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Antimicrobial and phytochemical analysis of methanolic leaf extracts of *Terminalia catappa* against some human pathogenic bacteria

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Abstract
Antibiotic resistance is one of the greatest threats of the 21st century. Scientists search for potential antimicrobial sources that can cope with antibiotic resistance. Plants of our community could be excellent source of drugs to fight off this problem. This study is focused on exploring antimicrobial properties of plants against some human pathogens. The antimicrobial potential of 20 methanolic plant extracts was screened against 8pathogenic bacteria by agar well diffusion method. The result indicated that the highest potential was observed in the methanolic extracts exhibited by leaves of *Terminalia catappa* against all pathogenic bacteria tested with zone of inhibition more than 20mm in all strains. The phytochemical screening of methanolic extracts of *T. catappa* revealed that they are positive for phyto chemicals; alkaloids, flavonoids, anthraquinones, tannins, steroids, phenols, quinones and saponins. The presence of these biologically active compounds in *T. catappa*, a valuable medicinal plant support that this plant is being used as medicine for curing various diseases in traditional medicinal systems and can also be employed in the treatmentof various ailements in medicine too.

Keywords: *Terminalia catappa*, antimicrobial potential, phytochemical screening, antibiotic resistance

Introduction
Antimicrobial agents are essentially important in reducing the global burden of infectious diseases [1]. Nature has provided a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine [2]. Thus the need to find new antimicrobial agents is of paramount importance.
Plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids, phenolic compounds, quinones and flavonoids, which have been found invitro to have antimicrobial properties [3, 4]. A number of phytotherapy manuals have mentioned various medicinal plants for treating infectious diseases as urinary tract infections, gastro intestinal diseases, respiratory diseases and cutaneous infections.
Terminalia catappa L. belongs to the family Combretacea. *T. catappa* is used primarily as an ornamental, shade and salt- tolerate street tree, but the leaves provide food for the tasar silkworm, and the seeds are edible like almonds with similar oils. *T. catappa* has been claimed to have therapeutic effects for liver related diseases [5]. In India, it is used as cardiac stimulant, its leaves are widely used as a folk medicine in South East Asia for the treatment of dermatosis and hepatitis [6]. More and more pharmacological studies have reported that *T. catappa* leaves and fruits have anti cancer, anti inflammatory, anti diabetic effects and hepato protective activities [7, 8] but the effective components and related mechanisms remain unknown.
The present study was carried out to analyze antimicrobial activity and biologically active compounds present in *T. catappa* methanolic leaf extracts against some human pathogenic bacteria (standard microbial cultures).

Material and Methods
Preparation of plant extracts: Twenty plant species were collected and predicting to posses bioactive compounds, plant species were collected based on the information available from literature [9, 10, 11] folklore [12] and through field observations. The plant materials were collected in and around Visakhapatnam district, Andhra Pradesh, India.
The collected plant materials were washed thoroughly with running tap water and finally with distilled water the material was chopped into small pieces and then air dried on a sterile blotter under shade for 20- 30 days.

The completely shade dried plant materials were coarsely powdered and allowed to Soxhlet extraction with methanol for 5-6 hours at temperature not exceeding the boiling point of the solvent and then filtered through Whatman no-1 filter paper. The extracted liquid obtqined was subjected to rotary evaporator and subsequently concentrated under reduced pressure (in vaccum at 4 ^{0}C). The residues obtained were designated as crude extracts, were labelled and stored in refrigerator for further study [13]. The dried plant extract residues obtained were redissolved in 0.1% Dimethyl Sulfoxide (DMSO) to get necessary concentration of crude extracts and filteration through a 0.45µm membrane filter and stored in sterile brown bottles in a freezer at 20 ^{0}C until bio-assayed.

