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POTENTIAL EFFICACY OF HERBAL MOUTHWASH AGAINST HUMAN DENTAL CARIES

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ABSTRACT

The main aim of this study was to find out the antibacterial activity of Aqueous extract of peel of *Punica granatum*, leaves of *Coleus aromaticus*, stem of and bark of *Azadirachata indica*, leaves of *Ocimum sanctum*, leaves of *Camellia sinensis*, bark of *Cinnamom verum*, Clove oil. For antibacterial test, Disc diffusion technique was used against Gram positive and Gram negative human pathogenic bacteria. Both the extract showed broad spectrum of inhibition by showing antibacterial effect for both Gram positive and Gram negative human pathogen bacterial strains. The Highest zone of inhibition were 25mm for Hot water *Punica granatum* peel extract of 100 µg concentration. The cold extract of *Punica granatum* demonstrated the strongest antibacterial activity with the MIC value of 0.19 µl, where *E.coli* 3 showed maximum inhibition against the extract. The results exhibits the scientific evidence for the centuries-old usage of this plant as a medicinal herb.

KEYWORDS: Leaf extract, Antibacterial activity, Mouth Wash, TLC, Phytoconstituent.

INTRODUCTION

Dental caries is a multifactorial, chronical bacterial disease, that causes demineralization and destruction of the hard tissues, usually by the production of acid bacterial fermentation of the food debris accumulated on the teeth surface. Worldwide, approximately 36% of the population have dental caries in their permanent teeth (Vos *et al.*, 2010). *Streptococcus mutans* is the most common cariogenic bacteria associated with dental caries. This bacterium has the ability to metabolize dietary sucrose and synthesize glucan by cell surface and extracellular glucosyltransferase (Shafer, Hine, Levy 2001).

Plaque may involve four or more different microorganisms combining forces to colonize the surface of the teeth. Remarkably, nature's own pomegranate fights the organisms' ability to adhere by interfering with production of the very chemicals the bacteria use as "glue" (Li *et al*., 2005). A study conducted at the Human Nutrition Center at Ohio State University in 2007 examined the effects of using a mouthwash containing pomegranate extract on the risk of gingivitis. *A. indica* extract has significantly reduced plaque index and bacterial count as compared to positive controls (chlorhexidine 0.2%). *Streptococcus mutans (S.mutans)* provide abbreviation in the saliva was found to be reduced significantly (Pai *et al*., 2004). Green tea mouthwash has been shown to effectively reduce plaque accumulation, and is free from side effects as of chemical mouthwashes (Moghbel, 2009). The anti-inflammatory and anti-infectious properties of tulsi make it a powerful treatment for gum disease (Biswas, 2005). Babool (*Accacia nilotica*) which has long been used for the treatment of skin, sexual, stomach and tooth problems. Coleus aromaticus is still quite unknown among Eastern herbalists. There is growing number of researches that are proving its effectiveness in fighting ailments. Cuban oregano leaves are simply eaten fresh for bronchitis, mouth and indigestion, flatulence, dyspepsia, epilepsy, rheumatism, kidney stones and helminthiasis.

MATERIALS AND METHODS

Collection of Plant material

Peel of *Punica granatum,* leaves of *Coleus aromaticus,* stem of and bark of *Azadirachata indica,* leaves of *Ocimum sanctum,* leaves of *Camellia sinensis,* bark of *Cinnamom verum,* Clove oil were collected from near by village around Erode. The plant material were washed with Tap water,air dried at room temperature and plant material were powder. Commercial mouthwash were purchase (Colgate Mouthwash, Chlorhexidine Mouthwash, Carysan oil, New JM Power) from the local market in erode.

Extraction of plant material (Chessbrough, 2000)

Aqueous extraction

10 gram of dried powder of Peel of *Punica granatum,* leaves of *Coleus aromaticus,* stem of *Azadirachta indica,* bark of *Azadirachata indica,* leaves of *Ocimum sanctum,* leaves of *Camellia sinensis,* bark of *Cinnamom verum,* was suspended in 100ml of Hot and Cold distilled water and the mixture was soaked for 24hours. The suspended solid was filtered through whatmann No.1 filter paper and kept in water bath at 60° C for 2hours. The dried crude extracts were stored at 4° C for further use.

