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IN VITRO TESTING OF ANTIMICROBIAL PROPERTIES OF LEMONGRASS, EUCALYPTUS AND THEIR SYNERGISTIC EFFECT

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Abstract- Herbal medicine represents one of the most important fields of traditional medicine all over the world. Plants produce certain bioactive compounds which are naturally toxic to microorganisms and so have been investigated as therapeutic agents. The present study was carried out so as to evaluate in vitro the antimicrobial effect of essential oils of two aromatic medicinal plants namely, Lemongrass and Eucalyptus. The in vitro evaluation was done for individual oils and combination of the two oils in 1:1 ratio against four bacterial pathogens of nosocomial infections namely *Staphylococcus aureus, Bacillus subtilis, Escherishia coli* and *Klebsiella pneumoniae*. The combination formula was used with two assumptions one that individual oil may show ineffectiveness if used repeatedly as seen in antibiotics and second the combined formulation may show wider range of antimicrobial activity. The antimicrobial activity of the plant essential oils was screened using Agar well diffusion method and the minimal inhibitory concentration (MIC) of the essential oils was determined by Broth assay method. The study showed promising results for the use of Lemongrass and Eucalyptus as antimicrobial agents.

Index Terms- Antimicrobial activity, Bioactive compounds, Drug resistance, Medicinal plants, Nosocomial infections.

I. INTRODUCTION

Medicinal plants are part and parcel of humans since the dawn of civilization. In India they form the back bone of several indigenous traditional systems of medicine. Today, pharmacological studies have acknowledged the value of medicinal plants as potential source of bioactive compounds. It has been estimated that between 60-90% of the populations of developing countries use traditional and botanical medicines almost exclusively and consider them to be a normal part of primary healthcare (WHO, 2002).

In herbal medicine, crude plant extracts in the form of infusion, decoction, tincture or herbal extract are traditionally used by the population for the treatment of diseases, including infectious diseases. Although their efficacy and mechanisms of action have not been tested scientifically in most cases, these simple medicinal preparations often mediate beneficial responses due to their active chemical constituents (Barnes et al., 2007). To promote the proper use of herbal medicine and to determine their potential as sources for new drugs, it is essential to study medicinal plants, which have folklore reputation in a more intensified way. In recent years, multiple drug resistance in both human and plant pathogens has developed due to indiscriminate use of synthetic drugs. This drives, the need to screen medicinal plants for novel bioactive compounds as plant based drugs are safe, biodegradable and have fewer side effects.

Lemongrass (*Cymbopogon citratus*) and Eucalyptus (*Eucalyptus globulus*) are very famous aromatic medicinal plants. They contain essential oils which are volatile, concentrated and hydrophobic liquids usually with pleasant and sometimes intensive odors. These essential oils contain bioactive compounds which are used mainly for medicinal purposes. They are applied directly on to the skin for treatment of different ailments and can also be consumed in very low concentrations. These compounds either act on different systems of animals including man, and/or act through interfering in the metabolism of microbes infecting them. The microbes may be pathogenic or symbiotic. In either way the bioactive compounds from medicinal plants play a determining role in regulating host-microbe interaction in favor of the host. These compounds exert a wide spectrum of biological activities such as antimicrobial, antiseptic, stimulant, carminative, diuretic, analgesic, etc.

Plant-derived extracts are available for sale in the local markets in India. The present study was an attempt to evaluate in vitro the antibacterial activity of the aromatic medicinal plants Lemongrass and Eucalyptus essential oils individually and in combination against the bacterial test cultures which especially cause nosocomial infections. Even though some reports are available on antimicrobial activity of these oils, the studies on multiple oil usage is negligible. One of the reasons for using it as combination formula is assumption that individual oil may show ineffectiveness if used repeatedly and irrationally as seen with irrational use of

antibiotics. Another reason is the combined formulation may show wider range of antimicrobial activity as well as better inhibition of pathogens. The microbial strains selected include Gram Positive bacteria- *Staphylococcus aureus, Bacillus subtilis* and Gram negative bacteria- *Escherichia coli* and *Klebsiella pneumonia*.

II. MATERIALS AND METHODS

II.1. Materials required:

II.1.1. Plant extracts:

Pure essential oils of Lemongrass and Eucalyptus were obtained from a local outlet in Hyderabad, India, which sells the plant extracts as therapeutic agents.

