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COMPARATIVE STUDIES ON *IN VITRO* ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *SESBANIA SESBAN* SEEDS AND *TEPHROSIACALOPHYLLA* LEAVES

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ABSTRACT

The current research involves the study of antimicrobial and antioxidant activities of methanolic extracts of *Tephrosiacalophylla* leaves (METcL), *Sesbania sesban* seeds (MESsS) and petroleum ether extract of *Sesbania sesban*seeds (PESsS) using *in-vitro* methods. The antioxidant activity of extracts was evaluated by using reducing power assay, H_2O_2 scavenging activity, superoxide radical scavenging activity using ascorbic acid as standard. The minimum inhibitory concentration (MIC) values and antimicrobial activity of extracts was screened by disc diffusion method against 8 microorganisms (gram positive and gram negative) using different antibiotics as standard. The results of this study showed that METcL, MESsS, PESsS possess a potential antioxidant activity and antibacterial activity in contrast to the standards.

INTRODUCTION

Oxidative damage is a result of excessive generation of ROS induced by various stimuli and unbalanced antioxidant protection capacity. ROS can damage cells by initiating chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or by altering membrane fluidity. Oxidative damage may lead to a variety of patho-physiological processes such as inflammation, heart disease, diabetes, parkinson's disease, genotoxicity and cancer[1, 2].Antioxidants may be defined as compounds that inhibit or de-lay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions[3]. Recently there has been a surge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing such free radical-induced tissue injury[4]. Natural antioxidants constitute a broad range of compounds including phenolic compounds, nitrogen compounds and carotenoids. In recent years, there has been increasing interest in finding antioxidants from natural sources, since they can protect the human body from free radicals and retard the progress of many chronic diseases[5]. Medicinal plants represent a rich source of antimicrobial agents in India and are widely used by all sections of people either directly as folk remedies or in different indigenous systems of medicine or indirectly in the pharmaceutical preparations of modern medicines.[6]. Although hundreds of plant species have been tested for antimicrobial properties, the vast majority have not yet been adequately evaluated[7]. *Tephrosia* is a large tropical and subtropical

genus belongs to the family Fabaceae or Leguminosae. The genus *Tephrosia* contains a wide variety of flavanoids and isoflavanoids. A new coumestan derivative Tephcalostan from the whole plant together with two known flavonoids, 7-*O*-methylglabranin and kaempferol 3-o-β-$_{D}$-glucopyranoside were isolated and characterized[8]. A benzil, Calophione A, 1-(6′-Hydroxy-1′,3′-benzodioxol-5′-yl)-2-(6″-hydroxy-2″-isopropenyl-2″,3″-dihydro-benzofuran-5″-yl)-ethane-1,2-dione and three coumestan derivatives, Tephcalostan B, C and D were isolated from the roots of *Tephrosia calophylla*[9]. A chemical compound named Betulinic acid (BA) has been isolated from the whole plant of *Tephrosia calophylla*[10]. The plant genus *Sesbania*, family Leguminosae, is one of the predominant genus comprised of about 60 species which are widely distributed throughout tropical and subtropical regions. *Sesbania sesban* is a shrub or short-lived tree up to 8 meter tall. Stem up to 12 cm in diameter, usually pubescent, sometimes becoming glabrous[11].The preliminary phytochemical studies on the leaf extracts of *Sesbania sesban* revealed the presence of Alkaloids, Terpenoids, Steroids, Tannins, phenolic compounds like saponins, Flavonoids, Amino acids and Glycosides[12]**.**

The aim of this study was to investigate the invitro antioxidant and antimicrobial properties of methanolic extracts of seeds of Sesbania sesban and leaves of Tephrosia calophylla, Pet ether extract of Sesbania sesban seeds.

MATERIALS AND METHODS

The plant materials such as leaves of *Tephrosia calophylla*, and seeds of *Sesbania sesban* were freshly collected from Talakona forest and nearby villages of Andhra Pradesh state. Micro organisms are selected and procured from the National Collection of Industrial Micro-organisms **(NCIM)** department of National Chemical Laboratory (**NCL)** Pune.

Preparation of Extracts: The freshly collected plant materials were washed, shadow dried and then dried in hot air oven at a temperature not more than 50°C. The dried materials were coarsely powdered using an electric blender. Powdered materials (500g) were then packed in soxhlet apparatus and successively extracted with petroleum ether, chloroform, ethyl acetate and methanol. Each time before extraction with the next solvent, the powdered materials were dried in hot air oven at below 50°C. Finally extracts were concentrated in rotary evaporator at a temperature not more than 50°C and then, dried under vacuum desiccator. The dried extracts were used for the screening of activities.