Microorganisms: Based on disease index eight bacterial strains were selected listed in table 1. All the eight microorganisms tested were purchased from microbial type culture collection and gene bank (MTCC) Chandigarh, India. All the pure cultures were obtained in lyophilized or freeze dried form are reconstituted in sterile water and produced a suspension of the microbial cells, inoculation was done with sterile inoculating loop to liquid broth medium. Liquid cultures are then incubated to allow cell replication and adequate growth of the culture, for use in bio assays. Following incubation, liquid cultures are refrigerated to store for furthur use. Typically, 24hrs will provide sufficient growth to allow visibly thick spread of the microbes for bio assay. The bacterial strains are maintained and tested on nutrient agar medium.

***In vitro* antimicrobial assays:** The development of simple *in vitro* prescreens could offer initial idea of the biological activity of plant extracts and its compounds. The antimicrobial activity of bacterial strains listed in Table 1 was performed by agar ditch/ well/ cup diffusion method [14, 15, 16] at desired concentration with DMSO solvent which did not affect the growth of microorganisms.

Table 1: Pathogen index

S.no	Pathogen	MTCC code	Disease
1	*Bacillus subtilis*	121	Septicemia, wound and burn infections
2	*Klebsiella pneumoniae*	39	Pneumonia, blood stream infections
3	*Streptococcus*	889	Scarlet fever, rheumatic fever
4	*Psuedomonas mirabilis*	425	Urinary tract infections
5	*Escherichia coli*	476	Urinary tract infections, pneumonia
6	*Micrococcus*	7527	Bacteremia, meningitis
7	*Enterococcus faecalis*	439	Urinary tract and nosocomical infections
8	*Enterobacter cloacae*	509	Skin infections, meningitis, bacteremia

Preliminary phytochemical screening: To detect various biologically active constituents present in the methanolic extracts the standard methods were followed [17, 18, 19, 20].

Determination of Alkaloids

a) **Dragendorff's test:** Initially 1ml of test sample solution was taken in a test tube and few drops of Dragendorff's reagent (potassium bismuth iodide solution) were added. A reddish brown precipitate was formed indicating the presence of alkaloids.
b) **Meyer's test:** 1ml of the test sample solution was taken into test tube and then few drops of Meyer's reagent (potassium mercuric chloride solution) were added. A white precipitate was formed indicating the presence of alkaloids.
c) **Wagner's test:** The test sample and Wagner's reagent was added in a test tube which gives brown precipitate indicating the presence of alkaloids.
d) **Hager's test:** 1ml of extract solution was taken into test tube and few drops of Hager's reagent (picric acid) was added. Yellow precipitate was formed reacting positively for alkaloids.
e) **Tannic acid test:** A few ml of 10%Tannic acid was added to 1ml test sample solution, a buff color precipitate was formed giving positive result for alkaloids.

Determination of Flavonoids

a) **Alkaline reagent test:** Few ml of test sample was taken and NaOH solution was added to form intense yellow color, which turns into colorless on addition of few drops of dilute acid indicating the presence of flavonoids.
b) **Lead acetate test:** when aqueous basic lead acetate was added to test sample produces reddish brown precipitate, indicating the presence of flavonoids.
c) **Zinc hydrochloride reduction test:** A mixture of zinc dust and concentrated hydrochloric acid were added to plant extract solution, immediate development of red color indicates the presence of flavonoids.
d) **Shinoda test (Magnesium hydrochloride reduction test):** To plant extract solution, few fragments of magnesium ribbon and concentrated hydrochloric acid were added drop wise development of reddish to pink color indicates presence of flavonoids.

Determination of Quinones

a) **Alcoholic KOH test:** The test sample treated with alcoholic KOH solution, the colors appears from red to blue indicates presence of quinones.
b) **NaOH test:** Few drops of NaOH were added to test samples blue color indicates presence of quinones.

