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A STUDY ON PHYTOCHEMICAL AND ANTIFUNGAL ACTIVITY OF LEAF EXTRACTS OF *TERMINALIA CATAPPA*

***SHIKHA MANDLOI, RENU MISHRA, RANJANA VARMA , BINU VARUGHESE AND JAGRATI TRIPATHI.**

Sri Sathya Sai College for Women , Bhopal. M.P. India

ABSTRACT

The antifungal activity of *Terminalia catappa* leaves was tested against five allergenic and pathogenic moulds. An initial screening of effectivity of different solvent extracts was done by phytochemical analysis and *in-vitro* antifungal activity. The antifungal activity was determined by both food poison technique and by cup-well diffusion method for five test organisms viz *Aspergillus niger, Alternaria alternata, Curvularia lunata ,Penicillium chrysogenum, and Trychophyton tonsurans* . Methanol extract showed significant antifungal activity. The MIC evaluated at three different concentrations, t was not concentration dependent activity. Most susceptible mould was *Curvularia lunata* and least susceptible was *Alternaria alternata* as compared with standard antifungals.Hplc analysis revealed presence of a known flavonoid component identified as quercitin.

KEYWORDS: ***Terminalia catappa,*** **phytochemical analysis, medicinal plants, bioactive compounds,antifungal activity**

SHIKHA MANDLOI
Sri Sathya Sai College for Women , Bhopal. M.P. India

***Corresponding author**

INTRODUCTION

The reduced number of herbal drugs available against pathogenic fungi makes it necessary to discover new classes of antifungals and compounds that inhibit their resistant mechanisms. This has led to the search for therapeutic alternatives, particularly among medicinal plants and compounds isolated from them that are used for their empirically antifungal properties [1]. Ethnopharmacological surveys performed around the world have mentioned that among the plant species belonging to combretaceae family, *Terminalia catappa* is the most requested medicinal plant. *Terminalia catappa* family Combritaceae (Tropical almond) has a vast natural distribution in near coastal areas of the Indian Ocean, through Tropical Asia and into Pacific Ocean. It is fast growing and easily propagated from seed that flourishes with minimal maintenance in suitable environments. [2].The decoction of leaves is used in Nigeria, as medicine against malaria and abdominal pains. In Togo & Benin root bark decoction is used in the treatment of various dermatosis. Similarly in Phillipines, leaves extract is used against Leprosis. [3] . The leaf, bark and fruit of this plant have long been used in folk medicine for antidiarrheic , antipyretic and haemostatic purpose in India, Phillippines, Malaysia and Indonesia. *T.catappa* leaf has been reported to possess antioxidative, hepatoprotective, antidiabetic, anti-inflammatory and anti-HIV reverse transcriptase activity.The presence of wide number of bioactive compounds with variety of biological activities in *T. catappa* attracted us to select this plant for the present study.[4] Phytochemical analysis of active plant extracts for qualitative identification of the major phyto constituents and anti fungal activity is also reported here.

MATERIALS AND METHODS

COLLECTION OF PLANT MATERIAL

Twigs of *Terminalia catappa* Linn were collected from Bhopal. The plant was identified from Govt. M.V.M. college with the help of the project report of Dr.Madhuri Modak , who has confirmed the identification from Botanical Survey of India, Allahabad.The leaves were separated washed and dried at room temperature. The dried leaves were powdered in a mixer blender and stored at room temperature in close containers in dark until used.A herbarium of the same was deposited at SNGGPG autonomous Girls college, Bhopal (M.P.).

SOURCE OF MICRO-ORGANISMS

Fungi *Aspergillus niger*, *Alternaria alternata, Curvularia lunata, Trychophyton tonsurans* and *Penicellium chrysogenium* selected were isolated from aeromycoflora of Bhopal based on their allergenic and pathogenic nature. Fungal mycelia from the culture were stained with *lactophenolcottonblue* and identified by using compound microscope at 40x objective (Olympus make) . The species were initially identified with reference to standard literature. For confirmation of identification cultures were sent to NCFT (National Centre of Fungal Taxonomy), Delhi.

EXTRACT PREPARATION

The powdered leaves (25g) of *T.catappa* was extracted separately to exhaustion in a soxhlet apparatus using ethanol, methanol, petroleum ether and acetone as solvent system. All the extracts were filtered through Whatman filter paper no.1 and then concentrated in hot air oven at low temperature 40^0-50^0C. A yield of 11.6% from ethanol,12.2% from methanol, 1.6% petroleum ether and 1.8% acetone solvent system were obtained. [5]

PHYTOCHEMICAL ANALYSIS OF LEAF EXTRACT

Specific qualitative tests were performed to identify bioactive compounds of pharmacological importance through standard methods as described by Harbone [6].

