Research Article

In vitro Evaluation of Antimicrobial Properties of Extracts of *Pterocarpus Santalinus* against Oral Pathogens and its Synergistic Effect with Ciprofloxacin and Fluconazole.

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Accepted on: 24-12-2012; **Finalized on:** 28-02-2013.

ABSTRACT

Crude extracts of *Pterocarpus santalinus* extracted with Petroleum ether, methanol, n-butanol, chloroform and ethanol were tested for antibacterial activity against 8 isolates viz. *Staphylococcus aureus*, *Streptococcus mutans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* known to cause oral infections. Zone of inhibition produced by different extracts against the selected strains was measured and compared with standard antibiotic Ciprofloxacin (1%) and Fluconazole (1%). The antibacterial sensitivity of crude extracts were tested by the disc diffusion test, and results showed that ethanol extracts and methanol extracts had average inhibition zones ranged from 18-23 and 10-16 mm in diameter, respectively while no activity was observed for other extracts. Minimum Inhibitory Concentrations (MIC) of ethanol extract was evaluated against these oral pathogens ranged from 4- 8 mg/ml while MIC of methanol extract ranged from 6-10 mg/ml. The synergy testing of ethanol extract was also carried out with known antimicrobial agents using agar well diffusion method. The results of conducted experiments using agar well diffusion demonstrated synergistic effects between antibiotics and plant extracts with significant reduction in the MICs of the test antibiotics against these strains. This study suggests the possibility of concurrent use of these antimicrobial drugs and extracts in combination in treating infections caused by these oral pathogens.

Keywords: Antimicrobial property, oral pathogens, plant extracts.

INTRODUCTION

Pterocarpus santalinus L.f. (Fabaceae) also called as Red sanders is an endangered and endemic to Andhra Pradesh. It grows well in hilly regions with hot and dry climate. *P. santalinus* is highly valued for its heavy, dark claret-red heartwood which yields 16% of red colouring matter to santalin. Santalin is used as coloring agent in pharmaceutical preparations and food stuff. Fruit extract have found many medicinal uses in treating inflammation, headache, skin diseases, chronic dysentery, etc. Traditionally it has been used in treatment of headache, skin diseases, fever, boils, scorpion-sting and to improve sight[1]. Previous chemical constituents revealed the presence of triterpene, isoflavone glucosides, savinin and calocedrin[2,3]. The plant wood has the potential to heal cuts, wounds and inflammation. It also aids in treating headache, skin diseases, fever, boils, scorpion string and to improve sight. The stem bark extracts of the plants have been found to exhibit antibacterial, antidiabetic, anti-hyperglycaemic activity and hepatoprotective activity. Despite several medicinal uses, the compounds constituting the plant extract have not been fully explored for their medicinal values[4-6] Therefore, the present investigation attempts to isolate and investigate the antimicrobial activities of *Pterocarpus santalinus* extracts. Dental caries is a multifactorial human disease that has widely affected many populations all over the world. Dental caries, also known as tooth decay or a cavity, is an infection that causes demineralization of the hard tissues (enamel, dentin and cementum) and destruction of the organic matter of the tooth, usually by production of acid by hydrolysis of the food debris accumulated on the tooth surface. All caries occurs from acid demineralization that exceeds saliva and fluoride remineralization, and almost all acid demineralization occurs where food (containing carbohydrate like sugar) is left on teeth. The effect of this herb against most of the oral pathogens is largely unexplored. So in the present study an attempt was made to see the antimicrobial activity of this herb against oral pathogens causing dental caries. Few studies have found that the efficacy of antimicrobial agents can be improved by combining them with crude plant extracts against different pathogens with crude plant extracts against different pathogens. But plant extracts as antimicrobials are rarely used as systemic antibiotics at present, this may be due to their low level of activity, especially against Gram-negative bacteria[7, 8]. Here we are trying to investigate an alternative approach to the treatment of bacterial infections by able approach to the treatment of bacterial infections by able to change the phenotype of a resistant pathogen to certain antibiotics to more susceptible pathogen to that antibiotics. This alternative approach deals with the use of synergistic effect between the antibiotics and extracts of this herb. This synergistic activity allows the use of both antibiotics and extracts together to ameliorate the diseases.