Preliminary Phytochemical Screening: Preliminary phytochemical screening was done using the specified protocols for the qualitative analysis of Alkaloids, carbohydrates, fixed oils, flavonoids, glycosides, phyto sterol/terpenes, saponins, and tannins/phenols[13, 14, 15].

METHODOLOGY

Ferric reducing antioxidant power (FRAP) assay: The reducing power of extracts was determined by the FRAP assay method of Gulcin et al., 2003[16]. Briefly, an aliquot of 1.0 ml of extracts at various concentrations was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide C (2.5 ml, 1% w/v, in water). The mixture was incubated at 50^0C for 20 min and the reaction was stopped by addition of 2.5 ml of trichloroacetic acid (10% w/v, in water), followed by centrifugation at 1036 g for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl3 (0.5 ml, 0.1%, w/v, in water), and the absorbance was measured at 700 nm against blanks that contained all reagents except the sample extracts.

Super oxide anion radical-scavenging activity: The measurement of O^{2-}scavenging capacity was assessed using the method of Duh et al. 1999[17]. The reaction mixture contained of 1ml of different concentrations of plant extract, 1ml of PMS (60ppm) solution, 1ml of NADH (450Mm). Incubate at 25^0C for 5 min. After incubation at ambient temperature, the absorbance was read at 560 nm. Assay of the antioxidant activity in plant extract was

based on IC_{50} expressed as µg/ml. The inhibition percentage of super oxide anion generation was calculated using the following formula:

$$\% \text{ Inhibition} = 1-(A_{sample}/A_{blank}) \times 100$$

Hydrogen peroxide scavenging activity: The hydrogen peroxide scavenging abilities of MES, PES and MET were determined according to the method of Ruch et al., 1989[18]. A solution of H2O2 (40mM) was prepared in phosphate buffer (pH 7.4).1ml of different concentrations of sample was added to a H2O2 solution (0.6 ml, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution was containing the phosphate buffer without H2O2. The percentage of H2O2 scavenging of samples and standard compounds was calculated as

$$\%\text{Scavenge}H_2O_2 = (A_0-A_1)/A_0 \times 100$$

Where A_0 is the absorbance of the control, and A_1 is the absorbance in the presence of the sample or standards.

Microbiological Sensitivity Studies: The inhibition of microbial growth under standardized conditions may be utilized for demonstrating the efficacy of antimicrobial agents.

Inoculum preparation: The selected bacteria from stock cultures are sub-cultured under aseptic conditions. For this, the test organisms were separated and streaked onto the fresh agar slants, incubated at 37^0 C for 24 hrs. The growth content of each slant was scrapped into 10 ml of sterile distilled water and a uniform suspension was prepared and cell count was adjusted to 104/ml. It was used as inoculums. All the tests were performed and their activities were expressed as the mean of Zone of inhibition diameter produced by the test organisms. From the prepared bacterial suspension 2% of inoculums per total volume of MH agar medium in the petridish were used for microbiological assays.

Determination of Minimum Inhibitory Concentration (MIC): MIC values of the antibiotics and plant extracts were determined using the standard method of the European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2000)[19]. Dilutions of the antibiotic ranging from 0.001-1.0 mg/ml in DMSO, dilutions of the extracts ranging from 0.05-25 mg/ml in DMSO were prepared and incorporated (added) into the wells of pre-sterilized and pre-inoculated Mueller Hinton agar medium plates with test organisms. Plates were incubated at 37^0C for 24 hrs under aerobic conditions. Results of MIC tests were represented in Table.

Sensitivity testing of the plant extracts

Diffusion method (cup-plate method): After determining the MIC values, for the further sensitivity tests, doses of test drugs were fixed as 250µg/ml, 500µg/ml, 750µg/ml and 1000µg/ml. And the dose of standards was fixed as 10µg/ml. Then microbiological assay was carried out by Diffusion method under aseptic conditions and then plates were kept for incubation at 37^0 C for 24 hrs. After incubation, cultures were examined for the growth of bacteria and zones of inhibition formed by them. The zones of inhibition were measured by using Antibiotic zone reader and results were represented in the table.