Determination of Anthraquinones;

a) **Borntrager's test:** 5ml of plant extract is to be shaken with 10ml benzene, filtered and 5ml of 10% ammonia solution was added to the filterate. When the mixture is to be shaken to appear a pink, red or violet color in the ammonical (lower) phase indicates the presence of free hydroxyl- anthraquinones.
b) **Modified Borntrager's test:** 2ml of test sample and 4ml of alcoholic KOH, dilute with 4ml of water and filter, then acidify with HCl. Next cool and shake well with 5ml of ether. To separate the ether into test tube and shake with 2ml of dilute solution of NH_4OH. Development of rose red to intense red color indicates of anthraquinones.

Determination of Glycosides

a) **Raymond's test:** To the test sample add 0.1 ml of a 1% solution of m-Dinitrobenzene in ethanol followed by 2 drops of 20% NaOH solution which gives violet color indicates presence of glycosides.
b) **Legal's test:** The test sample was treated with pyridine and sodium nitroprusside solution to developed blood red color.
c) **Kellar Kiliani test:** 1ml of concentrated H_2SO_4 was taken in a test tube then 5ml of extract and 2ml of glacial

acetic acid with one drop of $FeCl_3$ were added, formation of a blue color indicates presence of glycosides.

d) Concentrated Sulphuric acid test: When few ml of Concentrated H_2SO_4 was added to test sample gives reddish color indicates presence of glycosides.

e) Bromine water test: The extract sample treated with bromine water test solution gives yellow precipitate.

f) Molisch test: When naphthol and concentrated H_2SO_4 were added to test samples reddish violet ring at the junction of two layers was resulted, responding positive for the presence of glycosides.

Determination of Tannins

a) Gelatin test: Gelatin and water were added to test sample in a test tube to formation of white precipitate was resulted, indicating presence of tannins.

b) Mitchell's test: Iron and sodium citrate were added to test sample solution, a water soluble iron-tannin complex was formed, in case of tannins present. The complex of iron-tannin is insoluble in ammonium acetate solution.

Determination of Steroids

a) Salkowski test: Few ml of concentrated H_2SO_4 were added to extract sample in chloroform, a red color was appeared at the lower layer, which indicates the presence of steroids.

b) Libermann-Buchard test: The extraction sample was treated with few drops of acetic anhydride and were boiled, then few drops of concentrated H_2SO_4 was added from the sides of the test tube, shows a brown ring at the junction of the two layers and upper layer turns green which shows the presence of steroids.

Determination of Phenols

a) Ellagic acid test; When 5%glacial acetic acid and 5%sodium nitrite were added to extracts a muddy Niger brown color appears, which is a positive result for phenols.

Determination of Saponins

a) Froth test: A few ml of test sample taken into a test tube and add upto 20ml of water and shaken vigorously, then left to stand for 10min. A thick persistence froth was resulted indicates presence of saponins.

Results and Discussion

Evaluation of the antimicrobial activity of 20 different methanolic plant extracts was determined by agar well diffusion method against eight human pathogenic bacteria (table-2). It was observed that *T. catappa* was the most effective among the 20 plant extracts tested. It showed a significant zone of inhibition (ZOI) against all Gram positive and Gram negative bacteria tested (Table-2).

Table 2: Antimicrobial activity of plant extracts on pathogens

*Plant extract	DIZ (mm)							
	B. subtilis	***K. pneumoniae***	***Streptococcus***	***P. mirabilis***	***E. coli***	***Micrococcus***	***E. faecalis***	***E. cloacae***
Z. nummularia	-	8	-	6	-	-	7	8
C. fistula	-	-	-	-	-	-	-	-
D. metel	-	8	10	7	9	7	8	9
L. inermis	9	6	6	6	10	-	7	8
S. nigrum	-	7	9	8	8	9	8	9
A. marmelos	8	7	6	6	-	-	-	7
S. cumini	11	-	-	10	-	-	-	7
B. arundinacae	-	6	-	7	8	-	-	8
C. papaya	-	8	9	7	8	7	7	9
B. prionites	-	-	-	6	-	-	-	8
S. acuta	-	-	7	6	7	7	7	-
C. laburnifolia	-	7	-	8	8	8	-	10
T. patula	8	10	8	10	8	7	9	10
Eichornia	-	6	6	6	6	-	7	7
P. longum	-	-	-	8	9	9	-	10
T. cordifolia	-	6	-	-	-	-	-	6
T. catappa	33	25	28	25	27	33	32	38
M. heterophylla	-	-	-	6	8	6	-	8
T. f. graecum	-	7	6	7	6	-	7	8
P. guajava	-	-	-	-	8	-	-	-