Test for alkaloids (Mayer's test):- To 2.3 ml.of filtrate a few drops of Mayer's reagent was

added, appearance of ppt. indicates the presence of alkaloids. Wagner's test:- To 2-3 ml. filtrate was treated with few drops Wagner's reagent , reddish brown precipitate appeared.

Test for glycosides (Keller Killiani test) :- To the 2ml extract, a few drops of glacial acetic acid, one drop 5 % $Fecl_3$ and conc. H_2SO_4. were added, reddish brown color appeared at the junction of the two liquid layer and upper layer appeared bluish green.

Test for tannic and phenolic compound:- To 2-3 ml. of aqueous or alcoholic extract, added few drops of following reagent.

a. 5% $FeCl_3$ solution --- deep blue black colour.
b. Lead acetate solution --- white ppt.
Dilute Iodine solution --- red colour

Test for Saponin:- To 2 ml extract 5 ml. distilled water was added and heated to boil. Frothing appeared, showed the presence of saponin.

Test for Flavonoids:- To small quantity of residue, (0.1%) lead acetate solution was added. Yellow colour ppt. formed. Addition of increasing amount of sodium hydroxide to the residue showed decoloration.

Test for Steroid Salkowski reactions – To 2 ml. of extract add 2 ml. chloroform and 2 ml conc. H_2SO_4 and shaken well. Chloroform layer appeared red and acid layer showed greenish yellow fluorescence.

Test For Carbohydrate Molish test:- To 2-3 ml aqueous extract, add a few drops of α napthol solution in alcohol. Mix was shaken and conc. H_2SO_4 was added from sides of the test tube. Violet ring formed at the junction of two liquids showed the presence of carbohydrate in the extract.

Test for non- reducing polysaccharides (Starch) Iodine test : Mixed 3 ml test solution and few drop of dilute iodine solution. Blue colour appeared.

Tannic acid test for Starch : 20% tannic acid was added in the test solution. Precipitate appeared.

Test for Protein Biuret test: To 3 ml test solution added 4% NaOH and few drop of 1% $CuSO_4$ solution violet colour appeared. Millon's test: To 2 ml. of test solution, Millon's reagent was added , white precipitate appeared.

ANTIFUNGAL ACTIVITY STUDY

The antimicrobial activities of all the extracts were determined by Food poison technique (The fungitoxicity of the extracts in terms of percentage inhibition of mycelial growth was calculated by using the formula

% inhibition = dc-dt/dc x 100

Where:

Dc = Average increase in mycelial growth in control.

Dt = Average increase in mycelial growth in treatment.) [7] and Agar well diffusion method [8]

SCREENING OF SUSCEPTIBLE FUNGI

One gram of each of the dried evaporated solvent extract of all the solvents was dissolved in 10ml of the respective solvent. 500µl of each solvent extract was amended with 15µl of PDA medium before solidification. The medium having only solvent served as control. Test fungi were inoculated and after incubation at 25 ± 2^0C for two days the percent inhibition of mycelia growth was determined. The colony diameter was measured in millimeter. For each treatment three replicates were maintained,

MIC

Minimum inhibitory concentration of effective solvent extract was determined by cup well method[9].Three different concentrations of effective extract 100mg/ml,250mg/ml and 500mg/ml and 10mg/ml of standard were taken for the assay. The wells were filled with 50ul each of controls and test concentrations using a micro titre pipette.The plates were allowed to diffuse at room temperature for two hour.The plates were then incubated at 25^0C for two days and the zone of inhibition was measured.

THIN LAYER CHROMATOGRAPHY (TLC) OF LEAF EXTRACTS.

TLC for methanolic leaf extract that showed strong antimicrobial activity was carried out on prepared TLC microslides . [10] with Silica gel G. About 20 µl of plant extract was spotted on TLC chromatogram. A solvent system consisting of Chloroform : Ethyl Acetate (6 : 4 v/v**)** was used for separation of compounds.Individual Rf for

each spot was measured. TLC spots were visualized under UV light.

