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ANTIMYCOBACTERIAL, ANTICANDIDAL AND ANTIOXIDANT PROPERTIES OF TERMINALIA CATAPPA AND ANALYSIS OF THEIR BIOACTIVE CHEMICALS

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ABSTRACT

To find out the antibacterial antimycobacterial, anticandidal and antioxidant activities, phytochemical constituents and partial purification of Terminalia catappa and to detect the probable bioactive compounds. Methods: The extract was screened for its antimicrobial activity using eight bacteria by agar well diffusion method. Antimycobacterial activity of the extract was carried out using Mycobacterium smegmatis and Mycobacterium tuberculosis H37RV to find out the respective minimal inhibitory concentrations. Apart from this, Luciferase Reporter phage assay was done which is rapid and elegant method to determine the antimycobacterial activity. Anticandidal activity was also performed against various Candida species and Cryptococcus neoformens. The antioxidant study was done using 1-diphenyl-2 picryl hydrozyl (DPPH), ferric reducing power, free radical scavenging, hydroxyl radical scavenging, superoxide radical scavenging, and iron chelating and nitric-oxide radical scavenging assays. Further, the phytochemical constituents of the extract were analysed and bioactive guided fractionation was undertaken to find out the bioactive compounds through GC-MS analysis. Results: Antimicrobial activity of Terminalia catappa showed good inhibitory activity against both gram negative and gram positive bacteria. The extract showed good antimycobacterial activity against tested M. smegmatis and M. tuberculosis. It was further confirmed by Luciferase Reporter Phage assay which is a rapid and confirmatory test. Further, it was tested for promising activity against Candida species. Phytochemical analysis showed the presence of alkaloids, glycosides, phenolic compounds, tannins, flavonoids and amino acids. In addition to this, the plant showed a good antioxidant activity which is essentially needed for oxidative stress management. Partial purification of the plant compound showed the presence of twenty nine compounds in Gc- Ms analysis. Among them four compounds showed antituberculous activity. They are 1-Hexadecen, 1-Heneicosanol, 1-Nonadecene and Heptadecane 2,6,10,15 tetramethyl. Most of the other compounds showed antimicrobial and antioxidant activities. Conclusions: From this study, it is evident that the plant possesses good antimicrobial activity against pathogens including Mycobacterium and Candida species and good antioxidant activities which are helpful in oxidative stress management. Thus, the plant compound can be used in the treatment of various bacterial, mycobacterial and fungal infections. Hence, it can be used as a potential candidate for drug development against microbial infections; moreover its promising antioxidant property can confer protection to host against oxidative damages.

KEY WORDS

Terminalia catappa, antibacterial, antimycobacterial, anticandidal, phytochemical and antioxidant activities.

INTRODUCTION

Medicinal plants are used in the treatment of microbial infections and other human diseases traditionally from time immemorial. Until today herbal medicines are in use in India and in other parts of the world. This is because the plant derived compounds are less toxic when used in low concentrations and they do not have side effects when compared with the commercially available synthetic drugs. Synthetic drugs are not only giving side effects to human beings but they develop drug resistance on causative agents of tuberculosis, malaria and various diseases [1]. Recent studies have shown that plant derived compounds are useful in treating bacterial, fungal, viral, mycobacterial infections, etc. In recent years, multiple drug

resistance in human pathogenic microorganisms has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases [2]. In addition to this problem, antibiotics are sometimes associated with adverse effects on host including hyper-sensitivity, immune suppression and allergic reactions [3]. Therefore, there is an urgent need to develop alternative antimicrobial drugs for the treatment of infections obtained from various sources including medicinal plants. There were several bioactive compounds reported in plants [4]. There have been many Indian medicinal plants determined to have antimycobacterial activity [5]. 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 [6]. Terminalia catappa is commonly called as Badham (Tamil vernacular name) and almond in English. Tropical almond (Terminalia catappa) is a large, spreading tree distributed throughout the tropics in coastal environments. It is fast growing, easily propagated from seeds and can be easily maintained under suitable environmental conditions [7]. In Nigeria, the decoction of this plant is used to treat malaria and abdominal pains. In Togo and Benin, the decoction of root and bark is used to treat dermatosis. In Phillippines, the leaf extract is used against leprosy. The leaf bark and root of the plant is used for antipyretic and haemostatic purpose in India, Phillippines, Malaysia and Indonesia. The dried leaves were used for fish pathogen treatment, as an alternative to antibiotics. The various extracts of leaves and bark of T. catappa have been reported to have anticancer [8,9], anti-HIV reverse transcriptase [10] and hepatoprotective [11], anti-inflammatory [12], antimetastatic [13], antidiabetic [14] and aphrodisiac activities [15]. Apart from these, it has promising antioxidant activities which are due to many of the phytochemicals such as alkaloids, flavonoids,saponins, phenolic compounds, tannins, etc. In the present study, bioactive potentials including antimycobacterial, antibacterial, anticandidal and antioxidant properties of T. catappa were evaluated through various assays and discussed in detail.

