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Combating multidrug resistance of predominantly occurring oral pathogenic bacteria in periodontitis with medicinal plant extracts

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Abstract

Screening of certain medicinal plants for the antibacterial activity against predominantly occurring oral pathogens in periodontitis has been the focus of this study. The oral swabs of patients' suffering from periodontitis were the source of the pathogenic bacteria. Microorganisms co-inhabiting the site of infection were isolated, characterized and cultured. It was observed that the predominantly occurring microorganisms were *Prevotella intermedia*, *Porphyromonas gingivilis*, *Bacteroides forsythus*, *Actinobacillus actinomycetemcomitants* and *Fusobacterium nucleatum*. These pathogenic organisms were studied for their antibiograms and the drug resistance pattern studied. These multidrug resistant organisms were treated with the different concentrations of the methanolic and aqueous extracts of *Ochna gamblei* and *Psoralea corylifolia.*

The MIC studies were undertaken to adjudge the minimum concentration of the plant extract that inhibits the pathogens. Finally, the phytochemical analysis was performed to ascertain the bioactive phytochemical responsible for the antibacterial activity. Further studies aim towards determining toxicity parameters and bioavailability investigations with a view of generating a potential biotherapeutic drug to be effective and cost effective.

Keywords: periodontitis | MIC | multi drug resistance | phytochemical analysis | Nutrients

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Introduction

The etiologic agents of periodontitis have been identified as some specific microorganisms. More than 700 bacterial species or phylotypes, of which over 50% have not been cultured, have been detected in the oral cavity. Species belonging to the genera Gemella, Granulicatella, Streptococcus, and Veillonella are commonly found. There is a distinctive predominant bacterial flora of the healthy oral cavity that is highly diverse and is site and subject specific (Aas *et al.*, 2005). The bacteria, *Porphyromonas gingivilis*,

Bacteroides forsythus, Prevotella intermedia, Campylobacter rectus, Eikenella corrodens, Fusobacterium nucleatum, Actinobacillus actinomycetemcomitants, Treponema and Eubacterium species are the major components of dental plaque, which is host associated biofilm. Persistent plaque deposition causes inflammation of the gingiva which is called as gingivitis. When gingivitis is not treated, it can advance to periodontitis. In periodontitis, gums pull away from the teeth and form spaces called pockets that become infected.

The main goal of treatment is to control the spread of infection. Depending on the extent of the gum disease, the treatment varies. Therapeutic antibiotic along with surgery are the most opted form of treatment. But, due to high costs of antibiotics, the disastrous side effects and emergence of multi drug resistant strains of these pathogenic bacteria, a safe alternative is being investigated scientifically in this study. In the indigenous health care delivery system, numerous plant species and natural products derived from plants are used to treat diseases of infectious origin. Due to emerging antibiotic resistant infections, considerable attention has been paid to utilize eco-friendly and bio-friendly plant based products for prevention and cure of different human diseases since they are safe and effective. Studies have attempted to shed light on the antibacterial activity of some indigenous medicinal plants. Nonetheless, the investigations have primarily been restricted to screening only. Considering the high costs of the synthetic drugs and their various side effects, the search for alternative products from plants used in traditional system of medicine is justified. In order to promote herbal drugs there has to be an evaluation of therapeutic potentials of drugs (Geyid *et al.*, 2005). The medicinal plants, *Ochna gamblei* and *Psoralea corylifolia* are widely used by the traditional medicinal practitioners for the treatment of infectious diseases and hence have been put to systematic scientific investigation in this study.

Ochna gamblei belongs to the family Ochnaceae. It is a small, very pale, glaucous tree with sessile sometimes cordately based obtuse leaves. It is extensively used in the folk medicine as an anti-diarrheal, astringent and an anti- dysenteric. Bark of the tree is very thick; red colored and easily peeled off. Locally 5-10 g powder is given to patients suffering from diarrhea and hemorrhagic dysentery. (Moon *et al.*, 2009). *Psoralea corylifolia* belongs to the family Fabaceae. Isopsoralidin, a new crystalline material has been obtained from the seeds. Raffinose has also been isolated. The seed oil is anti staphylococcal at 0.5 µg/ml. The antibacterial activity of this plant has been reported. (Yin *et al.*, 2004; Newton *et al.*, 2000; Newton *et al..*, 2002)