HPLC analysis

The HPLC analysis of leaf extract was carried out using Waters(make) HPLC system, having Waters 486 UV visible detectors, equipped with rehodyne injector, HPLC cartridge system, Thermo C18 (250x4.6mm), 5µm column. A UV visible detector with variable wavelength was used for the separation of different bioactive compounds.Sample preparation for HPLC analysis :- Methanol extract of *T. catappa* was separated using a mobile phase of 50Mm KH_2PO_4 Buffer (pH-3 with OPA): Acetonitrile in the ratio of (30:70 v/v) to a flow rate of 1.0ml/min, with column temperature 25^0C. Injection volume was 20ul and detection was carried out at360nm**.**

STATISTICAL ANALYSIS

PHYTOCHEMICAL ANALYSIS

The results were stastistically analysed by Tukeys method using ANOVA software.

RESULTS AND DISCUSSION

RESULTS

Phytochemical characteristics of leaf extracts of *Terminalia catappa* in four different solvent tested are summarized in Table1. The leaf extracts in different solvents were screened for the presence of various bioactive phytochemical compounds.The analysis revealed presence of bioactive compounds such as terpenes, saponins, steroids, flavonoids, phenols and glycosides in ethanol fraction . The methanolic fraction contained alkaloid also. The therapeutic qualities of this plant can be attributed to the presence of chemical compounds like terpenes, phenols, saponins and flavonoids.

PHYTOCHEMICALS	METHANOL	ETHANOL	PET. ETHER	ACETONE
Alkaloids	+	-	-	+
Glycoside	+	+	-	-
Saponins	+	+		+
Tannic & Phenolic	+	+	-	+
Flavonoid	+	+	-	+
Steroid	+	+	-	-
Terpenoid	-	-	-	-
Sugars	-	-	-	-
Proteins	+	-	-	-

Table 1

Showing results of phytochemical analysis.

SUSCEPTIBILITY TEST AGAINST DIFFERENT ORGANIC SOLVENT EXTRACTS

The antifungal activity of *Terminalia catappa* was evaluated against five allergenic and pathogenic moulds. An initial screening of effectivity of ethanol, acetone, methanol and petroleum ether fraction on fungi was performed by food poison technique. Among the four extracts used, methanol extract for all five fungi showed significant antifungal activity.The percentage of inhibition of methanol extract was more than 50% and it was dose independent activity. Among the five fungi tested, *Curvularia* was found to be most sensitive and *Alternaria* to be least sensitive. It has been observed that ethanolic and methanolic fractions were effective against all the organisms in varying levels of sensitivity. However the extracts in acetone and petroleum ether fraction did not showed any remarkable antimicrobial activity. *Curvalaria lunata* was found to be most susceptible to ethanol fractions of *Terminalia catappa.* With an inhibition of 74% of growth where as *Trychophyton* and *Alternaria* were least susceptible. While comparing the solvents, methanol fraction was found to be more effective than ethanol fraction on all the fungi.In methanolic extract, *Aspergillus niger*, was most susceptible with 68.7% growth inhibition,

followed by *Penicellium* ,*Trychophyton, Curvalaria* and *Alternaria.* Results of antifungal analysis are shown in table 2 and fig 1.

Table 2
Showing results of screening of effective leaf extract of Terminalia catappa in various solvents.

S. No.	Leaf extracts	% of mycelial growth inhibition of various Fungi				
		Aspergillus niger	Alternaria alternata	Curvularia lunata	Penicillium chysogenum	Trychophyton tonsurans
1	Ethanol Extract	57.33 ± 0.33	24.66 ± 0.33	74.7 ± 0.28	50.8 ± 0.27	43.7±0.31
2	Methanol extract	68.7 ± .33	35.3 ± 1.45	60 ±0 .51	64 ± .15	61±0.25
3	Petrolium ether	64 ± 1.15	27.33 ± 0.18	36.67 ± 0.85	51 ± 0.57	61.67±1.53
4	Acetone	43.7 ± 0.31	31.7 ± 0.15	24.6 ± 0.33	43.7 ± 0.31	27.33±.18
5	Control Ketoconazole (10 mg/ ml)	63 ± 0.33	ND	ND	45 ± 0.57	71±0.33
6	Carbendazim (10 mg/ ml)	ND	75 ± .18	60 ± 0.33	ND	ND

Replicates = three
Result were expressed as mean ± SEM
All the results were found to be significant at p < 0.01 and p < 0.05