MATERIALS AND METHODS

(i) **Plant collection and preparation of extract:**

Fresh leaves of T. catappa were collected from Bharathidasan university campus washed in distilled water and shade dried at room temperature for 2 to 3 weeks, pulverized and stored in airtight container for further use. 200 grams of this powdered material was weighed and extracted with solvents methanol and ethyl acetate in 5:1 proportion and was mixed properly using a shaker and kept at 25^{o} C for overnight. Then, the mixture was taken and filtered using whatman filter paper no. 3 and the extract was vacuum dried and stored at 25^{o} C for further use.

(ii) **Bacterial strains:**

The reference strains used in this study were E. coli NCIM 2065, Staphylococcus aureus NCIM 5021, Bacillus subtilis NCIM 2063, Pseudomonas aeruginosa NCIM 5029, Vibrio cholerae MTCC 3904, Salmonella typhi, MTCC 3914, Proteus mirabilis MTCC 425, Klebsiella species, Methycillin resistant S. aureus (KKTCH), Mycobacterium smegmatis from IMTECH Chandigarh and M. tuberculosis H37 RV.

(iii) **Antibacterial activity:**

Antibacterial assay was conducted by Kirby-Bauer method [16]. The plant extract was taken and dissolved in DMSO with concentrations ranging from 0.5, 1 and 2.0 mg respectively. These strains were inoculated in sterile nutrient broth and incubated at 37^{o} C for overnight. The opacity of the organisms was compared with McFarland's turbidity standard 0.5 to make a dilution of 1.5×10^{8} cells [17]. Antibiotic controls for the tested organisms were also tested. Sterile nutrient agar plates were used for antimicrobial assay. Using sterile cotton swabs uniform lawns were prepared, different concentrations of plant extracts were loaded with careful attention, and plates were left for 30 minutes at room temperature, incubated at 37^{o} C for overnight and the experiments done in triplicates. The zones of inhibition were measured using a metric scale.

(iv) **Antimycobacterial activity:**

Initial screening was carried out using M. smegmatis which was procured from IMTECH Chandigarh, India. The bacteria was sub-cultured in nutrient broth and opacity was adjusted to McFarland's tube no 0.5 and different concentrations of the crude extract (0.5, 1 and 2.0 mg) were analyzed against the organism in triplicate and incubated at 37° C for overnight. Controls were used for the tested organism, and the zones of inhibitions were measured with metric scale. Then, it was further tested with M. tuberculosis H37RV strain.

(v) **Minimum inhibitory concentration against M. smegmatis:**

The broth dilution method was carried out in one ml with M. smegmatis (10^6 cells mL^{-1}) at different concentrations of test compound (250, 500, 750 and 1000 µg mL^{-1}) were done to find out MIC and incubated at 37° C. After overnight incubation, cells were centrifuged at 4000 RPM for 3 minutes. Then, they were suspended in 100 µL of nutrient broth, plated on nutrient agar plates and were incubated at 37° C for overnight. The plate with minimum concentration of the compound that showed no growth was considered as MIC.

(vi) **Minimum Inhibitory Concentration against M. tuberculosis:**

Approximately, 4to5 mg (one loopful) numbers of mycobacterial colonies diluted with sterile distilled water and made up to 5 mL. The opacity was adjusted to McFarland tube no 1.0 (equivalent to 10^7 to 10^8 cfu mL^{-1}). Further, two more log dilutions were made from the stock such as 10^{-2} and 10^{-4} using 3 mm calibrated wire loop, one loop full of culture was inoculated into each of labeled Lowenstein Jension tubes with careful attention and the tubes were incubated at 37° C for overnight.