Materials and Methods

Plant Collection and Processing: The bark of *Ochna gamblei* and leaves from *Psoralea corylifolia* was collected from urban fringe areas of Nagpur District (M. S., India). Voucher specimens have been deposited with the Department of Botany; RTM Nagpur University, Nagpur, India. The bark of *O. gamblei* and leaves of *P. corylifolia* were washed under running tap water and air dried under shade. After 15 days the dried bark of *O.*

gamblei and dried leaves of *P. corylifolia* were separately macerated in a mixer grinder to yield a fine powder which was sieved to yield particle size of 50-150mm. This dried powder (50g) was extracted in a Soxhlet apparatus using 100ml of petroleum ether (60-80^{0}C), chloroform (61^{0}C), methanol (78.5 ^{0}C) and water (80 ^{0}C)(Mukherjee, 2006). The extracts obtained were dried and stored in sealed tubes at 4 ^{0}C. The methanolic extracts were found to be more potent against multi drug resistant strains of uropathogenic bacteria such as *E.coli, S. aureus, K. pneumoneae* and *S. typhi* than the other solvent counterparts and hence used in this study (Moon *et al*., 2006).

Clinical Isolates: Clinical isolates of *Prevotella intermedia*, *Porphyromonas gingivilis*, *Bacteroides forsythus*, *Actinobacillus actinomycetemcomitants* and *Fusobacterium nucleatum* were obtained from swabs from periodontal pockets which are pathological deepening of the gingival sulcus. The bacterial cultures were maintained on Nutrient Agar (Himedia, Mumbai) at 4 ^{0}C and subcultured every two weeks.

Inoculum Preparation: Stock cultures of clinical isolates were maintained at 4^{0}C on nutrient agar slants. A working bacterial inoculum was prepared by inoculating a loop full of the clinical isolate into a 3 ml sterile nutrient broth tube and incubated at 37^{0}C for 24 hours. The turbidity was matched with 0.5 Mc Farland's Nephelometer Standard (WHO, 1983; *NCCLS*, 2000). Dilutions to the tube were done with sterile nutrient broth to get a cell density corresponding to 2 x 10^6 CFU/ml.

Media: Nutrient Agar (M001), Agar Agar Type I (RM666), Mueller Hinton Agar No. 2 (M1084) and Nutrient broth (M002) were procured from Hi-Media, Mumbai. The preparation of media was done strictly according to the manufacturer's instructions.

Antibiotic discs: Commercially available standard antibiotic discs were obtained from Hi-Media, Mumbai. The abbreviations and strength are given in brackets. The antibiotic discs used were Amoxicillin (Ac-30 mcg), Ampicillin (A-10 mcg), Chloramphenicol (C-30 mcg), Erythromycin (E-15 mcg), Penicillin-G (P-10 mcg), Kanamycin (K-30 mcg), Tetracyclin (T-30 mcg), Cephalexin (Cp-30 mcg), Ciprofloxacin (Cf-5 mcg), Co-trimoxazole (Co-25 mcg), Gatifloxacin (Gf-5mcg), Norfloxacin (Nx-10 mcg), Ofloxacin (Of-5mcg), Pe-floxacin (Pf-5 mcg), Sparfloxacin (Sc-5 mcg) and Streptomycin (S-10 mcg).

Antibiotic sensitivity test: The antibiotic sensitivity of the clinical isolates was studied by Bauer-Kirby disc diffusion method (Bauer *et al*., 1966). A sterile non-toxic cotton swab was dipped into the inoculum tube and rotated firmly against the upper inside wall of the tube to express excess fluid. This swab was now used to streak the entire agar surface of the plate three times turning the plate 60 ^{o} between each streaking. Five antibiotic discs were placed aseptically on each plate with enough spacing. All the plates were incubated at 37 ^{0}C for 18-24 hours. After incubation, plates were examined for zone of inhibition. Zones were measured and recorded as sensitive, resistant or

intermediate referring the zone size interpretive chart (NCCLS, 2002).