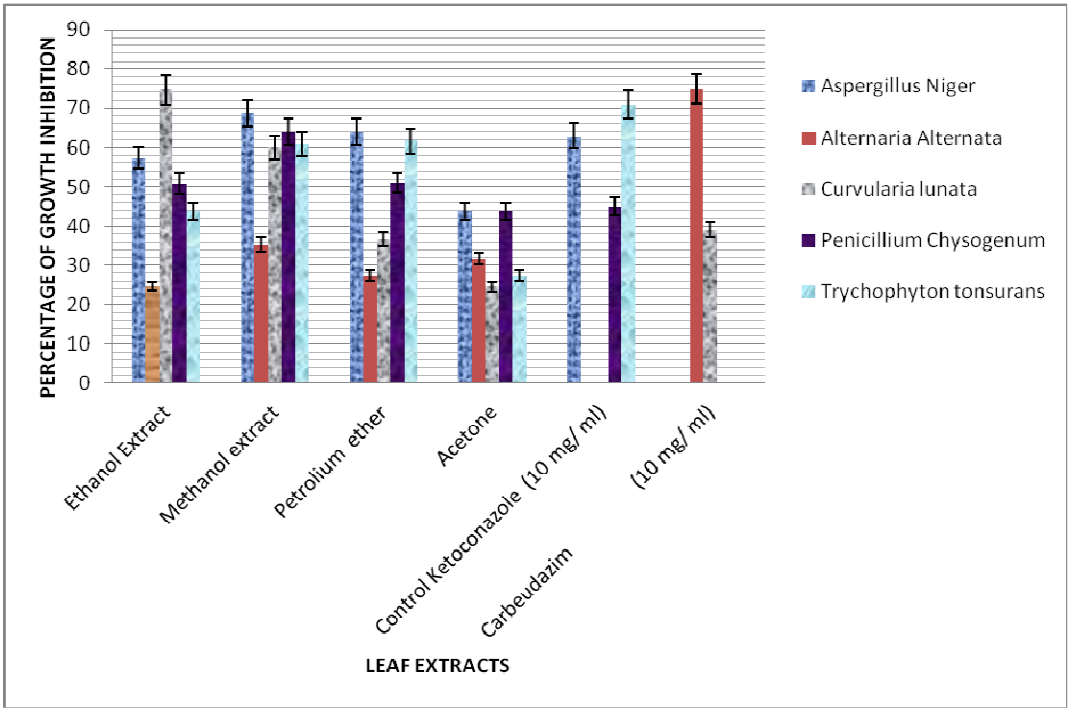

Figure 1
shows antifungal activity of leaf extracts of Terminalia catappa against various fungi.

MIC

Since, *Curvularia* was found to be more susceptible to ethanol and methanol fractions, MIC was determined by cup well method against it. Three concentrations of 100mg/ml, 250mg/ml and 500mg/ml were tested against the fungi. Significant antifungal activity was recorded in 250mg/ml fraction of ethanol and 100mg/ml of methanol of *T. catappa. Table 3.*

Sr. no.	Fungi	Ketoconazole /carbendazium diameter in mm	Ethanol mg/ml			Methanol mg/ml		
			100	250	500	100	250	500
1	A.niger	14.6±0.33	-	-	9.6±0.33	12.66±0.66	11.66±0.33	12.6±0.46
2	Alternaria alternate	23±0.12	-	-	13±0.57	-	-	19.66±0.33
3	Curvularia lunata	29.6±0.33	-	17.16±0.59	21±0.57	9.6±0.33	19.6±0.33	12.6±0.21
4	Trychophyton tonsurans	21.66±1.19	-	-	-	-	-	

Table 3

Inhibition zone diameter of ethanol and methanol extracts of Terminalia catappa

RESULTS OF TLC AND HPLC

Quercitin was isolated from the methanol extract and then detected on Tlc plate in comparison with standard flavonoid , quercitin.The retention factor value of 0.42 was found to be simillar to that of standard quercitin.Also, characterisation of methanolic extract through Hplc analysis, revealed that fraction of *T.catappa* leaves contain a bioactive component quercitin, one of the flavonoid component. The retention time of standard quercitin was recorded 6.235 min while the retention time of the sample was found to be 6.159 min.The amount of quercitin was calculated by extrapolating the value of area from the calibration curve.The percentage assay of quercitin after calculation was found to be 3.940. Table 4 and table 5.

The Rf value of TLC of T.Cattapa were found as follows.