(vii) **Luciferase Reporter Phage Assay against M. tuberculosis:**

Luciferase Reporter Phage Assay is a rapid and elegant test to find out the drug sensitivity of the compound against H37Rv strain and SHRE (Streptomycin, Isoniazed, Rifampicin and Ethambutol) resistant clinical isolate. The main stock of the plant compound was prepared with 10% DMSO so as to get 10 mg mL^{-1} stock solution. It was then filtered by using 0.45 µm solvent resistant filter. From the main stock two more dilutions namely 1 in 2 dilution and 1 in 10 dilutions were made. A thick suspension of the above mentioned strains were made in G7 H9 broth in a Bijou bottle which is equivalent to McFarland's opacity No 2.0 turbidity standard. Using 3 to 5 mm diameter glass beads a uniform suspension was made with G7H9 broth. The suspension was thoroughly mixed using a vortex mixer and the suspension was allowed to stand for few minutes. Then the suspension was transferred to another Bijou bottle. Four cryo-vials were arranged in cryo vial stand one for control another one for solvent control and third vial for 500 µg mL^{-1} and fourth vial for 100 µg mL^{-1}. 400 µL of G7H9 broth was transferred in the fourth vial and 350 µL into the remaining three vials. 50 µL of 10% sterile DMSO was added in the second vial (solvent control). Similarly, 50 µL of stock was added to the 3rd and 4th vials. 100 µL of M. tuberculosis H37Rv strain was added to all vials. Similarly, the test was repeated for SHRE resistant strains. All the vials were incubated at 37° C for 72 hours. After incubation 50 µL of phage phAETRC21 and 40 µL of 0.1 M $CaCl_2$ were added into all the vials. Further they were incubated at 37° C for 4 hours. The result is expressed in % reduction (in Relative Light Unit).

(viii) **Anticandidal activity:**

T. catappa was tested for anticandidal activity in Sabouraud dextrose agar plates. The organisms used were C. albicans, NCIM 3074, C. tropicalis NCIM 3118 C. krusei, C. glabrata, Cryptococcus neoformens and two more clinical isolates of C. albicans. These strains were inoculated into sterile Sabouraud dextrose broth and incubated at 37° C for overnight and the next day using sterile cotton swabs uniform lawns were prepared. The test was done by agar well method [18]. The concentrations of the plant extract were from 0.5, 1 and 2 mg. and the plates were incubated at 37° C. The zone of inhibition was measured using a metric scale.

(ix) **Phytochemical analysis:**

The plant extract was subjected to phytochemical analysis described by Brindha et al., (1981) [19] to find out the compounds present in it.

Test for alkaloids:

2 mL of hydrochloric acid was added to 0.5 mL of the plant extract. To this acidic medium, 1 mL of Dragendorff's reagent was added. These were mixed and allowed to stand till KNO_3 crystals out. An orange or red precipitate produced immediately indicates the presence of alkaloids.

Test for Tannins:

1 mL of the extract was taken in a test tube and then 1 mL of 0.008 M Potassium ferricyanide was added. 1 mL of 0.02 M ferric chloride containing 0.1 N hydrochloric acid was added and observed for bluish-black colour.

Test for Saponins:

The plant extract was mixed with 5 mL of distilled water in a test tube and it was shaken vigorously, and to this few drops of olive oil was added. The formation of stable foam indicates the presence of saponins.

Test for Flavonoids:

5 mL of dilute ammonia solution were added to a portion of the crude extract followed by addition of concentrated sulphuric acid. A yellow coloration observed in each extract indicated the presence of flavonoids. The yellow colour disappeared on standing.Test for amino acids:

2 mL of the plant extract was mixed with 1% ninhydrin in alcohol and tested for blue or violet colour development.

Test for Glycosides:

5 mL of the extract was treated with 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This was under layered with 1 mL of sulphuric acid. A brown ring at the interface indicates the presence of deoxy sugar characteristic of cardenolides.

Test for Phenols:

A small quantity of the extract was treated with 1% ferric chloride solution. Formation of green or purple, blue indicated the presence of phenol

(x) **Antioxidant Activity:**

Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay:

The DPPH radical scavenging activity was determined by the method described by Koleva et al., (2002) [20] 1.5 mL of 0.1 mM DPPH solution was mixed with 1.5 mL of various concentrations (10 µg to 500 µg mL^{-1}) of the plant extract. The mixture was shaken vigorously and incubated at room temperature for 30 minutes in the dark. The reduction of the DPPH free radical was Measured at 517 nm by a spectrophotometer. The solution without any extract and with DPPH and methanol was used as control. The experiment was done in triplicates. Gallic acid was used as positive control. Inhibition of DPPH free radical in percentage was calculated by the formula:

% Inhibition = [(A0-Ae)/A0]*100

Where Ao is the absorbance without sample, and Ae is absorbance with sample.

Determination of ferric reducing ability power:

The reducing power of the plant extract was determined according to the method of Oyaizu (1986) [21]. The extract (10- 100 µg) in 1 mL of distilled water was mixed with phosphate buffer [2.5 mL, 0.2 M pH 6.6] and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50° C for 20 minutes. 2.5 mL of 10% tri-cholro acetic acid was added to the mixture, and mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. Absorbance was measured at 700 nm using UV visible spectrophotometer (Shimadzu UV-2450)

% Inhibition = [(A0-Ae)/A0]*100

Where Ao is the absorbance without sample, and Ae is absorbance with sample. Ascorbic acid was used as positive control.

