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ANTIBACTERIAL ACTIVITY OF ECLIPTA PROSTRATA ON ORAL PERIODONTAL RED COMPLEX PATHOGENS -AN IN-VITRO STUDY

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ABSTRACT:

Introduction:
Periodontal disease is a chronic poly microbial infectious disease of oral cavity and potential independent risk factor for other systemic diseases. Red complex pathogens namely, Porphyromonas gingivalis, Treponema denticola and Tanneralla forsythia are the most important organisms causing periodontal disease. Eclipta prostrata is an Medicinal plants widely used as an alternative therapeutic agents for various infectious diseases in India, but its effectiveness on periodontal pathogens is yet to be evaluated.. Hence the present in vitro study was conducted to estimate the Antibacterial activity of Eclipta Prostrata (Acetone and Hexane extract) against oral periodontal Red complex pathogens

Materials and Methods:
Three species of bacterial strains - P. gingivalis, T. forsythia, Tanneralla denticola were selected for this study. Antimicrobial activity of the plant extract was determined by the disc diffusion method at 1mg/mL. Chlorhexidine is used as positive control and the respective solvent acts as negative control for determining the inhibition zone. Minimum Inhibitory Concentration (MIC) of the plant was estimated.

Results:
A significant zone of inhibition was noted in 100 µg/mL concentration of acetone and hexane extracts whereas there was no antibacterial activity at 25 µg/mL and 50 µg/mL concentration.
In both the extracts the highest zone of inhibition was noticed against P. gingivalis followed by Tannerella forsythia and the lowest against T. denticola. Hexane extract showed significant inhibition against the growth P. gingivalis and T. denticola with p- value 0.002 and 0.021 respectively. No significant difference was observed among the acetone and hexane extracts against Tannerella forsythia.

1. INTRODUCTION

Periodontal disease is a chronic poly microbial infectious disease that causes inflammation of the gingiva, periodontal ligament and alveolar bones that support the teeth. It is important to treat the disease on time since it not only affects the periodontal health but also the overall health of the patient. It has been proven that Periodontitis is a constant potential source for infection and is also an independent risk factor for other systemic diseases such as cardiovascular diseases, cerebrovascular diseases, peripheral arterial disease, respiratory diseases, low birth weight, diabetes, insulin resistance, rheumatoid arthritis, obesity, osteoporosis, and complications during pregnancy. [1,2,3]. A study conducted between the relationship between maternal periodontal disease and low birth weight babies showed that mother's periodontal disease can alone be a potential risk factor for low birth weight of the infant. [4]. A case of pyogenic liver abscess caused by periodontal bacteria has also been reported.[5] It has been proven in many studies that people with chronic systemic diseases are more prone to develop periodontal disease which in turn will affect the disease progression.

The overall prevalence of periodontal disease is 51% among the Indian population [6]. Sub gingival plaque of periodontal disease patients harbors more than 700 species and among these, Red complex pathogens namely, Porphyromonas gingivalis, Treponema denticola and Tanneralla forsythia are the most important organisms causing periodontitis. [2, 6].
Chronic microbial diseases seek frequent use of anti-inflammatory drugs and antibiotics to control the disease progression to prevent undue adverse outcomes of the disease. The extensive use of antibiotics increases the prevalence of anti-bacterial resistant strains which will render them ineffective and failure to control the bacterial disease [7].

Medicinal plants are alternative therapeutic agents which also show an impact in infectious diseases. One such herb, Eclipta prostrata belongs to the Astraceae family and is very common in tropical and subtropical regions. This herb has been used in the treatment of infectious disease - Hepatitis B in India and snake venom poisoning in Brazil. The plant extract of the Eclipta prostrata has been reported to have anti-inflammatory, antibacterial and antifungal properties. It is also effective against certain bacteria like Escherichia coli, Klebsiella pneumoniae, Shigella dysenteriae, Salmonella typhi, and Staphylococcus aureus [8,9,10,11] but its effectiveness on periodontal pathogens is yet to be evaluated. To the best of our knowledge there were no studies available in the literature to assess the antibacterial activity of Eclipta prostrata against Red pathogens.