Figure 1: Essential oils of Lemongrass, Eucalyptus and their mixture

II.1.2. Bacterial test cultures:

Pure cultures of the following Microorganisms were used for screening the antimicrobial properties of plant essential oils.

a) Staphylococcus aureus

b) Bacillus subtilis

c) Escherichia coli

d) Klebsiella pneumoniae

The pure cultures of these test bacteria were obtained from National Collection of Industrial Microorganisms (NCIM), NCL, India. The cultures were maintained on Nutrient Agar (HI Media, India) slopes at 4°C and sub-cultured before use.

II.1.3. Culture media:

a) Nutrient broth
b) Nutrient agar
c) Mueller Hinton Agar

II.2. Methods followed:

II.2.1. Agar well diffusion method for screening the antimicrobial activity of plant essential oils:

In vitro antibacterial activity was studied against four bacterial strains using Agar well diffusion method. In this method the antimicrobials present in the plant essential oil are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The Mueller-Hinton agar plates were seeded with the overnight broth culture of each test organism ($1.5x10^8$/ CFU/ml). Wells were prepared in seeded agar plates with 6mm diameter and 100 µl of each essential oil (125µg/µl concentration) was introduced in each well. The solvent used for preparing essential oil solution was absolute alcohol or ethanol. Solvent control well was run for every assay. All the inoculated plates were incubated at 37^0C for 24 hours in the incubator. The antimicrobial spectrum of the extract was determined in terms of diameter of inhibition zones. A zone of inhibition of 12mm (millimeter) or above was considered as sensitive and less than 12mm as resistant. The entire experiment was carried out under strict aseptic conditions. The samples were run in triplicates and each result is a mean of the three values obtained. The concentration for plant essential oils was selected based on MIC values that had previously been evaluated.

II.2.2. Evaluation of the synergistic effect of the two plant essential oils (phytochemicals):

This evaluation was done according to Agar well diffusion method on the four bacterial test cultures. The aliquots of 100µl of bacterial cultures grown in Nutrient broth for 18 hours ($1.5x10^8$/ CFU/ml) were spread plated on Mueller-Hinton agar medium

supplemented with 100µl of both the plant essential oils together (50µl + 50µl with 125µg/µl concentration). The methodology followed was same as above.

II.2.3. Broth assay method for determination of MIC:

Minimum bactericidal concentrations of the extracts were determined by a broth assay using Nutrient broth. The aim of a broth assay is to determine the lowest concentration of the assayed antimicrobial agent (minimal inhibitory concentration, MIC) that, under defined test conditions, inhibits the visible growth of the bacterium being investigated. MIC values are used to determine susceptibilities of test cultures to bioactive compounds at lowest concentrations. Four bacterial samples [*K. pneumoniae, S. aureus, B. subtilis* and *E. coli*] were grown in Nutrient broth for 6 hours. Next, 100µL (10^6 cells/mL) of each bacterial culture was inoculated in tubes with Nutrient broth supplemented with different concentrations of the phytochemicals in the ratio 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256 respectively, which were prepared from stock solution (with 1 gm/ml concentration) through serial dilution using ethanol as diluent. A control was maintained for ethanol. After incubation at 37 °C for 24 hours, the MIC of each sample was determined by looking for turbidity as a measure of the bacterial growth comparing the sample with the negative control which was non-inoculated Nutrient broth and the positive control which was Nutrient broth without any phytochemical.

III. RESULTS AND FINDINGS

Table 1: Results for Gram positive bacteria-*Staphylococcus aureus* and *Bacillus subtilis*

S.No.	Antimicrobial agent/ essential oil	Zone of inhibition (diameter in mm.)	
		Staphylococcus aureus	*Bacillus subtilis*
1.	Lemon grass	30	25
2.	Eucalyptus	20	22
3.	Lemon grass + Eucalyptus	20	30