Determination of hydroxyl radical scavenging activity:

The effect of plant extract on hydroxyl radicals was measured by Fenton reaction described by Yu et al., (2004) [22]. The reaction mixture contained 60 µL of 1 mM $FeCl_2$, 90 µl of 1 mM 1,10 phenanthroline, 2.4 mL of 0.2 M sodium phosphate buffer (pH7.8), 150 µL of 0.17 M H_2O_2, 525 µL of H_2O,and 1.5 mL of sample solution (10-500 mg mL^{-1}in respective solvents). The reaction was started by the addition of H_2O_2. After

incubation at 37° C for 4 h, the reaction was stopped by adding 750 µL of 2.8% trichloro-acetic acid and 750 µL of 1% TBA in 50 mM sodium hydroxide, the solution was boiled for 10 minutes, and then cooled in water. The absorbance of the solution was measured at 560 nm. Ascorbic acid (0.05-0.250 mg mL^{-1}) was used as a positive control. The ability to scavenge the hydroxyl radical was calculated using the following equation:

% Inhibition = [(A0-Ae)/A0]*100

Where Ao is the absorbance without sample, and Ae is absorbance with sample.

Determination of superoxide radical scavenging activity:

Superoxide scavenging activity of the plant extract was determined by the nitroblue tetrazolium reduction method as described by Sabu and Ramadasan (2002) [23]. The reaction mixture consists of 1 mL of nitroblue tetrazolium (NBT) solution (l M NBT in 100 mM phosphate buffer, pH7.4), 1 mL NADH solution (l M NADH in 100 mM phosphate buffer, pH 7.4) and 0.1 mL of different fractions and ascorbic acid (50 mM phosphate buffer, pH 7.4) was mixed. The reaction was started by adding 100 µL of (PMS) solution (60 µL MPMS in100 mM phosphate buffer, pH 7.4) in the mixture. The tubes were uniformly illuminated with an incandescent visible light for 15 minutes and the absorbance was measured at 530 nm before and after the illumination. The percentage inhibition of superoxide generation was evaluated by comparing the absorbance values of the control and experimental tubes. The abilities to scavenge the superoxide radical were calculated by using the following formula:

% Inhibition = [(A0-Ae)/A0]*100

Where Ao is the absorbance without sample, and Ae is absorbance with sample

Determination of chelating activity on Fe^{2+}:

The chelation of ferrous ions was estimated by the method of Dinis et al., (1994) [24]. The extract was assessed for its ability to compete with ferrozine for iron (II) ions in free solution. Extract (10-250 mg mL^{-1}), 2.5 mL was added to a solution of 2 mM $FeCl_2.4H_2O$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL); the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured at 562 nm against the blank performed in the same way using $FeCl_2$ and water. Ascorbic acid (0.625-5 µg mL^{-1}) served as the positive control. The percentage of inhibition of ferrozine-Fe_2+ complex formation was calculated using the formula:

% Inhibition = [(A0-Ae)/A0]*100

Where Ao is the absorbance without sample, and Ae is absorbance with sample.

Determination of nitric oxide radical scavenging activity:

Nitric oxide was generated from sodium nitroprusside and measured by Griess reaction described by Green et al., (1982) [25]. Sodium nitroprusside 5 mM in phosphate buffer solution was incubated with different concentration (10-500 µg mL^{-1}) of extracts at 25° C for 5 hours. Control without extract but with equivalent amount of buffer was treated in a similar manner. After 5 hours, 0.5 mL of incubation solution with 0.5 mL of Griess reagent. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthylethylene diamine was read at 546 nm with UVvisible spectrophotometer (Shimadzu UV-2450).

The nitric oxide radical scavenging activity was calculated using the following formula:

% Inhibition = [(A0-Ae)/A0]*100

Where Ao is the absorbance without sample, and Ae is absorbance with sample.

Compound purification by column chromatography:

The column (height 78 cm; radius 1.2 cm) was packed with Sephadex R LH- 20 gel (mobile phase methanol). The mobile phase was methanol 100%. About 2 g of crude extract was loaded in the column. The eluents were collected and separated by thin layer chromatography. Around 20 fractions were collected and they were tested for antimycobacterial activity using M. smegmatis by disc diffusion method. Four fractions showed good activity. Among the four fractions (F2, F3 and F4) fractions were taken into consideration. All these fractions were tested for antimycobacterial activity at concentration of 250 µg mL^{-1}. Except F1, others showed good activity. Slurry was prepared by mixing the fractions with silica gel mesh 60-120. Then, it was again separated by column chromatography (height 40 cm; radius 2 cm) using

non polar to polar solvents, petroleum ether, dichloromethane, chloroform, ethyl acetate, and methanol). The fractions were collected and separated in TLC with solvents in a proportion of 8:2:2 (butanol: acetic acid: water). Among the ten fractions, F4 showed good activity against M. smegmatis and M. tuberculosis. This fraction was further analyzed by GC-MS studies.

