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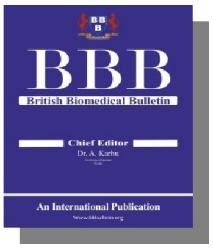
BRITISH BIOMEDICAL BULLETIN



Original

Cytotoxic and Radical Scavenging Potential of Indian Almond (*Terminalia catappa)* Leaf Extracts

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A R T I C L E I N F O

Received 27 Dec. 2013
Received in revised form 02 Jan. 2013
Accepted 04 Jan. 2013

Keywords:
Antioxidant; 2,
2-diphenyl -1- picryl hydrazyl (DPPH);
Ferric reducing antioxidant power (FRAP);
TPTZ,
Cytotoxic,
Brine shrimp.

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A B S T R A C T

Solvent leaf extracts of *Terminalia catappa* were evaluated for their cytotoxic and their radical scavenging potential. Brine shrimp assay was used for exploring cytotoxic activity, all the extracts namely hexane, DCM, chloroform, acetone and methanol were tested at the dose level of 25, 50, 100 and 200microgram /ml. A dose dependent response was obtained in all the extracts. Chloroform extract showed 100percent activity at the highest dose of 200µg/ml. Antioxidant activity was evaluated by one qualitative(TLC based) and two quantitative radical scavenging assays namely DPPH (2, 2- Diphenyl-1- picryl hydrazyl) assay and ferric reducing antioxidant power (FRAP) assay. In both assays acetone extract exhibited significant reduction of free radicals. The IC_{50} value of acetone extract was 36.9µg/ml. Ascorbic acid was used in these assays as reference compound. FRAP value of acetone extract was found to be 6.73 much higher than all the other extracts.

Introduction

Combretaceae family is well known for its medicinally important tree species like *Terminalia arjuna*, *T. bellerica* and *T. chebula*[9]. The leaves and bark of this plant is used for various medicinal purposes as it is rich in various chemical compounds like flavonoids, tannins, saponins and phytosterols[22]. In folk remedies the leaves of the plant are used for dressing of rheumatic joints, treat cough, asthma, dysentery, diarrhea, gonorrhea and skin ailments including scabies[12].

Medicinal plants have great importance in formulation of medicines due to enriched quality of phytochemicals produced by them as the byproduct of secondary metabolism. Antioxidants play important role in body defense system against reactive oxygen species (ROS) as they combine with reactive oxygen species and null their toxic effect[11]. Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide and other exogenous factors are generally the cause of several fatal diseases such as coronary heart disease, stroke, rheumatoid arthritis, diabetes and cancer[24]. Thus, any plant possessing antioxidant activity could be a potential lead for curing any of the above ailments. Cytotoxic activity is a good marker for detecting antibacterial, antimicrobial and parasitic leads from natural products. Brine shrimp assay has a good correlation with anti inflammatory and anticancer activity[16]; same has been used to depict the cytotoxic potential of *Terminalia catappa's* leaf extracts. Present study aims at exploring the cytotoxic and antioxidant potential of *Terminalia catappa* leaves.

Materials and Methods

Plant collection and extract preparation

Leaves were collected from medicinal germplasm garden of Regional plant resource centre, Bhubaneswar, Odisha. Leaves were thoroughly washed and dried in shade. Dried leaves were made into fine powder using Remi mixture grinder. Leaf sample was extracted using successive solvent extraction on polarity basis starting from hexane to methylene dichloride, chloroform, acetone and methanol using Soxhlet method. Extracts were concentrated under vacuum using Buchi rotavapour. The dried extracts thus obtained were stored in airtight screw cap vials till further use. Same were used for cytotoxic and radical scavenging assays.

Chemicals

All basic chemicals and solvents were of analytical grade. DPPH$^{\cdot}$ (1,1-Diphenyl-2-picrylhydrazyl radical), TPTZ (2,4,6-Tripyridyl-s-triazine) and Ferrous sulphate heptahydrate were procured from Hi-media, and solvents (n-hexane, chloroform, dichloromethane, acetone, methanol, benzene, ethyl acetate) were purchased from Spectrochem. Ferric chloride hexahydrate was purchased from S.D. fine chemicals. Ascorbic acid from Sigma chemicals Silica gel 60 F254 and sodium acetate trihydrate were purchased from Merck.

Biological evaluation

Extracts of both the species were subjected to following tests:

1. Cytotoxic activity using brine shrimp lethality assay[19]
2. Qualitative antioxidant activity by thin layered chromatography based DPPH assay[13]
3. Quantitative antioxidant activity using radical scavenging DPPH[14] and FRAP Assays[6] for both the tests Ascorbic acid was used as standard antioxidant.

Cytotoxic activity

Brine shrimp eggs were incubated in 1.8% saline for 24 hrs. Solvent extracts were tested at the doses of 25 to 200microgram/ml. After 24 hours live larvae were counted and

compared with the experimental control. Statistical analysis of all the tests was conducted using Student's T test and level of significance was ascertained for all the tests.