Moutsopoulos NM in his article stated that periodontal disease is mainly due to accumulation of supra gingival and sub gingival plaque which harbors 600 different aerobic and anaerobic bacteria, among these bacterias' Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia were clustered as red complex pathogens and they play an essential role in pathogenesis of periodontal disease. [2]
The reviews clearly shows that Eclipta prostrata is a potent antimicrobial agent and it is effective against various gram positive and negative microorganisms and review of literature also revealed that there are studies available on the antimicrobial activity of periodontal red pathogen Porphyromonas gingivalis, Treponema denticola and Tannerella forsythia. It is very well justified to conduct a study to assess the same and hence the present study was carried out to estimate the Antibacterial activity of Eclipta Prostrata (Hexane and Acetone extract of aerial part of the plant) on oral periodontal Red complex pathogens – Porphyromonas

gingivalis, Treponema denticola and Tannerella forsythia and to compare its antibacterial activity with chlorhexidine (one of the standard drugs for periodontal disease).

2. MATERIALS AND METHODS

The study was conducted after obtaining permission from the Institutional Review board and Institutional Ethical Committee.

Sample Preparation:
The plant samples' leaves were shade dried for 2-5 weeks and powdered finely in a mixture and sieved twice to obtain a fine powder.

Sample Extraction (Soxhlet Method):
About 50 g of dried powder was extracted with 200 ml of solvents. Acetone and hexane alone were used as solvents. 50g of coarse powder was inserted in the body of the Soxhlet extractor. The round-bottom flask is then filled with 250 mL of solvent. The Soxhlet extractor, round-bottom flask, and condenser were then attached to the setup using clamps and a stand. The condenser was linked to the rubber tube connected to the tap water for a continuous circulation of water. The isomantle was used to heat the solvent, which then began to evaporate as it passed through the apparatus to the condenser. The condensate was then dripped into the plant extract reservoir. When the solvent level reached the syphon, it spilled back into the flask, restarting the cycle. The procedure was set to run for 5 hours at 45-50°C. Finally, the extract was collected in the flask with the round bottom. Extract was kept in a desiccator till the remaining solvent was completely evaporated. The content of extractable matter was calculated in mg/g of air-dried material using digital weighing balance. The extract was stored in the refrigerator till further use.

TLC Analysis:
About 10µL of sample was placed on pre-coated silica TLC plate (merck), activated at 100° c and kept in an ethanol solvent system containing chloroform/ethanol/hexane(4:1:1) until it reached 3/4th of plate. The plates were exposed under UV 3665 nm and fraction Rf was calculated
R_f = distance of solute/ distance of solvent

FTIR Characterization:
10 mg of the dried extracts was encapsulated in 100 mg of KBr pellet to prepare translucent sample discs. The powdered sample of each extract was loaded in a FTIR spectroscope (PerkinElmer) with a scan range from 400 to 4000 cm−1 and a resolution of 4 cm−1. The peak values of the UV and FTIR were recorded.

Antibacterial Test:
Three species of bacterial strains - P. gingivalis, T. forsythia, Tanneralla denticola were selected for this study. Antimicrobial activity of the plant extract was determined by the disc diffusion method at 1mg/mL An overnight culture of each microbial isolate was emulsified with nutrient broth to a turbidity that was equivalent to 0.5 McFarland (10^5cfu/mL). In order to determine the antimicrobial efficacy of the extracts, aliquot of test culture (100 µL) was evenly spread over the surface of the solidified agar. Sterile discs loaded with extract at different concentrations were placed over agar surface. Chlorhexidine is used as positive control and the respective solvent acts as negative control for determining the inhibition zone. The plates previously loaded with respective extracts and test microorganisms were incubated

for 24 hrs to 48hrs at 37℃ for bacteria. After incubation, the inhibition zone was measured in millimeters.