*Plant extracts 100mg/ml concentration, 50µl in each well.
DIZ- zone of inhibition including 6mm well diameter, is the mean of 3 replicates.
-; indicates no inhibition.

The effectiveness of the extracts in tested bacterial strains was determined by measuring the minimum inhibitory concentration (MIC). MIC was performed for only those organisms which showed a zone of inhibition and were sensitive to the plant extracts. Methanolic leaf extracts of *T. catappa* upto 10mg-l (W/V) concentrations showed significant activity against all tested pathogens.

Analysis of biologically active compounds of leaf extract of *T. catappa* was done by using standard procedures [17, 18, 19, 20] and results were presented in Table-3. The result shows the presence of flavonoids, alkaloids, steroids, tannins, saponins, quinones, anthraquinones and glycosides. The presence of these secondary metabolites has been reported to account for the exertion of antimicrobial activity by plant. Chloramphenical and penicillin were used as positive controls and their inhibition zones against all bacterial strains were compared with inhibition zones of plant extracts.

Table 3: Phytochemical analysis of leaf extracts of *T. catappa.* L.

Phytochemical test	Test result
1. Alkaloids	
Dragendorff's test	+
Mayer's test	+
Hager's test	+
Wagner's test	+
Tanic acid test	+
2. Flavonoids	
Alkaline reagent test	+
Lead acetate test	+
Zn-HCl reduction test	+
Shinoda's test	+
3. Quinones	
NaOH test	+
Alcoholic KOHtest	+
4. Anthraquinones	
Borntrager's test	+
Modified Borntrager's test	+
5. Glycosides	
Raymond's test	+
Legal's test	+
Kellar Kiliani test	+
Conc.H_2SO_4 test	+
Bromine water test	+
Molisch test	+
6. Tannins	
Gelatin test	+
Mitchell's test	+
7. Steroids	
Salkowiski test	+
Libermann-Buchard test	+
8. Phenols	
Ellagic acid test	+
9. Saponnins	
Froth test	+

+; positive {presence of the constituent}

Tannins have been found to form irreversible complexes with proline rich proteins resulting in the inhibition of cell protein synthesis. Flavonoids inhibit nucleic acid synthesis, cytoplasmic membrane function, porin on the cell membrane, alteration of the membrane permeability and attenuation of the pathogencity. Quinines inhibit growth in microbes. Phenols inhibit protein synthesis in microbes. Glycosides causes cell lysis and disruption of cytoplasmic membrane of microbes.

This study can lead to the better understanding of the role played by the phytochemicals in inhibiting the growth of the pathogenic bacteria. The results of the present work clearly indicate that methanolic leaf extracts of *T. catappa* shows significant antimicrobial activity against all bacterial strains, it may be due to the presence of flavonoids, tannins, saponins and steroids [21, 22].

Conclusion

In conclusion methanolic leaf extracts of *T. catappa* showed significant antimicrobial activity against human pathogenic bacteria and presence of secondary metabolites responsible for antimicrobial action in the extracts. This suggests that constituents of the plant could be useful in chemotherapy. From the findings of this study, the following recommendations could be made; Firstly, there is a need to further isolate the active antibacterial agent(s) and secondly, it is necessary to determine toxicity of the active constituents, their side effects and pharmacokinetics effects.

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