Thin layer Chromatography:

Thin layer chromatographic plates were prepared by spreading the slurry uniformly on clean glass plates. The slurry was prepared by mixing the adsorbent, silica gel and water and the plates were dried and activated by incubating in an oven for thirty minutes at 120° C. The thickness of the adsorbent layer was typically around 0.10 to 0.25 mm for analytical purposes and around 0.5 – 2.0 mm for preparative TLC. The spots of samples were made by using capillary tubes. The mobile phase used was butanol: acetic acid: water in 8:2:2 proportion. The separated spots, were visualised by spraying with a mixture of 5% H_2SO_4 methanol, ninhydrin 200 mg dissolved 99 mL of acetone and l mL of acetic acid used and finally kept in UV illumination meter (Genei, India) (300 nm).

Gas Chromatography-Mass spectrometry GC-MS analysis:

The operating conditions of Thermo Scientific Ultra GC with following conditions Column DB 35 MS, Length 30 meter, breath 0.25 mm, Detector- FID-250° C ,Oven temperature- 85° C. Carrier gas Helium, Injection of sample 1 µL, Time- 35.31 minutes

Result:

(i)Antibacterial activity:

The plant extract was tested for its antibacterial activity with three different concentrations against many of the gram negative bacteria, and few of the gram positive bacteria. The result showed that the plant extract has promising activity against E. coli, P. mirabilis, P. aeruginosa, S. typhi and V. cholerae but less active against Klebsiella. The concentrations of 1 and 2 mg of extract showed promising activity than 0.5 mg concentration. The activity is more or less same with 1 mg and 2 mg than with 0.5 mg concentration. In the same way the plant extract showed good activity against gram positive bacteria such as S. aureus and Methycillin resistant S. aureus but less activity with B. subtilis. S. aureus showed good activity with all three concentrations but B. subtilis showed good activity with 1 and 2 mg than with 0.5 mg concentration which is shown in (Table 1).

Table 1 Antibacterial activity of *T. catappa*

Organism	Zone of inhibition (mm) Concentration in (mg mL^{-1})		
	0.5	1.0	2.0
E. coli	12	15	20
Klebsiella	3	5	6
Proteus mirabilis	9	15	20
Pseudomonas aeruginosa	10	13	15
Salmonella typhi	7	10	15
Vibrio cholerae	20	22	20
Bacillus subtilis	6	10	15
Staphylococcus aureus	15	15	15
Methycillin resistant Staphylococcus aureus	18	20	20

(ii) Antimycobacterial activity:

The plant extract was tested for its antimycobacterial activity against M. smegmatis with three different concentrations namely 0.5, 1 and 2 mg. Here, all three concentrations showed promising activity. The MIC at 500 µg mL^{-1} concentration the extract showed good inhibitory activity against the organism. Similarly the extract was tested for its activity with M. tuberculosis H37RV strain by MIC method showed good inhibitory activity at 1280 µg mL^{-1} concentration which is shown in Table 2.

Table 2 Antimycobacterial activity of T. catappa:

S.No.	Bottle	Concentration (µg mL-1)	Result
1	1	L.J slant without drug	2+
2	2	10	2+
3	3	20	1+
4	4	40	1+
5	5	80	28
6	6	160	28
7	7	320	24
8	8	640	20
9	9	1280	0

MIC: 1280µg mL-1

(iii) Luciferase reporter phage assay:

The plant extract was tested for its antimycobacterial activity by this method which is an elegant and rapid method to find out the sensitivity of the compound against H37RV and SHRE (Streptomycin,Isonized, Rifampicin and Ethambutol) resistant clinical strains .With H37RV strain the extract doesn't show any activity, but when tested with SHRE resistant strain the plant extract showed 42.29 % of reduction when tested with100 µg mL^{-1}, but no activity with 50 µg mL^{-1}. Here the result is expressed in percentage of reduction in relative light units. Result is shown in Table 3.

Table 3 Luciferase Reporter Phage Assay

Test Organism: S, H, R&E resistant *M. tuberculosis*

Name of the plant	**% Reduction in RLU**	
Terminalia catappa	50 µg mL-1	100 µg mL-1
	0	42.29
Control Isoniazid(0.2µg mL-1	33.25	33.25
Test Organism: H37RV strain of *M. tuberculosis*		
Name of the plant	**% Reduction in RLU**	
Terminalia catappa	50 µg mL-1	100 µg mL-1
	0	0
Control Isoniazid(0.2 µg mL-1)	81.46	81.46

(iv) Anticandidal activity:

The plant extract was tested for its anticandidal activity with three different concentrations namely 0.5, 1 and 2 mg against many of the candidal strains namely C. albicans, C. tropicalis, C. krucei, C. glabrata and two clinical strains of C. albicans showed good activity. Here all the three concentrations showed good activity with the extract except C. tropicalis which showed good activity with 1 mg and 2 mg concentrations only. In the case of C. neoformans lesser activity was shown when compared with other candidal strains which are shown in Table 4.