Minimum Inhibitory Concentration (MIC)-Resazurin-Based Turbidimetric Assay:
Both micro dilutions were performed precisely according to the Clinical and Laboratory Standards Institute (CLSI) protocol. Different Concentrations of plant extract were prepared by using two fold initial concentrations of substance and diluted to obtain 1.56, 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/mL of concentration. All the tubes were inoculated with 10µL (1.5 x 106 cell/ml) of test pathogen and incubated at 37℃ overnight. Tube without culture served as control. After an overnight incubation at 37 °C, 5 µl resazurin (6.75 mg/L) was added to all wells and incubated at 37 ℃ for another 4 h. Changes in color were observed and recorded. The lowest concentration prior to color change was considered as the Minimum Inhibitory Concentration (MIC).

Statistical Analysis:
SPSS version 20 was used for statistical analysis. Tests were carried out in triplicates and the mean values were calculated from the triplicate values. The values are expressed as the Mean ± SD; differences between groups were considered to be significant if $p<0.05$.

3. RESULTS AND DISCUSSIONS

The increased use and misuse of antibiotics in recent times for various indicated and non-indicated diseases has increased the antibiotic resistance of various strains of bacteria. This provoked the interest of physicians and researchers in studying the effectiveness of ancient herbs as an alternate or adjuvant system to effectively handle the different microbial strains.

Eclipta prostrata, a herb well-known for its antimicrobial property, is prevalent in various parts of the world like India, Srilanka, Malaysia, Australia, etc. Many researchers in different parts of the world extensively studied its antimicrobial activity, anti-tumorous effect and anti-hemorrhagic property [10]. This herb played a significant role in ancient medicine to treat various diseases like liver cirrhosis, infective hepatitis, enlarged spleen, Urinary tract infection and many skin disorders. Eclipta prostrata is found to be effective against various strains like E.coli, Klebsiella and Salmonella typhi [6,7,8,9].

Periodontal disease is a microbial disease that leads to bleeding gums and alveolar bone destruction which greatly hinders the chewing ability. Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola are called Red pathogens and are considered as significant microorganisms of periodontal disease. Chronic increased microbial load of these pathogens in the periodontal tissues pave the way for many systemic diseases as well. Mechanical removal of the microbial colonized plaque and administration of chlorhexidine mouthwash are the conventional treatment modes recommended for initial periodontal disease; but chlorhexidine when used in long term produces many adverse effects like teeth discoloration, loss of taste and altered oral microbiome. Hence we got the interest of analyzing the efficacy of the ancient herb, Eclipta prostrata against the periodontal pathogens and thereby improving the overall periodontal health of the patients. On reviewing various literature in Google and Pubmed search engines, there was no research article available that effectively analyses the antibacterial activity of Eclipta prostrata against these Red pathogens. Hence the present study was conducted to analyze the effectiveness of the same.

Acetone and Hexane extracts of Eclipta prostrata plant extract was subjected to antibacterial activity test by disc diffusion method against P. gingivalis, T. denticola and Tannerella forsythia in different concentrations of 25µg/mL, 50µg/mL and 100 µg/mL and chlorhexidine

drug was used as the standard. A significant zone of inhibition was noted in 100 µg/mL concentration of acetone and hexane extracts whereas there was no antibacterial activity at 25 µg/mL and 50 µg/mL concentration.

In both the extracts the highest zone of inhibition was noticed against P. gingivalis (17.33±0.58 in acetone and 22.00±1.00 in hexane) followed by Tannerella forsythia (14± 1.00 in acetone and 16±1.00 in hexane) and the lowest against T. denticola (12±1.00 in acetone and 15±1.00 in hexane). Hexane extract showed significant inhibition against the growth P. gingivalis and T. denticola with p- value 0.002 and 0.021 respectively. No significant difference was observed among the acetone and hexane extracts against Tannerella forsythia. The results of Antibacterial activity of Acetone and Hexane extract are shown in **Table 1, Graph 1 & Figure 1.**

The results were subjected to statistical analysis and Mann Whitney U test was used to compare the antibacterial activity (zone of inhibition) of Eclipta prostrata and standard chlorhexidine drug against the red pathogens. Eclipta prostrata showed significant zone of inhibition against all the three red pathogens but chlorhexidine was found to be more effective than the Eclipta prostrata, but in case of P. gingivalis, an important and significant periodontal pathogen, the hexane extract of Eclipta prostrata at 100µg/mL showed a statistically significant antimicrobial activity which is very similar to the chlorhexidine. This result gives us a promising hope to use Eclipta prostrata as an alternate drug for chlorhexidine against P. gingivalis. [**Table 2 & 3.]**

