature as it is bestowed with enormous antimicrobial substances, used to treat various illnesses ranging from common cold to cancer.[8,9,24-28] Literature reports have proved its effectiveness against a wide range of bacteria, fungi and viruses.[3,8-21,26,27,29,30] It is known to inhibit Gram-positive organisms to a greater extent than Gram-negative organisms as their high lipid content cell walls act as a diffusional barrier making it less susceptible to the antibacterial agents.[11,13]

In most of the reported studies, investigations have been limited to assessing only the antibacterial activity of traditional medicinal plants against oral pathogens.[2,22] In order to determine the anticariogenic potential of plant extracts, it is essential to evaluate its effect on virulence factors of S mutans.[31,32] Thus we have evaluated the efficacy of ocimum sanctum at different levels.

An array of extractants have been tried to solubilize anti-microbials from plants like water or alcohol (methanol/ ethanol), which is used for the preparation of large quantity of crude extracts.[16] Alcoholic and aqueous extract of tulsi are recommended in most of the traditional medicinal systems as their antibacterial effectiveness was supposed to be more efficient.[26] Thus here we tried extracting ocimum sanctum using water, methanol and ethanol and found methanol extract to be more sensitive against S mutans, in contrast to Aggarwal et al[8] ethanol OS extract. The extract preparation method differed in both the studies, while there exists no standardized extract preparation protocol in the literature. In addition, the present study could not be corroborated with other studies in the literature as different solvents gave varied results when tested against different microorganisms.[26,29] Also, the essential oils in OS are

found to be more soluble in alcohol than distilled water. While OS methanol extract was found to exhibit comparatively higher activity against Bacillus Subtillis, E Coli[16], Strep Pyogenes, S Aureus, Bacillus Cereus, Pseudomonas Aeruginosa Klebsiella Pneumonia, Shigella, Enterobacter, Salmonella typhi[10,12] and Proteus vulgaris[14] than other organic and aqueous extracts.[9] The presence of eugenol in the methanol extract was observed by HPLC analysis[16], which is known to demonstrate broad spectrum antibacterial effect; these results coincide with the present study. In addition, increase in the antibacterial activity of OS methanol extract and essential oil was observed with increased concentration[12] against tested strains analogous to that observed against S mutans in our study.[27]

The antibacterial efficacy of ocimum sanctum is due to its rich phytochemical constituents. Though its chemical composition is complex[26], on phyochemical screening Ursolic acid, Flavonoids, Apigenin, Polyphenols, Anthocyanins, Luteolin, Terpene, Eugenol, Oleanolic acid, Rosmarinic acid, Linalol, β-caryophyllene [33], Phenol derivatives (Carvcrol, Thymol or sesquiterpene alcohols) have been found. Among these it has been proved that its eugenol, ursolic acid and phenol derivatives are known to exhibit broad spectrum antimicrobial activity.[13,24] In addition, its overall effects are due to the synergistic interaction of its active components, which cannot be fully replaced by its purified isolated compounds.[26,34,35] Ali and Dixit study has found that its flavonoids (orientin and vicentin) show evidence of antibacterial properties.[32] Its essential oil is known to exert membrane damaging effects to microbial strains and stimulate leakage of potassium ions leading to cytoplasmic damage.[27]

Agar diffusion method estimates the potency of the antimicrobial substance qualitatively. It measures the diameter of the zone of inhibition (ZOI) as the size of the growth-free zone determines whether the bacterium is susceptible, resistant, or intermediate to the leaf extract.[21] Analogous to other studies[2,8,22], the zone of inhibition of the leaf extracts were found to be lesser when compared to chlorhexidine, nevertheless it still could be regarded as a naturally safer alternative, considering its higher safety margin. Aggarwal et al[8] found higher zones of inhibition at 4% (10 µl, 20 µl, 30 µl, 50 µl,75µl) concentration of ethanol extract. Kulkarni and Damle have found chlorhexidine (0.12%) mouth rinse to be more efficient in reducing S mutans count in saliva than sodium fluoride and triclosan.[36] It is the most researched antimicrobial agent, thus with the available evidence it could be considered as a standard against which the newer ones could be tested[8]. Hence it was used as a positive control for assessing anticariogenic potential of OS extract.