Table 4 Anticandidal activity of T. catappa

Organism	Zone of inhibition (mm)		
	Concentration in mg mL^{-1}well		
	0.5	1	2.0
C. albicans	12	8	18
C. tropicalis	7	10	10
C. krucei	15	15	20
C.glabarata	17	19	20
Cryptococcus neoformens	5	5	8
C. albicans CLS1	12	13	12
C. albicans CLS 2	13	15	17

CLS Clinical strain

(v) Phytochemical analysis:

The phytochemical analysis of the plant extract showed the presence of alkaloids, glycosides, saponins, phenols, tannins, flavonoids and amino acids Table 5.

Table 5 Phytochemical analysis:

S.No	Phytochemicals	Extract
1	Alkaloids	+
3	Glycosides	+
4	Saponins	+
5	Phenols	+
6	Tannins	+
7	Flavonoids	+
8	Aminoacids	+

(vi) Antioxidant studies:

DPPH Scavenging activity:

DPPH method is a simple, rapid, sensitive and reproducible assay used for evaluating the antioxidant activity of plant extract. Here the DPPH scavenging ability of the extract is nearer to that of the control. Here the plant extract ranging from 10 to 500 µg mL^{-1} is used. The IC_{50} value of the plant extract is 42 µg mL^{-1} and for Gallic acid which is used as a control, the scavenging ability is 50.46 µg mL^{-1}.

FRAP Assay:

The reducing power of the plant extract was evaluated by FRAP assay which is an easy, inexpensive and reproducible assay for antioxidant activity. Here ascorbic acid is used as positive control. The extract ranging from 10 to 100 µg is used here. The ferrous reducing power of the plant extract is 54.9 µg mL^{-1} for the positive control is 34.3 µg $mL^{-1.}$ The reducing power of the plant extract in this test is significantly higher than that of the control.

Hydroxyl radical scavenging activity:

The hydroxyl radical scavenging assay is a relevant one. Here the 10 to 500 mg mL^{-1} concentration of the extract is used here. Ascorbic acid is used as positive control. The scavenging ability of the plant extract is 96.6 µg mL^{-1} and for the control it is 35 µg $mL^{-1.}$ The radical scavenging ability of the plant extract is significantly higher than that of the control.

Superoxide radical scavenging activity:

The superoxide radical scavenging assay is more relevant than the other methods described here. The scavenging activity is significantly higher than that of the positive control. The IC 50 value is 59 µg mL^{-1} whereas for the control the value is 30.5 µg mL^{-1}.

Iron chelating activity:

Here in this experiment ferrozoine quantitatively forms complexes with Fe^{2+}. Ascorbic acid is used as positive control. Different concentrations of the plant extract ranging from 10-250 mg mL^{-1} are used here. The chelating ability of the plant extract is 62 µg mL^{-1}

and for the positive control it is 35 µg mL^{-1}. Here also the chelating ability of the extract is significantly higher than the control.

Nitric oxide radical scavenging activity:

Here the plant extract was evaluated against the scavenging activity of nitric oxide. Different concentrations ranging from 10-500 µg mL^{-1} were used. The radical scavenging activity of the plant extract is 89 µg mL^{-1} and for the positive control it is 69.7 µg mL^{-1}. Here also the scavenging activity is significantly higher than the control.

Compound purification:

The compound was purified by column chromatography followed by thin layer chromatography and was further analysed by GC-MS. From the GC-MS result of F4 fraction compounds namely 1-Hexadecen, 1-Heneicosanol, Heptadecane, 2, 6, 10, 15-tetramethyl and 1-Nonadecene showed antituberculous activity and most of the remaining compounds showed antimicrobial and antioxidant activities.