The Minimum Inhibitory Concentration for Acetone extract was found to be 3.125 µg/ml, 50 µg/ml and 100 µg/ml for P. gingivalis, T. denticola and Tannerella forsythia respectively. MIC of Hexane extract of P. gingivalis, T. denticola and Tannerella forsythia was found to be 100 µg/ml, 6.25 µg/ml and 12.5 µg/ml respectively. Minimum Inhibitory Concentration is described in **Table 4 and Table 5**.

4. CONCLUSION

Periodontal disease is the common dental problem encountered in all age groups. The disease is due to the accumulation of local factors and also due to the influence of systemic diseases. The dental plaque harbors pathogenic bacteria which initiates soft tissue and hard tissue destruction. Frequent use of antibacterial agents in periodontal disease increases the number of resistant microorganisms. Herbal medicine is recapturing its place in recent times to treat various diseases because of their less adverse effects, cost effectiveness and ready availability. One such herb is Eclipta prostrata and it is extensively used in treating various microbial diseases due its antibacterial property. The present study assessed the antibacterial activity of the herb against red pathogens of periodontal disease. The results of the study showed that this herb has significant antibacterial property against all three red pathogens (P. gingivalis, T. denticola and Tannerella forsythia) and on comparing the extracts, hexane extract was found to be better than the acetone extract. The outcome of the study encourages the method of using ancient Indian herbs as an adjunct therapy in treating periodontal diseases.

Tables and Graphs:

Table 1: Antibacterial activity (zone of Inhibition) of Eclipta prostrata

Mean and standard deviation of Diameter of zone of Inhibition (in mm)

Group (100 µg/mL)		N	Mean	Std. Deviation	P-VALUE
P. gingivalis	Acetone	3	17.33	0.58	.002
	Hexane	3	22.00	1.00	
T. denticola	Acetone	3	12.00	1.00	.021
	Hexane	3	15.00	1.00	
Tannerella forsythia	Acetone	3	14.00	1.00	.070
	Hexane	3	16.00	1.00	

Table 2: Mann Whitney U test was used to compare the Antibacterial activity of Acetone extract of Eclipta prostrata and chlorhexidine (CHX)

Concentration		N	Mean	Std. Deviation	p-value
P. gingivalis	100	3	17.33	0.58	.000
	CHX	3	25.00	1.00	
T. denticola	100	3	12.00	1.00	.000
	CHX	3	22.00	1.00	
Tannerella forsythia	100	3	14.00	1.00	.002
	CHX	3	20.00	1.00	

Table 3: Mann Whitney U test was used to compare the Antibacterial activity of Hexane extract of Eclipta prostrata and chlorhexidine (CHX)

Concentration		N	Mean	Std. Deviation	P-VALUE
P.gingivalis	100	3	22.00	1.00	.070
	CHX	3	24.00	1.00	
T. denticola	100	3	15.00	1.00	.004
	CHX	3	20.00	1.00	
Tannerella forsythia	100	3	16.00	1.00	.021
	CHX	3	19.00	1.00	

Table 4: Minimum Inhibitory Concentration (MIC) of Acetone extract by viability test

Pathogen at Con µg/ml	1.56	3.125	6.25	12.5	25	50	100	200
P. gingivalis	+	-	+	+	+	+	+	+
T. denticola	+	+	+	+	+	-	+	+
Tanneralla forsythia	+	+	+	+	+		-	+

(+ indicates viability and - indicate no growth(MIC)

Table 5: Minimum Inhibitory Concentration (MIC) of Hexane extract by viability test

Pathogen at Con µg/ml	**1.56**	**3.125**	**6.25**	**12.5**	**25**	**50**	**100**	**200**
P. gingivalis	+	+	+	+	+	+	-	+
T. denticola	+	+	-	+	+	+	+	+
Tanneralla forsythia	+	+	+	-	+	+	+	+