Activity Testing of Methanolic Extracts of Plant: A suspension (0.1 ml) of the test organisms from the 18 hour cultures was thoroughly mixed with 20 ml of sterile Mueller Hinton Agar maintained at 45-50 ^{0}C. The seeded M.H. Agar was poured in presterilized petri plates and set aside. After solidification, the seeded agar was punched with a flamed (sterile) 10mm cork borer in order to obtain a well of 10mm diameter in the center of the petri plate. 100 µl of the methanolic plant extract is loaded into the well accurately with a micropipette (with presterilized tips) to obtain concentration of 20, 40, 60, 80 and 100mg/ml. The petri plates were delicately handled and kept in refrigerator for 30 minutes and then at room temperature for 30 minutes which facilitated diffusion of the plant extract. The petri-plates were then incubated at 37 ^{0}C for 24 hours (Perez *et al*., 1990). The zone of inhibition was measured with HiAntibiotic ZoneScale0, HiMedia, Mumbai. 2% MeOH and sterile distilled water were used as negative controls.

Chemical prospection: The respective fractions of bark of *O. gamblei* and leaf of *P. corylifolia* were submitted to phytochemical tests in order to detect the presence of sterols, alkaloids, saponins, flavanoids, cardiac glycosides, cyanogenetic glycosides, anthroquinones, tannins, phenol, proteins, amino acids and carbohydrates. These tests are based on visual observation of color modification or precipitate formation after addition of specific reagents.

Minimal Inhibitory Concentration (MIC) Determination: MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MIC determination is important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. Clinically, the minimum inhibitory concentrations are used not only to determine the amount of antibiotic that the patient will receive but also the type of antibiotic used, which in turn lowers the opportunity for microbial resistance to specific antimicrobial agents.

In this study, the determination of MIC was done by the Agar Dilution Method (NCCLS, 1990). Stock solutions of 100mg/ml of the methanolic extract of selected plants were prepared in DMSO-Tris buffer (3:7). 100 µl to 3000 µl of this stock solution was added to 20 ml sterile M.H. Agar to achieve final concentration of 0.5 mg/ml, 1mg/ml, 2mg/ml, 5mg/ml, 8mg/ml, 10mg/ml and 15mg/ml. These were poured in petridishes and allowed to solidify. The reverse side of the plate was divided into 10 checker board blocks by glass marker to accommodate bacterial cultures. A bacterial inoculum of all the test organisms was prepared.

Results and Discussion

The antibiogram shows the pattern of resistance obtained after performing antibiotic sensitivity tests which are shown in Table 1. The clinical isolates were found to be resistant to one or more than one antibiotic. A

sensitivity test performed with commonly used sensitivity test disks resulted in the appearance of multiple drug resistance phenotypes of the bacteria tested. A comparison of data in the inhibition zones of pathogenic bacteria showed that ampicillin, amoxicillin, kanamycin, ofloxacin and kanamycin were resistant against all of the bacterial strains tested.

Table 2 shows the antibacterial activity of aqueous and methanolic extracts *O. gamblei* and *P. corylifolia* against the 5 microbes. The extracts were tested at 10, 30, 50 and 100 mg/ml. Clinical isolates of *Porphyromonas gingivilis* which show resistance to commonly used antibiotics like Amoxicillin, Penicillin, Cephalexin, Streptomycin etc., when treated with MeOH extract of *Ochna gamblei* and *Psoralea corylifolia* show a zone of inhibition of 24 mm and 18 mm diameter at 100 mg/ml concentration thereby suggesting the potential role of the bioactive phytochemical for antibacterial activity.

In this study, the clinical isolates of *Prevotella intermedia* show resistance against most commonly used antibiotics such as Amoxicillin, Co-trimoxazole, Cephalexin, Ampicillin, Kanamycin, Penicillin etc. Interestingly, the MeOH extracts show commendable activity against multi-drug resistant *Prevotella intermedia* strains. Worth mentioning is the extraordinary activity shown by both the plants at 100 mg/ml concentration. The clinical isolates of *Campylobacter rectus* show resistance to more than one antibiotic as clearly seen from the resistance pattern in Table 1. The MeOH extracts of *O. gamblei* bark are effective through 30 mg/ml to 100mg/ml concentration. The clinical isolates of *Actinobacillus actinomycetemcomitants* show a common resistance pattern for Am, A, P, and Cp. The MeOH extracts of *O.gamblei* showed more potential as an antibacterial agent at all the concentrations tested whereas *F. nucleatum* also shows sensitivity towards both the plants.