Statistical Analysis: Results are expressed as mean ± S.E.M. of three determinants. Comparisons among the groups and IC_{50} values were determined by nonlinear regression analysis using Graph Pad Prism, Version 5.0 (Graph Pad Software, San Diego, CA, USA).

RESULTS & DISCUSSION

Phytochemical extraction: The extraction process of Tephrosia calophylla leaves and Sesbania sesban seeds have revealed that, Tephrosia calophylla was given higher yield (13.4%) in methanolic extraction whereas Sesbania sesban has given higher yields in both pet ether (10%) and methanolic (12.3%) extracts. Hence methanolic extracts and pet ether extracts were selected for the further studies.

Preliminary phytochemical Studies: The phytochemical screening subjected to detect the presence of some secondary plant metabolites following standard procedure shown in Table 1.

Table: 1 List of Test Organisms, Their Ncim No. And Standard Antibiotics Used

S.No.	Microbes	NCIM No	Antibiotics
1	Staphylococcus aureus	2602	Tetracycline
2	Streptococcus griseus	2183	Tetracycline
3	Bacillus subtilis	2480	Rifampicin
4	Staphylococcus epidermidis	2493	Neomycin
5	Corynebacterium species	5212	Tetracycline
6	Proteus vulgaris	2813	Erythromycin
7	Salmonella typhimurium	2501	Erythromycin
8	Escherichia coli	2981	Chloramphenicol

Table 2: - Preliminary phytochemical screening of *tephrosiacalophylla* leaves and *sesbania sesban* seeds extracts.

Phytochemical constituents	**METcL**	**MESsS**
Alkaloid	+	-
Carbohydrates	+	+
Glycosides(Anthraquinone, cardiac)	-	+
Saponin glycosides	+	-
Proteins	+	+
Volatile oils	+	+
Fats and fixed oils	-	+
Steroids	-	-
Flavonoids	+	+

METcL : Methanolic extract of *Tephrosiacalophylla* leaves
MESsS : Methanoic extract of *Sesbaniasesban* seeds
+ : Indicates the presence of phytochemical constituents
- : Indicates the Absence of phytochemical constituents