(+ indicates viability and - indicate no growth(MIC)

Graph 1: Antibacterial activity of Acetone and Hexane extract of Eclipta prostrate

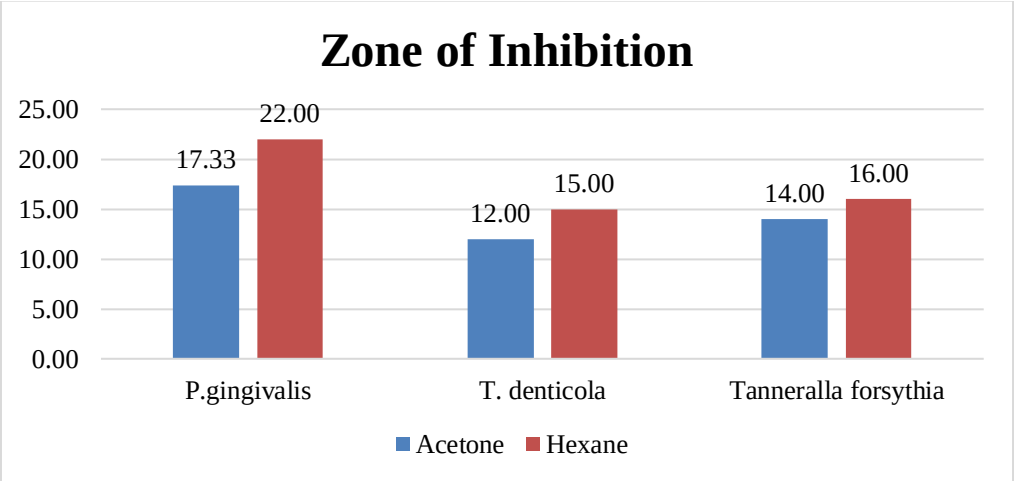
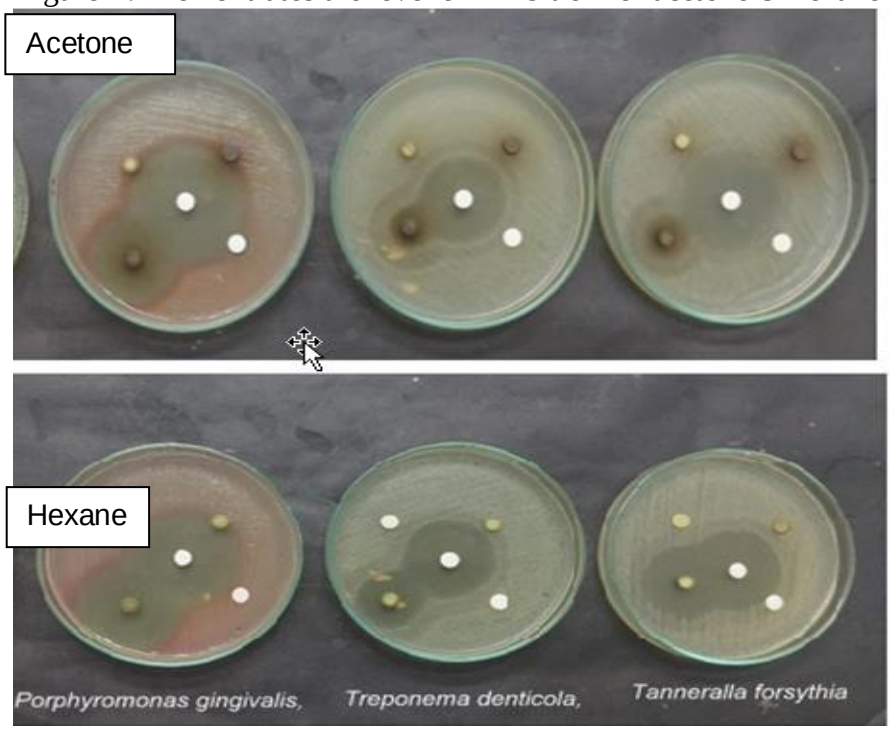


Figure-1: Demontrates the level of inhibition for acetone & hexane



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