Table 3 shows the phytochemical profiling for the two plants. *Ochna gamblei* showed the presence of sterols and alkaloids in its methanolic counterparts while *P. corylifolia* showed the presence of sterols, alkaloids tannins in it methanolic extracts though at a lesser concentration. The chemical prospection of these plant extracts and fractions have indicated the presence of various secondary metabolite classes (Table 3) that are known to present different therapeutic applications. The activity of the plant extracts relates to the respective composition of the plant bioactive phytochemical. Sterols, terpenoids and tannins present in the respective extracts of plants were capable of affecting the growth of the tested bacterium. The terpenoids exhibit activity against the test microorganisms acting as either protein denaturing agents, solvents or dehydrating agents. Terpenoids act upon bacterial membranes by bringing about the inhibition of electron transport, protein translocation, and phosphorylation steps and other enzyme-dependent reactions. The plant extracts clearly demonstrate antibacterial properties, although the mechanistic processes are poorly understood. These activities suggest potential use as chemotherapeutic agents.

Table 4 shows the MIC of the two plants against the tested microorganisms. The Minimum Inhibitory Concentrations results of the selected plants prove that the plant extracts exhibit bacteriostatic activity at high dilution rates. The overall results reveal that the plants or plant extracts can be effectively used as therapeutic agents. The reason for the inhibitory effect of these extracts is presumably due to the presence of bioactive phytochemicals which inhibit the growth of bacteria. This study has highlighted some plants which are worthy of further investigation for their antibacterial activities to assess the *in vivo* and *in vitro* activities of these extracts against pathogenic strains of microorganisms.

Conclusion

From the results of antibacterial screening of *Ochna gamblei* and *Psoralea corylifolia*, it is clear that both the plants display significant antibacterial activity. Further research in this study focuses on the isolation of bioactive phytochemicals and also inducing the callus to produce higher concentrations of bioactive phytochemicals which are responsible for the antibacterial activity and to combat the multi drug resistance shown by the human oral pathogenic bacteria. It can be used as antibacterial supplement in the developing countries towards the development of new therapeutic agents. Additional *in vivo* studies and clinical trials would be needed to justify and further evaluate the potential of these plants as antibacterial agents in topical or oral applications.

		Zone of inhibition in mm diameter																	
	S.No	Microorganisms	A	Am	C	E	P	K	T	Cp	Cf	Co	Gf	Nx	Of	Pf	Sc	S
1		*Porphyromonas gingivilis*	18	22	24	19	17	19	22	15	33	24	29	32	32	30	32	12
2		*Prevotella intermedia*	32	33	26	27	22	26	27	29	28	29	19	19	26	26	32	15
3		*Camphylobacter rectus*	20	22	25	22	25	22	22	16	32	20	26	28	26	22	30	12
4		*Actinobacilius actinomycetemcomitants*	18	20	21	20	18	17	20	14	22	18	20	20	30	30	30	16
5		*Fusobacterium nucleatum*	<10	<10	13	13	<10	15	15	11	20	12	21	24	24	27	21	<10

Table 1: Antibiotic sensitivity of micro-organisms

Sr. No	Test Microorgani	C 1	C 2	Type of	Diameter of zone of inhibition in mm Conc. of extract is in mg/ml									
					OCHNA GAMBLEI				*PSORALEA CORYLIFOLIA*					
					10	30	50	100	10	30	50	100		
1.	*P.gingivitis*	--	--	MeOH	18 + SD 0	19+ SD1	21+ SD 0	24+ SD 0	12+ SD 0.5	14+ SD 0.25	16+ SD 0.25	18+ SD 0.25		
		--	--	Aq	14± SD 0.5	15± SD1	16± SD 1	23± SD1	10± SD 0	11± SD 1	12± SD 0.5	14± SD 0.5		
2.	*P.intermedia*	--	--	MeOH	19⊥ SD 1	25⊥ SD1	24⊥ SD 0	29⊥ SD1	19⊥ SD1	24⊥ SD 0	26⊥ SD 0.25	28⊥ SD 0.25		
		--	--	Aq	16± SD 1	19± SD1	19± SD 0	21± SD 0	0± SD 1	12± SD 1	16± SD 0.5	18± SD 1		
3.	*C. rectus*	--	--	MeOH	14± SD 1	20± SD 0.5	21± SD 0.5	27± SD 1	12± SD 0.5	14± SD	15± SD 0.5	17± SD 0.25		
		--	--	Aq	0± SD 0	10± SD 0	12± SD1	13± SD 0	10± SD 0.5	12± SD 0	14± SD 0.25	16± SD 0.25		
4.	*A. actinomycete-*	--	--	MeOH	19± SD 0.5	21± SD 1	22± SD0	28± SD 1	13± SD 0	16± SD 1	18± SD 1	22± SD 0.25		
		--	--	Aq	0± SD 0	16± SD 0	19± SD 1	22± SD 0.5	0± SD 0.5	11± SD 0.25	14± SD 0.25	15± SD 0.5		
5.	*F. nucleatum*	--	--	MeOH	12± SD 1	14± SD 0	16± SD 0	18± SD 0.5	11± SD 0	14 ± SD 0.25	16± SD 1	18± SD 0.5		
		--	--	Aq	0± SD 0.5	10± SD 0	12± SD 0.5	14± SD 0	0± SD 0.5	10± SD 0.25	12± SD 0.5	14± SD 1		