Bacterial strain isolation and characterization

A total of 11 Teeth samples were isolated from Erode karthi dental Hospital and 9 Teeth sample were isolated from Erode Thiliraj dental Hospital. Bacterial isolates were maintained at 4° C at nutrient agar slant. A clinical pathogens were characterized by using Bergey's manual of systematic bacteriology

Antibiotic susceptibility test (Bauer *et al*., 1966)

3 *Streptococcus mutants, 3 E.coli,* and *3 S. aureus* cultures were selected for the further study. The antibiotic sensitivity test for each isolate was carried out on Nutrient Agar by disc diffusion method. Commercially available antibiotic disc such as Amikacin (30µg), Erythromycin (30µg), Ciprofloxacin (30µg), Streptomycin (30µg), Vancomycin (30µg), were placed onto nutrient agar plates swabbed with bacteria. The plate was incubated at 37° C and the zone of inhibition was observed after 24hours.

Antibacterial activity of plant extract

Agar well diffusion technique (Perez, 1990)

The extract was tested for antibacterial activity by standard agar well-diffusion method against pathogenic bacteria each 3 of *Streptococcus mutants, E.coli,* and *S. aureus.* The pure cultures of bacterial pathogens were sub cultured on nutrient broth. Culture was swabbed uniformly using sterile cotton swabs, and then 100µl of plant extract solution was loaded into the wells. After incubation at 37°C for 24 hours, the different levels of zone of inhibition was measured.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of plant extract was determined by serial dilution method in the nutrient broth, it can be done in Microtitterplate method. The inoculum was prepared from fresh overnight broth culture in nutrient broth. Plates were incubated for 24 hours at 37ºC. MIC was recorded as lowest extract concentration demonstrating no visible growth in the broth.

Preparation of synergistic mixture for Antibacterial activity (Finney, 1971)

The synergistic mixtures were prepared with different types of combination by *Punica granatum, Camellia sinensis* and *Ocimum sanctum*. It was prepared with a combination of different plant such as 1:1 ratio of *Punica granatum* and *Camellia sinensis*, *Camellia sinensis* and *Ocimum sanctum*, *Punica granatum* and *Ocimum sanctum*, and 1:1:1 ratio of *Punica*

granatum, Camellia sinensis and *Ocimum sanctum* the antibacterial activity of synergistic mixture was performed by well diffusion method.

Screening of Phytochemical Compounds

The Aqueous extracts of the *Punica granatum, Coleus aromaticus,* stem of *Azadirachta indica, Azadirachata indica, Ocimum sanctum, Camellia sinensis, Cinnamom verum,*were subjected to qualitative phytochemical tests for the identification of various active constituents, using the methodology Harborne 1998. The following major pharmaceutically valuable phytochemical compounds were analyzed as Alkaloid, Flavonoid, Tannin Triterpenoid, Saponins, Cardiac Glycosides, Phylobotannins, Steroids, Coumerins, Anthocyanins, Oil and fat.

Proximate analysis of the Plant Powder

The Ash and crude fiber content at Powder was determined by A.O.A.C in 1990. The total flavanoid content was estimated by the Bhom and Kocipai-Abyazan in 1994.

Column chromatography

Air dried powder peel of *Punica granatum* was soaked in methanol for overnight. 0.5 g of crude extract was used for small column (6mm x 2mm) chromatography. Silica gel (mesh 60-120) was used as column packing material. The column was eluted using a series of solvent systems: Hexane + Acetone (70+30%), Hexane + Acetone (40+60%), Acetone + Ethyl acetate (75+25%), Acetone + methanol (50+50%), Ethyl acetate + Methanol (10+90%) (0.175, 0.3014, 0.565, 0.755, 0.913). Each series solvent was added 10ml and fraction was collected 2ml up to 5 fractions. Collected fractions were applied on TLC plate using Acetone: Methanol: Acetic acid (75:08:50µl).

Phytochemical analysis using Thin Layer Chromatography (TLC) (Darabpour *et al.*, 2011)

The ethyl acetate, acetone, hexane, methanol extracts of the most effective parts of Peel of *Punica granatum* was analyzed by TLC; the presence of different constituent types in screened extracts was established by adding 0.5µl of the extracts at 100mg/ml concentration on pre coated silica gel plate. The plate was developed in a chamber saturated with solvent system: Acetone: Methanol: Acetic acid (75:08:100µl).

Preparation of Mouthwash: Herbal mouthwash was prepared by using 1:1:1 ratio of cold water extract of *Punica granatum*, *Camellia sinensis* and *Ocimum sanctum*. 1% of sodium chloride and lemon were added for reducing the bitter taste.