MATERIALS AND METHODS

Preparation of extracts

The sample of *Pterocarpus santalinus* was collected and air-dried for two weeks at room temperature (25±2°C) and pulverized with a grinder into smooth powder for solvent extractions. Solvent extractions were carried out

in Petroleum ether, methanol, n-butanol, chloroform and ethanol. The extractions were carried out with slight modifications to method of Ghalab Adwan, *et. al*. by immersing 10 g of dried powder in 50 ml of each solvent, then keeping it at room temperature for 24 hours. At the end of each respective extraction, extract was filtered using Whatman filter paper. The filtrate was concentrated by keeping in boiling water bath. The resulting residue was then dissolved in DMSO and used for the bacterial susceptibility test[7, 8]

Collection and maintenance of oral pathogens

The pathogens viz. *Candida albicans* (MTCC 3017), *Candida tropicalis* (MTCC 184), *Candida (Torulopsis) glabrata* (MTCC 3019), *Candida Parapsilosis* (MTCC 1965), *Streptococcus mutans* (MTCC 1943), *Staphylococcus aureus* (MTCC 3160), *Pseudomonas aeruginosa* (MTCC 2295), *E. coli* (MTCC 43) were procured from the Microbial Type Culture Collection (MTCC) center, IMTECH, Chandigarh, India. With slight modifications to method to the method of Monali Gajbhiye *et.al.*, these cultures were grown on potato dextrose agar (PDA) and Nutrient agar (NA) medium at 25°C for 3 days (1 day for bacterial culture) and maintained at 4°C in a refrigerator[9].

Assay for antimicrobial activity

The antibacterial activities of the crude plant extracts were evaluated by agar well diffusion method. One ml each of the test bacterial isolates standardized at 10^7cell/ml from a 24 hour broth culture was aseptically spreaded over solidified Muller Hilton Agar plates. Wells of equal distance were dug on the seeded plates with previously sterilized cork borer (4mm). Each well was filled up with the crude extracts. The plates were incubated at 37°C for 24 to 48 hours. The sensitivities of the test organisms to the crude and fractions extract were indicated by clear zone around wells. Where applicable, the halos were measured with a transparent ruler and expressed as the degree of sensitivity[10].

Minimum Inhibitory Concentration

MICs were carried out with slight modification to the method described by Hirasawa *et. al.* (1999). Different concentrations (1, 2, 4, 8, 16, 32 mg/ml) were prepared using DMSO. Again, the agar well diffusion method was used. The test was carried out in triplicate and the mean was recorded[11].

Antimicrobial agents and antibiotic inhibition

Two antibiotics were used for evaluation of synergism assays. The antibiotics used were 1% Ciprofloxacin (for bacteria) and 1% Fluconazole (for fungus). The agar well diffusion method was adopted and zones of inhibition were measured using zone scale.

Determination of synergistic activity of extracts with antibiotics

Antibacterial activity was measured using a well diffusion method according to the National Committee for Clinical Laboratory Standard[10]. Briefly, Petri plates containing approximately 25-30 ml of Mueller Hinton agar medium were inoculated using a cotton swab with a 4-6 h old culture of the bacterial strains. Wells (6 mm diameter) were punched in the agar and filled with 30 µl of plant extracts or antibiotics and in case of synergism effect 30 µl of each has been added into well. The plates were incubated at 37°C for 18-24 h. The antibacterial activity was assessed by measuring the inhibition zone diameter was added (mm) around the well. The average of three replicates for each extract, antibiotic and combination has been calculated. Synergism effect was considered when combinations exhibited with enlargement of combined inhibition zone size by 0.5 mm[12].

Statistical analysis

The correlation coefficient is calculated for zones of inhibition of extracts alone and for the zones of inhibition due to both extracts and antibiotics. The standard deviation, z-test and t-test are calculated to analyze the data statistically.

RESULTS AND DISCUSSIONS

In vitro testing for antimicrobial activity using well-diffusion method demonstrates that *Pterocarpus santalinus* contain the bioactive compounds. The extracts were prepared in Petroleum ether, methanol, n-butanol, chloroform and ethanol. The ethanol and methanol extracts of *Pterocarpus santalinus* have higher antimicrobial activities than Petroleum ether, n-butanol and chloroform extracts. The diameters of inhibition zones are tabulated in Table 1. The best activity was shown by extract of ethanol and is shown in figure 1. Minimum Inhibitory Concentrations (MIC) of both the extracts was evaluated against these oral pathogens which ranged from 4- 10 mg/ml. Minimum Inhibitory Concentrations are tabulated in Table 2. To check the synergistic activity of the extract with Ciprofloxacin and Fluconazole, these antibiotics were also subjected to well diffusion to observe the zones of inhibition. The diameters zones of inhibition are tabulated in Table 3. To observe the synergistic effects between extract and antibiotics, the extracts and antibiotics both were subjected to well-diffusion. The results are tabulated in Table 4. The results were analyzed statistically and various statistical parameters were calculated. The correlation coefficient was calculated for diameters of inhibition due to extract of ethanol and diameters of inhibition due to synergistic effect of both extract of ethanol and antibiotics. It was found to be-0.095(not corrected). The correlation coefficient (corrected) was found to be-0.128. The value of t-test was found to be-0.318 and that of z-test was 0.252. The results show that the extracts have bioactive compound which inhibits the growth of various oral pathogens. The spectrum of the antimicrobial compound present is found to be broad as it is inhibiting bacterial as well as fungal species.