Values are mean inhibition zone (mm) ⊥ S.D of three replicates.

Table 2: Antibacterial activity of methanolic and aqueous extracts of *Achna gamblei* and *Psoralea corylifolia*

	Ochna gamblei					*Psoralea corylifolia*				
Tests	**E**	**C**	**A**	**M**	**Aq**	**E**	**C**	**A**	**M**	**Aq**
Sterols										
Salkowski's test	-	-	+	+++	++	-	-	+++	++	+
Liberman test	-	-	+	+++	++	-	-	+++	++	+
Liberman Burchard test	-	-	+	+++	++	-	-	+++	++	+
Alkaloids										
Dragendorf's reagentt	-	-	+	+++	++	+++	-	-	++	+
Mayer's reagent	-	-	+	+++	++	+++	-	-	++	+
Wagner's test	-	-	+	+++	++	+++	-	-	++	+
Hager's test	-	-	+	+++	++	+++	-	-	++	+
Tannic acid test	-	-	+	+++	++	+++	-	-	++	+
Scheibler's test	-	-	+	+++	++	+++	-	-	++	+
Saponins										
Foam test	-	-	-	-	-	-	-	-	-	-
Flavonoids	-	-	-	++	-	++	-	-	-	-
Cardiac Glycosides										
Keller-Killiani test	-	-	-	++	-	-	-	-	-	-
Legal's test	-	-	-	++	-	-	-	-	-	-
Cyanogenetic glycosides										
Grignard's test	-	-	-	-	-	-	-	-	-	-
Anthroquinones										
Bortranger's test	-	-	+	++	-	-	-	-	-	-
Tannins										
Ferric Chloride test	-	-	-	-	-	-	-	-	++	+
Lead acetate test	-	-	-	-	-	-	-	-	++	+
Potassium dichromate test	-	-	-	-	-	-	-	-	++	+
Gelatin solution test	-	-	-	-	-	-	-	-	++	+
Bromine water test	-	-	-	-	-	-	-	-	++	+
Phenols										
Ferric Chloride test	-	-	-	-	-	-	-	-	-	-
Nitric acid test	-	-	-	-	-	-	-	-	-	-
Phthalic acid test	-	-	-	-	-	-	-	-	-	-
Proteins										
Biuret test	-	-	-	++	++	-	-	-	-	-
Xanthoproteic test	-	-	-	++	++	-	-	-	-	-
Millon's test	-	-	-	++	++	-	-	-	-	-
Amino acids										
Ninhydrin test	-	-	-	++	++	-	-	-	-	-
Carbohydrates										
Molisch test	-	-	+	++	++	++	++	-	-	-
Barfoed's test	-	-	+	++	++	++	++	-	-	-
Fehling's test	-	-	+	++	++	++	++	-	-	-

E: Petroleum Ether; C: Chloroform; A: Acetone; M: Methanolic; Aq: distilled water
* - denotes absence of the phytochemical tested; *+ denotes concentration of phytochemical tested as low; * ++ denotes concentration of phytochemical tested as moderate; * +++ denotes concentration of phytochemical tested as good; * ++++ denotes concentration of phytochemical tested as high

Table 3: Phytochemical analysis of plant extracts

Sr. No.	Microorganisms	MIC in mg/ml of extracts	
		O. gamblei	*P. corylifolia*
1	*Porphyromonas gingivilis*	*10*	5
2	*Prevotella intermedia*	>15	8
3	*Camphylobacter rectus*	1	8
4	*Actinobacilius actinomycetemconitants*	>15	5
5	*Fusobacterium nucleatum*	10	8

Table 4: The MIC of *O. gamblei* and *P. corylifolia*

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