Fig:1 Determination of Reducing power

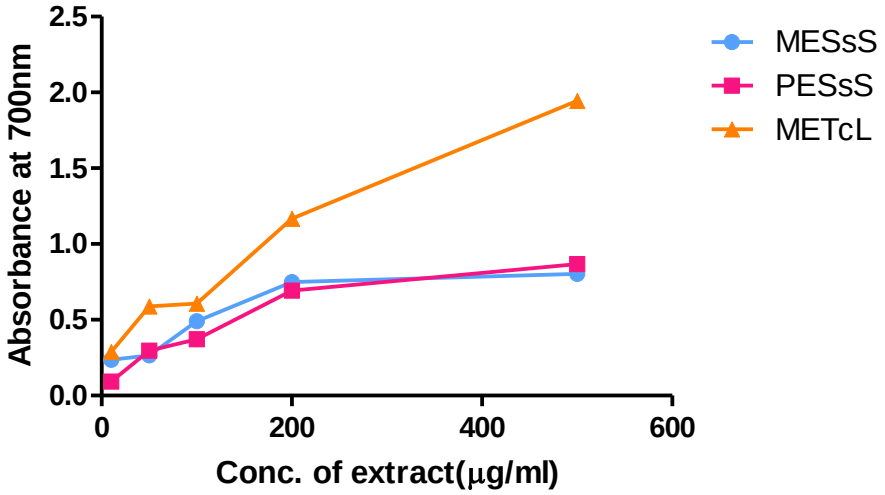

Fig: 2 Determination of hydroxyl radical scavenging activity

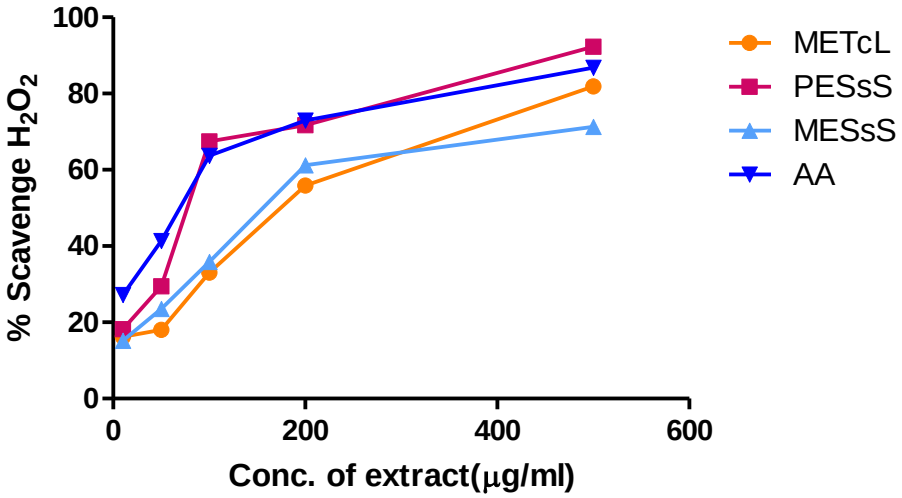

Fig:3 Determination of superoxide radical scavenging activity

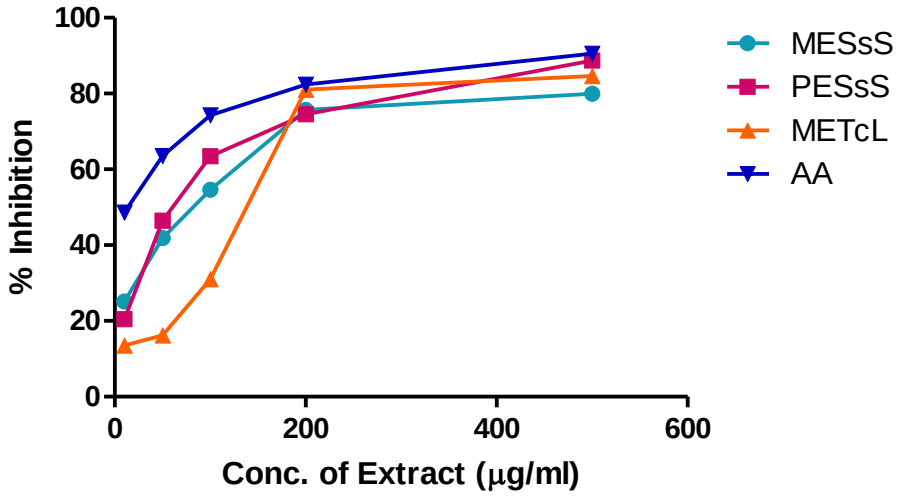

TABLE: 4 MIC values of me*tcl*, *pesss*, messs and antibiotics.

Test organism	Minimum inhibitory concentration (mg/ml)							
	TET	**NEO**	**ERY**	**CHL**	**RIF**	**METcL**	**PESsS**	**MESsS**
Staphylococcus aureus	0.004	--	--	--	--	-	-	4.5
Staphylococcus epidermis	--	0.412	--	--	--	4.0	4.0	4.0
Corynebacterium species	0.016	--	--	--	--	5.0	5.0	5.0
Bacillus subtilis	--	--	--	--	0.001	4.5	5.0	4.0
Streptococcus griseus	0.016	--	--	--	--	-	-	1.0
E.coli	--	--	--	0.008	--	-	-	0.5
Proteus vulgaris	--	--	0.412	--	--	0.5	0.5	0.5
Salmonella typhimurium	--	--	0.128	--	--	-	-	0.5

Table 3: Zone Of Inhibition of Antibiotic and Plant Extracts

Microorganisms	DMSO	METcL				PESsS				MESsS				Antibiotic
	Conc.(µg/ml)													
	50µl	250	500	750	1000	250	500	750	1000	250	500	750	1000	200
	Zone of Inhibition (mm)													
Staphylococcus aureus	6.1	-	-	-	-	-	-	-	-	12	12.8	13	14.4	21
Staphylococcus epidermis	6.5	12	14.8	15.8	15.6	13.8	16	16.8	19.4	12.2	13.4	15.2	14.6	26.4
Coryne bacterium species	6.5	13.4	17.2	17.4	18.2	23	29.2	31.2	32.6	11.4	13.8	16.8	16.4	35
Bacillus subtilis	6.2	12.8	16.8	17.2	18.8	14.6	14.8	16.8	17.4	14	14.4	14.8	14.8	27
Streptococcus griseus	6.5	-	-	-	-	-	-	-	-	14.4	14.8	15.5	19.2	21
E. coli	6.8	-	-	-	-	-	-	-	-	14.6	15.4	15.8	16.4	17.2
Proteus vulgaris	6.9	10.8	12	12.4	13.4	12.6	13	13.8	14.6	13	13.5	14.2	14.8	15.2
Salmonella typhimurium	6.3	-	-	-	-	-	-	-	-	13.4	13.6	13.8	14.6	16.6

Conc. : Concentration,
DMSO : Dimethyl sulphoxide(solvent used),
METcL: Methanolic extract of *Tephrosiacalophylla* leaves,
PESsS : Petroleum ether extract of *Sesbaniasesban* seeds and
MESsS: Methanoloic extract of *Sesbaniasesban* seeds.