RESULT AND DISCUSSION

A total 20 dental samples were collected from patient diagnosed with dental caries by a physician. Out of 20 isolates recovered and identified prevalence of *Streptococcus* sp. Each 3 isolates like *S. mutans, Staphylococcus, E.coli* were taken for further study. The various biochemical characterization and Gram staining tests were performed for identification of bacteria. Essam *et al*., 2014 collected 75 plaque sample for his study and 35 isolates are expected to be belonging to *S. mutans.* The antibiotic of Vancomycin (30µg) produced 21.35mm zones, Erythromycin (30µg) produced 27.15mm zones, Ciprofloxacin (30µg) produced 26.25mm zones, Amikacin (30µg) produced 31.05 zones, Streptomycin (30µg) produced 25.05mm zones against *S. mutans, Staphylococcus, E.coli*. Gamal *et al* 2014 reported the susceptibility of *S. mutans* isolate taken antibiotics revealed that highly sensitive to vancomycin with percentage 95%, penicillin. *S. mutans, Staphylococcus* and *E.coli.* the result revealed that inhibition zone around the extract varied from Colgate Mouthwash 13.2mm, Chlorhexidine Mouthwash 20.2mm, Carysan Oil 16.2mm, New JM power 8.35mm. Similar results found by Jain *et al.,* 2015 reported for Chlorhexidine. The highest zone of inibition around the well varied peel of *Punica granatum* hot extract were observed at 25.35mm. Leaves of *Coleus aromaticus* hot extract was 13.3mm. Stem of *Azadirachta indica* hot extract were attained at 14.25mm. Bark of *Azadirachata indica* hot extract were obtained at 18.45mm. Janani *et al.,* 2013 reported aqueous extract of *Punica granatum* showed 24mm zones for *S.aureus* as shown in Table 1 and fig 1.

Table. 1.

S.no	Code	Zone of inhibition (mm)	
		Hot Extract	Cold Extract
1	A	22.15 ± 0.21	18.25 ± 0.35
2	B	19.05 ± 0.07	18.35 ± 0.49
3	C	21.25 ± 0.35	20.1 ± 0.14
4	D	19.2 ± 0.28	18.15 ± 0.21
5	E	19.1 ± 0.14	18.3 ± 0.42
6	F	20.2 ± 0.28	12.2 ± 0.28
7	G	25.35 ± 0.49	22.45 ± 0.63
8	H	22.25 ± 0.35	23.35 ± 0.49
9	I	19.4 ± 0.56	16.45 ± 0.63

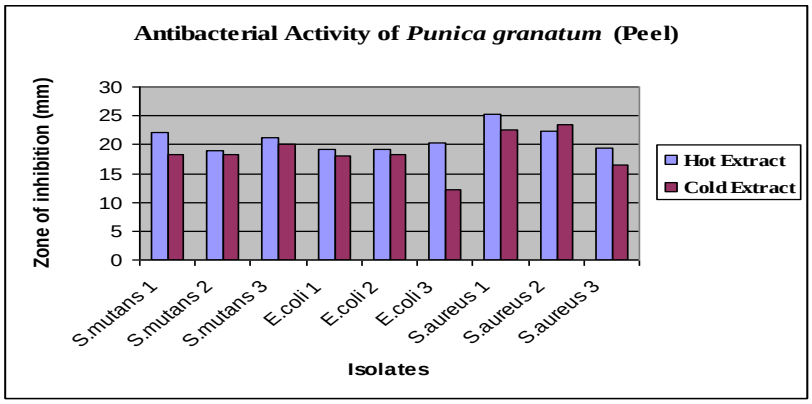
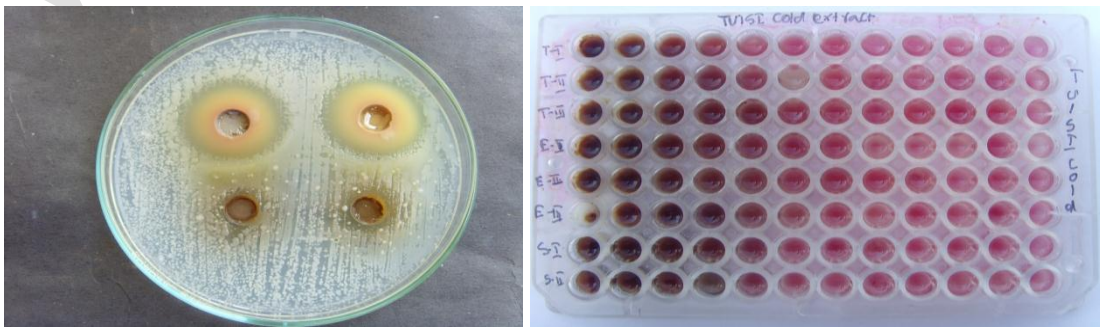


Figure. 2.