TLC based antioxidant activity

Qualitative testing of antioxidant activity was done using the standard protocols[13]. TLC was conducted using three solvent systems namely EMW (ethyl acetate: methanol: water in the ratio of 40.5:4:4), CEF (Chloroform: ethyl acetate: formic acid in the ratio of 5:4:1) and BEA (Benzene:ethanol: ammonium hydroxide in the ratio of 90:10:1). After running of the plate up to 8cms they were dried and were sprayed with 0.2% 2,2, diphenyl-1-picryl-hydrazyl. Antioxidant bands were detected as yellow spots against a purple background.

Radical scavenging DPPH assay

Radical scavenging DPPH assay were conducted using the standard protocols[13.] 1.0mM solution of DPPH in methanol was prepared and 500µl of this solution was added to 4.0ml of extract solution (hexane, DCM, chloroform, acetone & methanol) in methanol at different concentrations (9.8 - 1250µg/ml). After 30 minutes incubation, the absorbance was measured at 517nm. Ascorbic acid at various concentrations (9.8 - 1250µg/ml) was used as reference compound.

Lower the absorbance of reaction mixture indicates higher free radical scavenging activity. The capability to scavenge DPPH free radical was calculated using the following equation.

Percentage of Inhibition (%) = Acontrol – Atest / Acontrol X 100

Where *Acontrol* is the absorbance of control reaction and *A test* is the absorbance of sample extracts. The antioxidant activity of the leaf extract was expressed as IC_{50} and compared with standard. The IC_{50} value was defined as the concentration (in µg/ml) of extracts that scavenges the DPPH radicals by 50%. IC50 of the extracts was compared with the standard antioxidant ascorbic acid.

FRAP assay

Antioxidant activity was analyzed by Ferric reducing antioxidant power assay as per the protocol of Ahmed and Beigh[2]. FRAP assay was performed based on the reduction of Fe^{3+} – TPTZ to a blue coloured Fe^{2+} – TPTZ. The FRAP reagent was prepared by mixing 300mM acetate buffer (pH 3.6), 10mM TPTZ and 20mM $FeCl_3$. $6H_2O$ in a ratio of 10: 1: 1, at 37°C. Different concentrations (0.625 – 10mg/ml) of sample extract were prepared with methanol. A total of 100µl of sample and 300µl of methanol was pipetted in to test tubes. FRAP reagent (3ml) was added to the same test tubes and incubated at 37°c for 4 min. Ascorbic acid was used as standard antioxidant compound for FRAP assay. $FeSO_4$.$7H_2O$ was prepared (0.1–1.0mM) and tested using same procedure for plotting standard calibration curve. Absorbance was measured at 593nm.FRAP value was calculated according to the equation[13] The FRAP values thus obtained were described as mmol/g Fe(II) equivalents.

FRAP (mM) = 0-4 min of ΔA593 nm of test sample / 0-4 min of ΔA593 nm of standard sample × [standard] mM

Three replicates of each sample were taken and data was analyzed and significance was ascertained.

Results and Discussion

Cytotoxic assay

The brine shrimp lethality assay represents a simple and result oriented bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and anti-tumor properties [16].Besides same assay has been used along with other antimicrobial assays for biological

assessment of medicinal plants[19]. A number of novel compounds have been discovered as lead using brine shrimp assay guided fractionation, some of these are 2,4-thiazolidinediones[3],diaryl sulfonyl ureas etc[4].

A thorough assessment of medicinal plant is possible if all the extracts ranging from non polar to polar extracts are explored for depicting its biological activity which does not leave any scope of leaving potential lead present in the plant, so in this study five extracts namely hexane, DCM, chloroform, acetone and methanol extracts were tested for both the activities. Activity of all the extracts ranged from 14-100%, with maximum activity in polar extracts chloroform to methanol.

The highest percentage lethality was obtained in chloroform extract i.e. 100% at 200µg/ml. The percentage lethality was maximum in higher dose i.e. at 200µg/ml for each extract, All the extracts showed dose dependent response (Fig 1). This study is in confirmation with Pawar and Pal[21] where chloroform extract along with methanol extract showed good antimicrobial activity against five micro organisms, same trend was here as methanol extract also showed an inhibition of >70% at the highest dose. As chloroform extract has shown very good cytotoxic activity and same has been reported in antimicrobial activity assay, needs elaborate exploration for the isolation of potent molecules. Other species namely *Terminalia glaucescens* has also shown antimicrobial properties[1], this could be due to the presence of cytotoxic potential of the genus as shown in the present study. Micorbes are unicellular organism, any molecule active against them is bound to be cytotoxic in nature, Combrataceae family is known for its antimicrobial potential[7], this study has strengthen the use of *Terminalia catappa* leaf extracts. A very significant cytotoxic potential of chloroform extract has paved the way for further investigations.