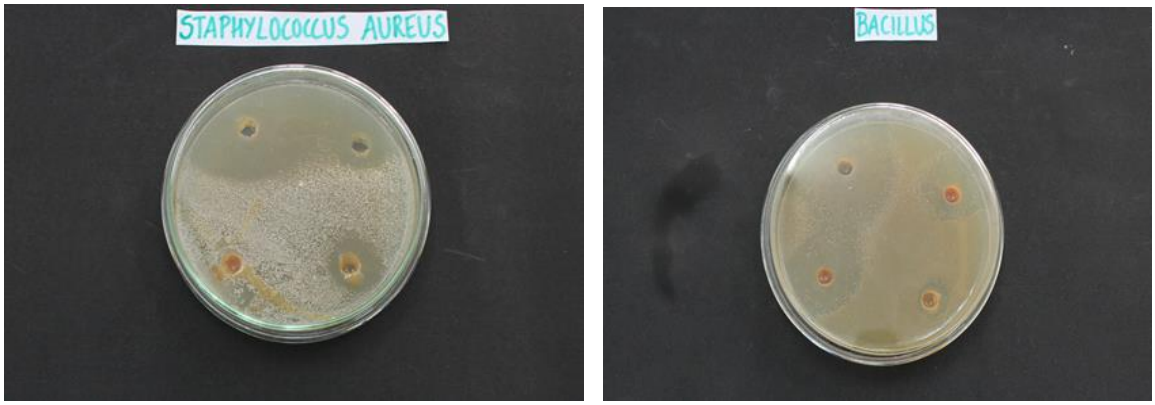

Figure 2: Zones of inhibition in Gram positive bacteria

Table 2: Results for Gram negative bacteria-*Escherishia coli* and *Klebsiella pneumonia*

S.No.	Antimicrobial agent/ essential oil	Zone of inhibition (diameter in mm.)	
		E. coli	*Klebsiella pneumoniae*
1.	Lemon grass	25	25
2.	Eucalyptus	15	20
3.	Lemon grass + Eucalyptus	15	26