Table 1: Antimicrobial activity of extracts of *Pterocarpus santalinus* against oral pathogens

Extracts of *Pterocarpus santalinus*	Diameter of inhibition zones (mm.)*							
	Pa	*Ec*	*Sa*	*Sm*	*Cg*	*Cp*	*Ca*	*Ct*
Petroleum ether	-	-	-	-	-	-	-	-
Chloroform	-	-	-	-	-	-	-	-
Ethanol	-	21±0.46	21±0.56	23±0.33	22±0.53	18±0.28	23±0.66	-
n-butanol	-	-	-	-	-	-	-	-
Methanol	-	10±0.5	13±.52	12±0.85	10±0.78	14±0.42	16±0.57	-

Abbreviations: *Pa: Pseudomonas aeruginosa, Ec: Escherichia coli, Sa: Staphylococcus aureus, Sm: Streptococcus mutans, Cg: Candida glabrata, Cp: Candida parapsilosis, Ca: Candida albicans, Ct: Candida tropicalis.*
*Mean diameter± Standard deviation

Table 2: MIC of extracts of *Pterocarpus santalinus* against oral pathogens

Extracts of *Pterocarpus santalinus*	Minimum Inhibitory Concentration(mg/ml)							
	Pa	*Ec*	*Sa*	*Sm*	*Cg*	*Cp*	*Ca*	*Ct*
Petroleum ether	-	-	-	-	-	-	-	-
Chloroform	-	-	-	-	-	-	-	-
Ethanol	-	4	6	4	5	8	7	-
n-butanol	-	-	-	-	-	-	-	-
Methanol	-	6	6	8	7	10	9	-

Abbreviations: *Pa: Pseudomonas aeruginosa, Ec: Escherichia coli, Sa: Staphylococcus aureus, Sm: Streptococcus mutans, Cg: Candida glabrata, Cp: Candida parapsilosis, Ca: Candida albicans, Ct: Candida tropicalis.*

Table 3: Diameters of Inhibition zones due to antibiotics

Strains	Ciprofloxacin	Fluconazole	Diameters of inhibition zones(mm)***
Pseudomonas aeruginosa	Added	-	32±0.21
Escherichia coli	Added	-	30±0.23
**Staphylococcus aureus*	Added	-	-
Streptococcus mutans	Added	-	27±0.67
***Candida glabrata*	-	Added	-
Candida parapsilosis	-	Added	28±0.76
Candida albicans	-	Added	29±0.87
Candida tropicalis	-	Added	26±0.43

**Staphylococcus aureus* is resistant to Ciprofloxacin; ***Candida glabrata* is resistant to Fluconazole; ***Mean Diameter ± Standard Deviation

Table 4: Diameters of Zones of inhibition due to synergistic effect of extract to the antibiotics

Strain	Ciprofloxacin	Fluconazole	Extract	Diameters of Inhibition (mm.)*				
				PE	Ch	Et	n-but	Met
Pseudomonas aeruginosa	Added	-	Added	32±0.21	32±0.21	32±0.21	32±0.21	32±0.21
Escherichia coli	Added	-	Added	30±0.23	30±0.23	22±0.33	30±0.23	12±0.34
Staphylococcus aureus	Added	-	Added	-	-	23±0.56	-	15±0.56
Streptococcus mutans	Added	-	Added	27±0.67	27±0.67	24±0.87	27±0.67	13±0.65
Candida glabrata	-	Added	Added	-	-	23±0.53	-	11±0.53
Candida parapsilosis	-	Added	Added	28±0.76	28±0.76	20±0.45	28±0.76	16±0.76
Candida albicans	-	Added	Added	29±0.87	29±0.87	26±0.76	29±0.87	18±0.78
Candida tropicalis	-	Added	Added	26±0.43	26±0.43	26±0.43	26±0.43	26±0.43

Abbreviations PE: Petroleum ether, Ch: Chloroform, Et: Ethanol, n-but: n-butanol, Met: Methanol; *Mean Diameter ± Standard Deviation