Classical Antimicrobial assays like MIC and MBC are used to quantitatively determine bacteriostatic and bactericidal effects of the test material on pathogens. These tests are usually rapid and could be carried out with minimal cost.[37] In our study, methanol OS extract showed MBC value at 6.4 mg/ml and MIC value at 3.2mg/ml against S. mutans. In the premier study[8], 10, 20, 30, 50, 75 µl with 15 different concentrations (0.5,1 ,1.5,2,2.5 ,3,3.5,4,4.5, 5,6,7,8,9,10) of ethanol extract was used than 20µl with 2.5,5,10,20 concentrations of methanol extract used in our study. This inhibition produced by the extract against S mutans could be due to various extrinsic and intrinsic factors, different solvents and concentrations tried.[2,22]

An important step during caries formation is the adherence of microorganism to tooth surface, its prevention could inturn reduce its virulence.[31,32] We observed sucrose dependent inhibition of S mutans adherence with increasing concentration of OS extract, which could have lead to the drop in aggregation also. We had used the glass surface to mimic the hard surface of the tooth similar to Prabhu[31] and Hasan[32] et al study.

Adherence is known to occur due to the hydrophobic interactions between the (S mutans) cells and the adhering surface (tooth). Thus adherence and hydrophobicity of S mutans are interrelated in caries cascade. In the present study, the reduction in cell surface hydrophobicity of S. mutans in a concentration-dependent manner could be attributed to the binding of active components of the OS extract to the proteins associated with the cell surface of S mutans, enabling them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable, while extensive leakage from bacterial cells could lead to death. Besides less hydrophobic strains are known to implant worse in oral cavity.[31,32]

Acidogenecity (ability to generate acid) and acidurance (to function at low pH) are the two key virulence traits of S mutans.[31] We observed that there was a significant change from acidic to neutral pH with gradual increase in concentration of the extracts but after 24 hours the lower extract concentrations showed slight reduction in the pH. Nevertheless this extract still has the potential to produce a cariostatic effect.

Glucans are produced by S mutans through an enzyme glucosyltransferases (GTFs), which plays a pivotal role in the conversion of sucrose to a sticky substance called glucan[34]. In this study, glucan synthesis reduced in the presence of OS extract in a concentration dependent manner. Flavonoids are known to exhibit anti GTFase activity, thus here OS flavonoids could have played a role in the inhibition of GTF which might hinder S mutans endurance in the oral cavity.

In the present study, no significant change in the growth pattern at MIC value was noticed. It could be interpreted that OS extract did not have much effect on S mutans

growth while it played a role only in inhibiting its virulence factors.

This medicinal herb has been used for several thousand years without any adverse effects. Scientific studies have shown that aqueous extract did not produce any acute toxic symptoms in mice (100% survival) at doses 5g/kg body weight (bw) with LD value 6200 mg/kg bw and alcoholic extract was well tolerated (80% surival) upto 4g/kg bw with LD value 4600 mg/kg bw. Thus researchers have proved that it has high safety margin with low toxicity.[28,38]

The present study is unique as till date there are no studies assessing OS effect on virulence factors of S mutans, so it could not be directly compared with any other study, but to some extent pioneer study by Aggarwal et al[8] was compared. Other studies have been discussed only to elicit OS antibacterial potential.

OS inhibited virulence factors of S mutans, thus it could be suggested as a potential antistreptococcal agent. Barely, few studies have been done using this aromatic herb in dentistry, which could be due to the minimal attention it has received in dentistry, as it lacks published studies. Standardization of the plant extracts preparation method, concentrations tried, dosage and the evaluation in in- vitro and clinical trials will aid comparison between studies, in order to achieve better outcome.

CONCLUSION

Ocimum sanctum, time tested premier medicinal herb has shown anti streptococcus activity authenticating anticariogenic property by inhibiting acid production, cell surface hydrophobicity and adherence. It is too early to arrive at a conclusion as it demands further invivo and long term clinical trials so that it can be tried as a safer, cost-effective, and acceptable anticariogenic mouthrinse in children.

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