Methanolic extract of Tephrosia calophylla revealed the presence of alkaloids, carbohydrates, steroids, volatile oils, flavonoids, saponin glycosides,and proteins. While methanolic extract of Sesbania sesban showed presence of carbohydrates, flavonoids, glycosides, volatile oils, proteins, fats and fixed oils.

Antioxidant activity

Determination of reducing power: The plant extracts could reduce the most Fe^{3+} ions which show their reducing capacity. Increase in absorbance of the reaction indicates reducing power. All the three extracts in the present study showed their reducing ability by increased absorbance with concentration.

Hydroxyl radiacal scavenging activity: Hydrogen peroxide itself is not very reactive, but can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells[21]. Scavenging of H_2O_2 by extracts may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water. The extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. Figure 2 shows that PESsS showed significant scavenging activity (H_2O_2) than that of other two extracts i.e., MESsS, METcL when compared to ascorbic acid. The IC_{50} Value for scavenging of H_2O_2 for MESsS was 18 0 ± 0.61 µg/ ml The IC_{50} Value for scavenging of $H_2O_2$for PESsS was 80.35 ± 0.051 µg/ ml The IC50 Value for scavenging of H2O2 for METcL was 124.0 ± 0.36 µg/ ml while IC_{50}value for ascorbic acid was 87.57 ± 0.091 µg/ ml.

Superoxide Radical scavenging activity: In the PMS–NADH–NBT system, superoxide anion derived from dissolved oxygen by PMS–NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture[22].Fig. 3 shows the percentage

inhibition of superoxide radical generation by three plant extracts and comparison with ascorbic acid. The percentage inhibition of superoxide generation by PESsS was found to be significant than that of MESsS, METcL when compared to ascorbic acid.The IC50 Values for scavenging of superoxide generation for MESsS, PESsS, METcL, ascorbic acid were found to be 87.61 ± 0.74 µg/ ml, 69.31 ± 0.33 µg/ ml, 122.40 ± 0.60 µg/ ml and 76.4 ± 0.49 µg/ ml respectively.

Microbiological studies

Minimum Inhibitory Concentration (MIC) values: The MIC values of different extracts and antibiotics were determined and represented in the table 4. MIC values of extracts against gram negative bacteria are less compared to gram positive bacteria and those of MESsS are less than that of other two extracts against Bacillus subtilis and Proteus vulgaris and MESsS has shown broad range action against all selected microorganisms Standard MIC value of Antibiotics and test drugs ranges between: 0.001-1.0 mg/ml and 0.05-20mg/ml respectively **(--)** denotes the test was not performed; (**–)** denotes the result was negative.

Microbiological Sensitivity Test: In the present study, three extracts of two different plants were tested for antimicrobial activity against 8 microbial pathogens (5 Gram positive and 3 Gram negative). The results of microbiological sensitivity test were represented in the table 3. METcL, PESsS showed nil activity towards Staphylococcus aureus, Streptococcus griseus, E. Coli and Salmonella typhi. MESsS showed activity against all the tested organisms. The maximum zone of inhibition 32.6 mm was observed with PESsS (1000µg/ml) against Corynebacterium.

CONCLUSION

On the basis of the results obtained in the present study, it was observed and concluded that the *Sesbania sesban* seed and *Tephrosia calophylla* leaves extracts, which contains flavonoids and related phenolic compounds have exhibited potential antioxidant and antibacterial activities. These in vitro assays indicate that selected plant extract especially pet ether extract of *Sesbania sesban* seeds is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. However, the active components responsible for the anti-oxidative activity are currently unclear. Therefore, further investigations need to be carried out to isolate and identify the antioxidant compounds present in this plant extract. Furthermore, the in vivo antioxidant activity of this extract needs to be assessed prior to clinical use. This study is a preliminary evaluation of antimicrobial activity of the plants. It indicates that several plants have the potential to generate novel metabolites. The plants demonstrating antibacterial activity may help to discover new chemical classes of antibiotics that could serve as selective agents for the maintenance of animal or human health and provide biochemical tools for the study of infectious diseases

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