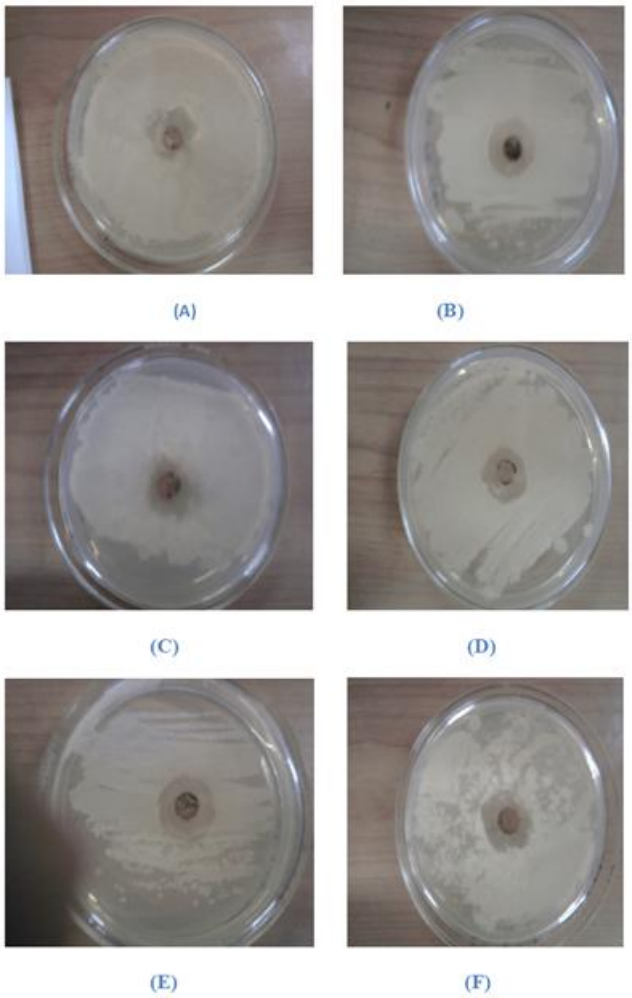

Figure 1: Antimicrobial activity shown by ethanol extracts against: (A) *Candida albicans* (B) *Candida glabrata* (C) *Candida parapsilosis* (D) *E. coli* (E) *Staphylococcus aureus* (F) *Streptococcus mutans*.

Manjunatha *et. al.* observed that the stem bark extract shows maximum activity against *Enterobacter aerogenes, Alcaligenes faecalis, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus* . They also observed that the leaf extract shows maximum activity against *Escherichia coli, Alcaligenes faecalis, Enterobacter aerogenes* and *Pseudomonas aeruginosa*. Both extracts exhibited concentration dependent activity[3,5]. Balaraju *et. al.* carried out a study to evaluate the antimicrobial activity of leaf extract from *P. santalinus*. Hexane, ethyl acetate and methanol extracts were obtained by cold percolation method. Result indicated that P. santalinus exhibited significant antimicrobial activity at all the dosage tested (1.25 mg/disc, 2.5 mg/disc and 5 mg/disc). Ethyl acetate and methanol extracts were found to be active towards drug resistant strains. This study showed that leaf extract of *P. santalinus* can be a potential source of new antimicrobial agents for the tested drug resistant bacterial strains and fungi[13]. Stella *et. al.* studied that *Pterocarpus santalinus* extracts inhibited the growth of gram positive bacteria and gram negative bacterium. Maximum inhibitory activity was observed against *Bacillus subtilis* (0.312 mg/ml), however no activity was found against *Pseuomonas aeuroginosa* and *Klebsiella pneumonia*[14].

CONCLUSION

Extracts of *Pterocarpus santalinus* in this study demonstrated a broad-spectrum of activity against both bacteria and fungi due to presence of some bioactive substances. Bioactive substances from this plant can therefore be employed in the formulation of antimicrobial agents for the treatment of various bacterial and fungal infections causing oral infections. Isolation, identification and purification of these bioactive components and determination of their respective antimicrobial potencies and toxicological evaluation with the view to formulating novel chemotherapeutic agents should be the future direction for investigation. This study also suggests the possibility of concurrent use of these antimicrobial drugs and extracts in combination in treating infections caused by oral pathogens.

Acknowledgement: The authors are grateful to the Hon'ble Vice-Chancellor, Kurukshetra University, Kurukshetra and the Director, University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra for providing infrastructure facilities to carry out research work.

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Source of Support: Nil, **Conflict of Interest:** None.