Extract	Chloroform : Ethyl Acetate (6 : 4 v/v)
Terminalia cattapa (Methanolic)	2.60/6 = 0.43

Table 4

Result of Assay of Quercetin in methanol Extract

Name of Extract	% Assay
Terminalia cattapa (Methanol)	3.940

Table 5

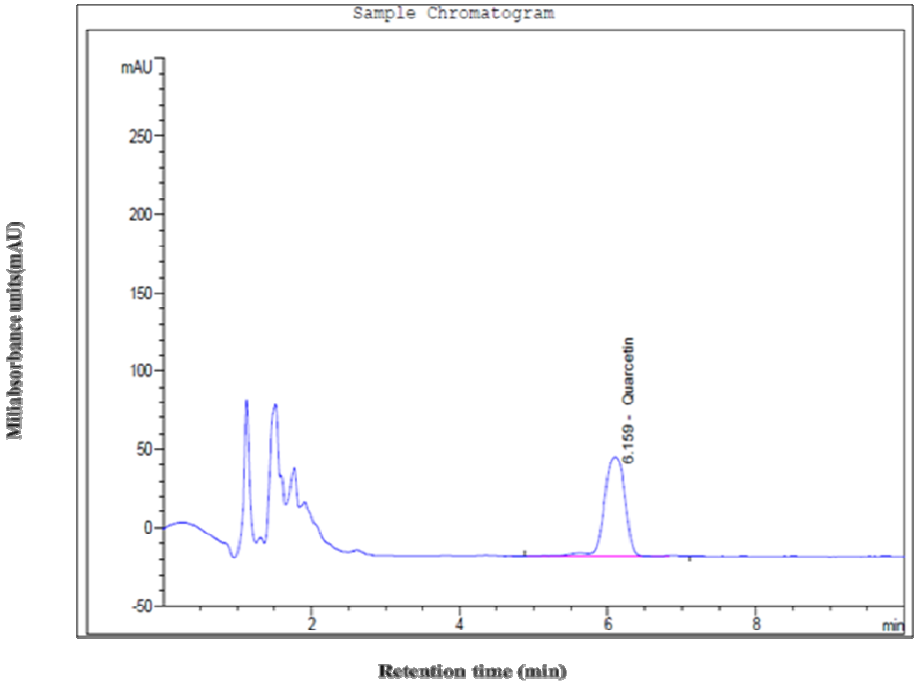

Figure 2
Sample Chromatogram of Extract Terminalia cattapa (Methanol)

DISCUSSION

The leaf, bark and fruit of this plant have long been used in folk medicines for antidiarrheic, antipyretic, haemostatic purposes in India. Phytochemical screening of ethanolic extracts of *T. catappa* shows the presence of glycosides, steroids, flavonoid, tanins, phenolic compounds and saponins. The earlier work on methanolic leaf extracts of *T. catappa* has also reported the presence of these components.[11,12] Earlier report on phyochemicl analysis of pericarp of *T. chebula* fruit has also shown alcohol to be best extractant of bioactive components. [13] The presence of antifungal activity in alcoholic fraction of *Terminalia* leaf extracts finds agreement with the work of several other workers.[14-19] Hydroalcoholic extracts of *T.catappa* and *T. mantaly* have been reported to inhibit the *in vitro* growth of *Aspergillus fumigatus.*[20]Several studies have reported the presence of bioactive compounds that are responsible for the medicinal properties of the plant that is used for treatment of different ailments. Some important antimicrobial phytoconstituents of T.catappa leaf reported are tannins (terflavins,tergallagin),flavonoids (rutin) and triterpinoids. [21,22] TLC and HPLC of methanolic leaf extracts showed the presence of quercitin (flavonoid) as active constituent. These findings correlate with the other findings where an average proportion of flavonoids was reported from various fractions of same plant.[23] In yet another work monomeric flavonoids were detected as active constituents in *E. officinalis.*[24]The present study on antifungal screening also justifies the traditional uses of *T.catappa* in treatment of various ailments. Study of the synergistic interaction of active phytocompounds with antibiotics is required to exploit the use of this plant extracts in the combination therapy of infectious diseases caused by multidrug resistant organism.

CONCLUSION

Since growth of *Curvularia lunata, Aspergillus niger* ,*Penicellium* and *Trychophyton tonsurans* was suppressed by more than 50%, the

alcoholic extracts are effective against them. This study has highlighted *T. catappa* as a potential antifungal agent against C*urvularia lunata* under *in vitro* conditions. It is concluded that antifungal activity of methanolic leaves extract of *T.cattapa* and its active constituents would be helpful in interacting various kinds of human allergies and plant diseases

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