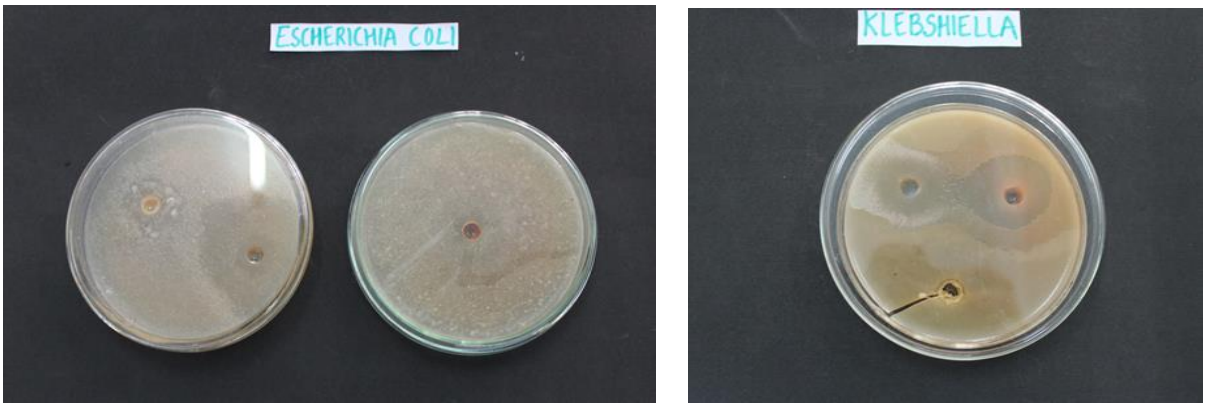

Figure 3: Zones of inhibition in Gram negative bacteria

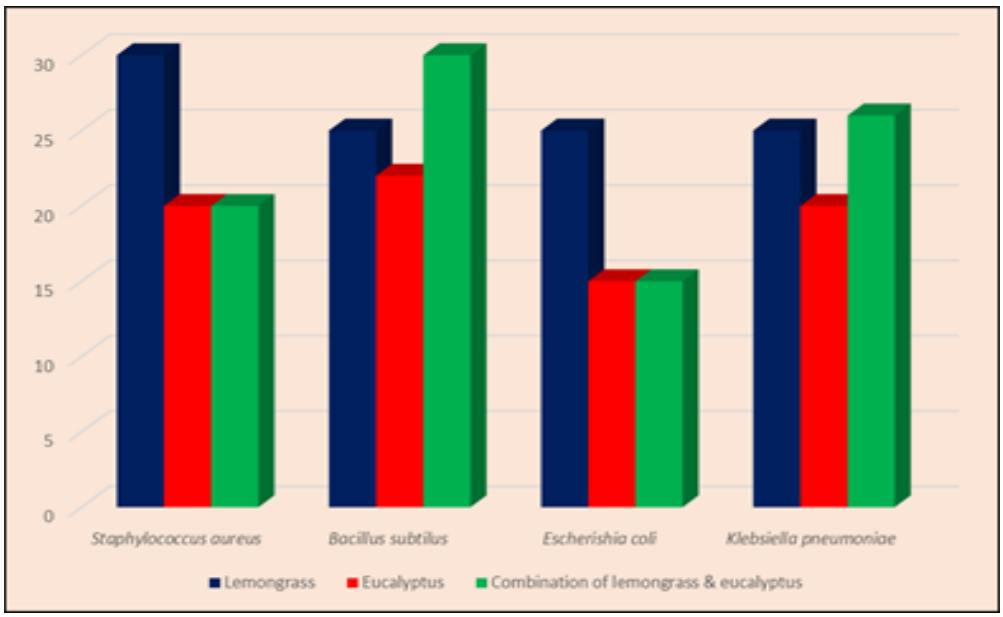

Figure 4: Comparison of antimicrobial activity of test oils

Table 3: MIC of Lemongrass

S.No.	Dilutions of Lemongrass essential oil	Concentrations of essential oil (mg/ml)	Turbidity			
			Staphylococcus aureus	*Bacillus subtilis*	*E. coli*	*Klebsiella pneumoniae*
1	1:2	500	-	-	-	-
2	1:4	250	-	-	-	-
3	1:8	125	-	-	-	-
4	1:16	62.5	+	+	+	+
5	1:32	31.25	+	+	+	+
6	1:64	15.625	+	+	+	+
7	1:128	7.813	+	+	+	+
8	1:256	3.906	+	+	+	+

[+: Presence of turbidity indicating growth; - : Absence of turbidity indicating no growth]

Figure 5: Tube Dilution Assay of Lemongrass

Table 4: MIC of Eucalyptus

S.No.	Dilutions of Eucalyptus essential oil	Concentrations of essential oil (mg/ml)	Turbidity			
			Staphylococcus aureus	*Bacillus subtilis*	*E. coli*	*Klebsiella pneumoniae*
1	1:2	500	-	-	-	-
2	1:4	250	-	-	-	-
3	1:8	125	-	-	-	-
4	1:16	62.5	+	+	+	+
5	1:32	31.25	+	+	+	+
6	1:64	15.625	+	+	+	+
7	1:128	7.813	+	+	+	+
8	1:256	3.906	+	+	+	+

[+: Presence of turbidity indicating growth; - : Absence of turbidity indicating no growth]

Figure 5: Tube Dilution Assay of Eucalyptus

IV. DISCUSSION

Among four bacterial strains tested, two were Gram positive bacteria namely, *Staphylococcus aureus* and *Bacillus subtilis* and two were Gram negative bacteria namely, *Escherishia coli* and *Klebsiella pneumoniae*. Gram positive and Gram negative bacteria show difference in their sensitivity towards antibiotics which are used as therapeutic agents .This difference is attributed to differences in their cell wall composition. Gram negative bacteria have thin layer of peptidoglycan and has outer membrane made up of lipoproteins and lipopolysaccharides. Gram positive cell wall has thick peptidoglycan layer and lacks outer membrane.

The plants extracts used in the study namely, Lemongrass and Eucalyptus showed high inhibitory effect on both Gram positive and Gram negative bacteria. Lemongrass showed highest inhibition zones for all the test cultures, when tested by agar well diffusion

assay (Kirby Bauer method). Both the herbal oils showed slightly higher inhibitory effect on growth of Gram positive bacteria and moderate effect on Gram negative bacteria. The combination of two oils in 1:1 ratio showed higher inhibitory activity against *Bacillus subtilis* and *Klebsiella pneumoniae* but did not have any beneficial inhibitory effect for *Staphylococcus aureus* and *E.coli.*

Gram positive and Gram negative test cultures were equally sensitive to both the oils individually as well as in combination and did not show difference because of the Gram character. Thus these oils may have its action on proteins, cell membranes or any other enzymatic processes.

The MIC for Lemongrass and Eucalyptus oil for all the test cultures was found to be 125 mg/ml and below that it was not effective in inhibiting the growth of any of these cultures .The extracts were tested using ethanol as solvent. Ethanol at this concentration did not have any inhibitory effect when tested as control.

The pathogens used like, *Staphylococcus aureus, E.coli, Klebsiella pneumonia* are the leading nosocomial infective agents. The routes of pathogen transmission are many and varied. Spread is by direct or indirect contact with animate or inanimate objects, and may be horizontal or vertical (ref. [7]).

Wide scale use of antibiotic led to microbial drug resistance, an adaptive response in which microorganisms are able to tolerate any amount of drug that would ordinarily be inhibitory (ref. [8]). Therefore, the use of essential oils from plants seems to be the practical alternative or a supportive treatment process. The combined therapy may become useful in blocking the ways of microbes to develop resistance to antimicrobial agents.

V. CONCLUSION

Our study of in vitro testing of antimicrobial activity indicates promising results for the use of Lemongrass, Eucalyptus & Lemongrass and Eucalyptus as combined oil preparation as therapeutic agents against *Staphylococcus aureus, E.coli, Klebsiella pneumonia and Bacillus subtilis.*

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