DISCUSSION

Terminalia catappa is naturally widespread in tropical and subtropical zones of Indian and Pacific and planted extensively throughout the tropics. The leaves of Combretaceae family are widely used as a folk medicine is Asia. A lot of pharmacological studies have been reported that the leaves and fruits of the plant have anticancer, antioxidant, anti-Hiv reverse transcriptase, anti-inflammatory, antidiabetic and hepatoprotective activities. The juice of the leaves is used externally to treat scabies and leprosy and internally to cure colic and headache. In India leaf bark and fruit have long been used as folk medicine for antidiarrheic, antipyretic and homeostatic purposes. Phytochemical study of the plant showed the presence of alkaloids, glycosides, saponins, phenols, tannins, amino acids, reducing sugars and steroids. Earlier studies made on T. catappa leaves also showed the presence of the same phytoconstituents [26,27]. These bioactive compounds are usually responsible for the medicinal properties of the plants which are helpful to treat different diseases. Some of the important phytoconstituents are tannins (terflavin, tergallagin), flavanoids (rutin, quercitin) and triterpenoids [28,29]. Punicalin and punicalagin which are isolated from T. catappa are used as anti-Aids compounds [30,31]. Tannin and flavanoid glycosides exhibit significant free radical scavenging effect [32,33]. The plant showed promising antimicrobial, antifungal [34,35] activities. Ethanolic leaf extract of T. catappa showed anti-inflammatory effect on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema in both acute and chronic animal models [36]. Tender leaves of the plant also showed analgesic effect [37]. Apart from this it is shown that the alkaloid extract of the leaves of the plant showed promising antimalarial activity [38]. Alkaloids have been associated with medicinal uses for centuries and one of its common biological properties is their cytotoxicity [39]. Several workers have reported analgesic, [40] antioxidant, [41] antispasmodic and antibacterial activities [42] of alkaloids. Glycosides are known to lower the blood pressure according to many reports [43]. Saponins have the property of precipitating and coagulating red blood cells. Some of the characteristics of saponins include the formation of foams in aquous solutions, haemolytic activity and cholesterol binding properties [44]. Saponins are known to produce inhibitory effect on inflammation [45]. Phenolic compounds possess biological properties such as antiaging, anticarcinogenic, anti-inflammatory and cardiovascular protection and improvement of endothelial function as well as inhibition of angiogenesis and cell proliferation activities. Several studies have described the antioxidant properties of medicinal properties which are rich in phenolic compounds. Natural antioxidants mainly come from plants in the form of phenolic compounds which are in the form of flavanoids and phenolic acids etc. Tannins bind to prolin rich protein and interfere with protein synthesis. Flavanoids are hydroxylated phenolic substances known to be synthesized by plants in response to microbial infection and they have been found to be antimicrobial substances against a wide array of microorganisms in vitro. Their activities are probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall. They also have effective

antioxidant and strong anticancer activities. [46,47,48].

The Gc-Ms study of the plant extract indicated the presence of twenty nine compounds among which four major compounds namely 1-Hexadecene, 1-Heneicosanol, 1-Nonadecene, and Heptadecane 2, 6,10,15 tetramethyl showed antituberculous activity along with other pharmacological activities. [49,50,51,52]. 1-Hexadecene is an alkene having antimicrobial, antifungal, antioxidant activitites along with antitubercular activity [53,54,55,56,57]. 1-Heneicosanol and Heptadecane 2, 6,10,15 tetramethyl are compounds which showed antitubercular activity along with other pharmacological activities [58,59]. 1-Nonadecene is a long chain fatty acid showed antitubercular, antifungal, antimicrobial and antioxidant activitites along with it also acting as a neutraceutical and flavouring agent [60,61]. Majority of the remaining compounds showed antioxidant, antimicrobial activity and many other biological activities. Glycerol 1, 2-diacetate is used as a carrier solvent, topical antifungal agent for superficial mycoses, used as food additive and is used in preparation of perfumery and cosmetics [62]. Phthalic acid, butyl tetradecyl ester is pthalic acid derivative and is a plasticizer compound showed antitumour, anti-inflammatory and antimicrobial activity [63, 64,65]. Dibutyl phthalate is a bioactive ester produced by bacteria, fungi, algae and also used as antifungal and antibacterial agent [66, 67, 68,69]. It is PH and thermo tolerant antimetabolite of proline [70]. It eliminates tumour cells on bone marrow, and acts as a purging agent in autologous bone marrow transplantation, stimulates adipogenesis and glyceroneogenesis, affects the differentiation of Human Liposarcoma [71]. Phthalic acid butyl undecyl ester is an alcoholic compound used to cure cardiovascular and cerebrovascular diseases, and is anti-inflammatory and antibacterial agent [72]. Apart from this it acts as Cathepsin B inhibitors. Hoang et al isolated two cathepsin B inhibitors from the culture supernatant of marine Pseudomonas species PB01[73].The inhibitors were identifed as dibutyl phthalate and di-(2-ethylhexyl) phthalate, which exhibit dose-dependent cathepsin B with IC_{50} values of 0.42 and 0.38 mM, respectively. The substances inactivate the pericellular cathepsin B of murine melanoma cells. Cathepsin B (EC3.4.22.1), which belongs to the papain superfamily, is a cysteine proteinase with a cysteine residue in its active site. This enzyme promotes the growth, invasion, and metastasis of cancer cells by catalyzing the degradation of the interstitial matrix and basement membranes; this allows cancer cells to invade locally and to metastasize. Cathepsin B also plays an important role in a variety of pathologies, including inXammation, pancreatitis, osteoarthritis, tumor angiogenesis, apoptosis, and neuronal diseases [74,75,76,77,78,79,80]. In addition, this enzyme markedly enhances infection by the Ebola virus by converting the 130-kDa viral glyco protein GP1 to a 19-kDa species [81]. Because of the role of cathepsin B in disease development, including cancer cell proliferation and virus infection, studies of cathepsin B inhibitors from marine isolates of Pseudomonas should be intensified. Apart from the above functions, Dibutyl phthalate is also helps in proteolytic and antiproteolytic balance [82]. 5-Methyl-1-phenylbicyclo [3.2.0] heptane is used as anti HIV agent, used as nucleoside reverse transcriptase and non nucleoside reverse transcriptase inhibitors and is also used as protease inhibitors [83,84]. 10-Nonadecanone is a ketone, showed good anticancerous, antimicrobial and antioxidant activity [85, 86]. The plant extract of T. catappa when tested with 1 mg and 2 mg concentration is susceptible to gram negative bacilli like E. coli, P. mirabilis, P. aeruginosa, S. typhi and V. cholera and resistant to Klebsiella. But when tested with 0.5 mg concentration of the extract P. mirabilis and S. typhi showed resistant activity. Similarly, the plant extract showed good activity with S. aureus and methycillin resistant S. aureus with all three concentrations. But B. subtilis showed resistance with 0.5 mg concentration and significant activity with 1 and 2 mg concentrations. Regarding anticandidal activity the extract showed significant activity with C. albicans, C. tropicalis, C. krucei, C. glabrata and two more clinical strains of C. albicans. Apart from this, the extract showed lesser activity with C. neoformens. In general in Combretaceae family species like T. catappa, T. bellerica showed good antibacterial activity. T.