A – *S.mutant* 1, B- *S.mutant* 2, C- *S.mutant* 3, D – *E.coli* 1, E – *E.coli* 2, F- *E.coli* 3, G- *S.aureus* 1, H - *S.aureus* 2, I - *S.aureus* 3.

The maximum zone of inhibition from Bark of *Cinnamom verum* hot extract were obtained 17.35mm. Clove oil 50µl were observed at 19.05mm and 100µl was 23.35mm. Charu gupta *et al.*, 2011 stated clove oil produced (13mm) maximum zone against *S.mutans.* Leaves of *Ocimum sanctum* hot extract from 10.4mm to 15.4mm and leaves of *Ocimum sanctum* cold extract from 7.4mm to 17.05mm. Pooja agarwal *et al.*, 2010 reported that the 75 µl of ethanol extract of *Ocimum sanctum* showed upto 25mm zone of inhibition against *S. mutans.* Leaves of *Camellia sinensis* hot extract was 24.15mm.

The minimum inhibitory concentration of hot and cold extract of *Punica granatum, Coleus aromaticus, Azadirachta indica* (stem and bark) *Ocimum sanctum, Camellia sinensis, Cinnamom verum* and Clove oil. 0.19µl of cold extract of *Punica granatum* showed maximum bactericial activity for *E.coli* 3. Cold extract of *Ocimum sanctum* showed moderate bactericial activity for selected isolates (50µl). Cold extract of *Camellia sinensis* inhibited the bacterial growth at the concentration of 3.12 µl. Subhas chandrappa *et al.*, 2010 reported MIC value of hot water extract of *Coleus aromaticus* ranged from 46 – 62 µg/ml.



Antibacterial activity of plant extract Minimum inhibition concentration(MIC)

Based on the antibacterial activity and MIC results three plant extracts (Peel of *Punica granatum*, leaves of *Ocimum sanctum*, leaves of *Camellia sinensis*) were chosen for further study. The selected herbs were used at the concentration of 1:1:1 ratio. The synergistic effect of selected plant extracts. Based on the zone of inhibition results *Punica granatum* with *Camellia sinensis* and *Ocimum sanctum* got maximum zone of inhibition compared to other synergistic mixtures as shown in fig 2.

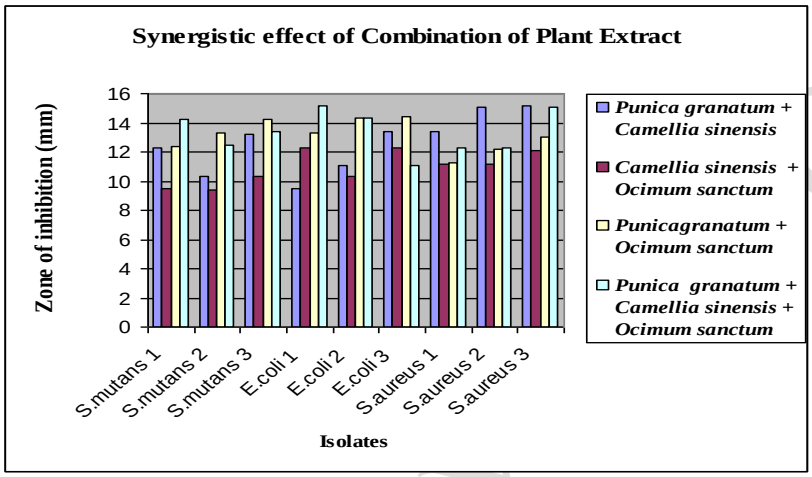


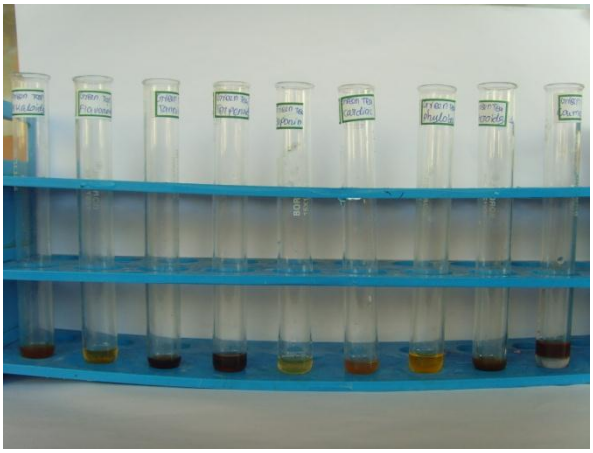
Figure. 2.