chebula is used as customery traditional medicine used by villages and tribals of many states in India including fever, cough diorrhoea, gastroenteritidis, skin diseases, candidiasis [87,88] and antifungal diseases [89] antiviral and anticarcinogenic activities [90]. Similar results were shown by [91,92,93] T. catappa showed significant antimycobacterial activity with both M. smegmatis and H37RV strain of M. tuberculosis by both and minimum inhibitory concentration methods. This was further confirmed by Luciferace reporter phage assay which is a faster, reliable and elegant test. In this test, the extract showed good activity against SHRE resistant clinical strains of M. tuberculosis but showed no activity with H37RV strain. If the active compound will further be purified and tested for its activity it may show improved activity. Hence this extract can be used in single or in combination with other TB drugs.

Similarly T. chebula showed potent antioxidant activity and it is also a probable radio protector [94]. The antioxidant studies and reducing power of the compound was done by many experiments in this study. The DPPH method is one of the most widely used chemical methods to determine antioxidant capacity because it is considered to be practical, fast, and stable [95]. The DPPH radical scavenging effect of the extract is significant. Here the IC_{50} value of the plant extract is lower than Gallic acid which means the antioxidant activity is significantly higher. Thus, the plant T. catappa has potent antioxidant activity which serves as a source for the bio-chemo therapy. The FRAP assay is a direct assay which measures the reducing ability of the plant. Here the IC_{50} value of the plant extract is nearly double to that of the ascorbic acid which is acting as a control. This also indicates the higher amount of phenolic and flavonoid content present in the plant extract. The higher the presence of phenolic content in the compound indicates the presence of higher antioxidant activity of the plant [96]. The hydroxyl radical scavenging activity also indicates that the plant shows good antioxidant activity where the scavenging activity of the plant extract is double than ascorbic acid which serves as control. Regarding superoxide radical scavenging activity the IC_{50} value is double when compared with ascorbic acid which is used as the control. Similarly the iron chelating activity is also more or less double to that of the ascorbic acid. In the case of nitric oxide scavenging activity the IC_{50} value of the plant is considerably higher than the control. Hence, as all the antioxidant tests conducted here proved that the plant Terminalia catappa showed good antioxidant activity which can be used an alternate source against natural and oxidative illness. In total, the antioxidant activity and presence of phenolic and flavonoid contents of Terminalia catappa indicated that the plant can be used for therapeutic and industrial activities. From the results obtained from the above experiments, it is concluded that there are many potential compounds isolated from plant Terminalia catappa which can be used for therapeutic and industrial purposes.

Conflict of interest statement

We declare that we have no conflict of interest.

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