Qualitative and Quantitative Phytochemical Analysis

As a result of this study it was found that the peel extract of *Punica granatum* generally revealed good phytochemical constituents. Lali Growther *et al*., 2012 also reported the TLC analysis of *Punica granatum* as shown in Table 2 and 3.

Table. 2.

Parameters	***Punica granatum***	***Camellia sinensis,***	***Ocimum sanctum***
Alkaloids	+	+	+
Flavonoids	+	+	-
Tannis	+	-	+
Terpenoids	+	+	+
Saponins	+	+	-
Cardica Glycosides	-	-	-
Phylobotannis	-	-	-
Steroids	+	+	+
Coumarins	+	+	+
Oil and Fat	-	-	-
Ash (g)	0.48 ± 0.33	1.23 ± 0.98	0.9 ± 0.6
Flavonoids (g)	0.62 ± 0.47	0.82 ± 0.52	0.78 ± 0.43
Fibre (g)	0.65 ± 0.45	0.78 ± 0.58	1.52 ± 1.07



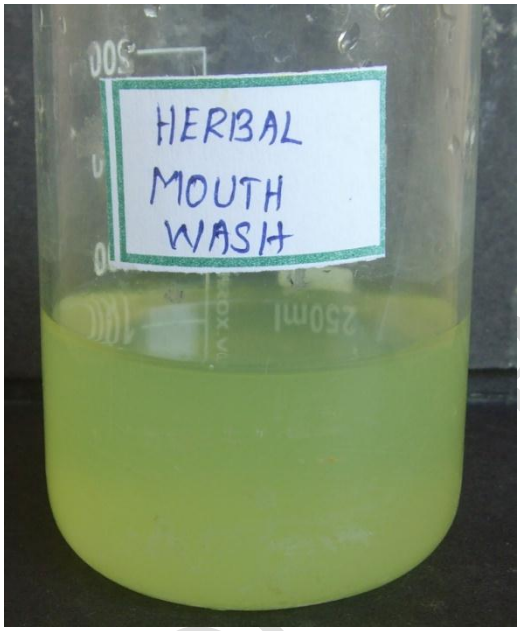
Qualitative Phytochemical Analysis Quantitative Phytochemical (Fiber content)

Table. 3: Column and Thin layer chromatography.

S. No	Solvent	Fraction	Rf value	Identification of the compound	TLC Spot Observation
1	Hexane + Acetone 7 : 3	F1 to F3	F1=1.15 F2=1.80 F3=1.31	Alcohol	Yellow spot
				Carboxylic acid	Yellow-green on a blue back ground
2	Hexane + Acetone 4 : 6	F4 to F7	F4=1.125 F5=1.069 F6=1.80 F7=1.916	Alcohol	Yellow spot
3	Ethyl acetate + Acetone 2.5 : 7.5	F8 to F11	F8=2.095 F9=1.607 F10=1.533 F11=1.643	Alcohol	Yellow spot
				Carboxylic acid	Yellow-green on a blue back ground
4	Methanol + Acetone 5 : 5	F12 to F14	F12=1.704 F13=2.647 F14=1.305	Alcohol	Yellow spot
				Carboxylic acid	Yellow-green on a blue back ground
5	Methanol + Ethyl acetate 9 : 1	F15 to F17	F15=1.184 F16=1.394 F17=1.382	Alcohol	Yellow spot
				Carboxylic acid	Yellow-green on a blue back ground
				Phenolic compound	Bluish black colour
				Carbohydrate	Purple colour

Column Chromatography (Fraction) Phenolic Compound

The stability analysis of synergistic mixtures (mouthwash) were tested by using Agar well diffusion method at 8th day and 16th day. It was prepared with a combination of cold water extract of *Punica granatum*, *Camellia sinensis* and *Ocimum sanctum* at 1:1 ratio. Upto 16th day the synergistic herbal mouth wash was highly effective against dental pathogens**.**



CONCLUSION

The aqueous crude extracts of Peel of *Punica granatum,* leaves of *Coleus aromaticus,* stem of *Azadirachta indica,* bark of *Azadirachata indica,* leaves of *Ocimum sanctum,* leaves of *Camellia sinensis,* bark of *Cinnamom verum,* were exhibited antibacterial activity against *Streptococcus mutants, E.coli,* and *S. aureus.* Possible antimicrobial substances contained in the extracts including Tannins, Alkaloids and Terepenoid. It therefore suggests that constituents of the plant extracts could serve as a source of drugs useful in the chemotherapy of enteric diseases.

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