Antioxidant assays

A number of assays have been used to test for the antioxidant activity but most widely used methods are radical scavenging assay as good antioxidant is one which has the capacity to combine with the free radical species generated by the metabolic processes to render them neutral and reduce their toxic effect[14].

Qualitative DPPH assay on TLC

The DPPH method measures electron-donating activity of other compounds in the mixture and hence provides an evaluation of antioxidant activity due to free radical scavenging. Any molecule that can donate an electron or hydrogen to a mixture will react with and bleach DPPH. DPPH is reduced from a purple compound to a light yellow compound. Hexane, chloroform and dichloromethane extracts of *T. catappa* leaf showed fewer bands in comparison with the more polar solvents namely acetone and methanol (Table 1). Same assay has been used for the isolation of antibacterial compounds by Kotze and Eloff[13] based on their antioxidant profile. All the three solvents showed good amount of separation. Maximum numbers of bands were obtained in acetone extract in CEF solvent.

DPPH free radical scavenging assay (Quantitative)

The DPPH free radical has been used to test the potential of compounds as free radical scavengers of hydrogen donors and to investigate the antioxidant activity of plant extracts[11]. The reduction of DPPH radical was determined by decrease in its absorbance induced by antioxidants at 517nm. Concentration of sample at which the inhibition percentage reaches 50% is its IC_{50} value. The lower the IC_{50} value, the higher is the antioxidant activity of the tested sample. In the present work, five solvent extracts of leaf sample of *T. catappa* L. along with

ascorbic acid (Standard) were evaluated for their DPPH free radical scavenging activity. All the five extracts investigated showed varied levels of DPPH scavenging activity. Ascorbic acid standard showed IC_{50} value in between 19.53 and 39.06µgml^{-1} and its IC_{50} was 29.7µgml^{-1}. Among all the five extracts, acetone and methanol extracts showed good DPPH radical scavenging activity (Fig 3). The IC_{50} value of acetone extract ranged between 19.53 and 39.06µg ml^{-1} and its IC_{50} value was 36.9µg ml^{-1}. The IC_{50} value of methanol extract ranged between 39.06 and 78.125µgml^{-1} and its IC_{50} value was 39.2µg ml^{-1} (Fig 3). The acetone extract showed better DPPH free radical scavenging activity than methanol and other extracts showed IC_{50} value greater than 1250µg ml^{-1}. DPPH free radical scavenging activity increased in increasing order of polarity of the solvent extracts (Fig 2). Thus, qualitative and quantitative tests indicated towards the similar trend, which suggested that antioxidant potential of polar extracts is far better than the non polar ones. This study is in confirmation with an earlier study in which petroleum ether which is a non polar was found lacking in antimicrobial potential whereas chloroform and methanol extracts were potent antimicrobials[21].

Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power assay was used to evaluate the antioxidant potential of different solvent extracts of *T. catappa* leaf along with reference antioxidant compound ascorbic acid. Principally, FRAP assay treats the antioxidants in the sample as reductants in a redox-linked colorimetric reaction. This assay is relatively simple and easy to conduct. FRAP assay measures the reducing potential of antioxidant to react on ferric tripyridyltriazine (Fe^{+3}-TPTZ) complex and produce blue colour of ferrous form [5] which can be detected at absorbance 593 nm. Antioxidant compounds which act as reducing agent exert their effect by donating hydrogen atom to ferric complex and thus break the radical chain reaction. The higher the absorbance, the higher is the antioxidant activity which is indicated by the high FRAP value.

In this present study, the highest FRAP value of 6.73 ± 0.12 was obtained from *T. catappa* leaf acetone extract while lower value of 2.51 ± 0.007 was obtained from hexane extract (Fig 3). The antioxidant potential of *T. catappa* leaf extracts were in the increasing order of chloroform < hexane < DCM < methanol < acetone (Fig 3). In the FRAP assay a linear increase in reducing power was observed over the concentration range 0.625 – 10 mg/ml sample and ascorbic acid (Fig 3). The acetone and methanolic extract has potent reducing power and equivalent to ascorbic acid.

The leaves of the *T. catappa* species are known for their pharmacological activities as they contain many phytochemical which can act as antioxidants. In this paper we have shown that various solvents extracted antioxidant compounds from the leaves of plant belong to *T. catappa* species. Acetone and methanol extracted the highest number of antioxidant compounds based on DPPH – TLC analysis. Qualitative DPPH assay on TLC was successfully used in this study to systematically assess the total antioxidant activity of the *T. catappa* leaf extracts. The quantitative DPPH free radical scavenging assay gave comparable results between all extracts of the *T. catappa* plant. The results showed that acetone and methanol extracts were both potential DPPH free radical scavengers and can be comparable to pure antioxidant compound i.e. ascorbic acid. The FRAP assay also gave the result which showed acetone extract of *T. catappa* leaf has highest FRAP value than other extracts and can be comparable with ascorbic acid. The graph of FRAP assay also enlighten the fact

that antioxidants present in the extracts of *T. catappa* were good reducers of free radicals. Overall results have produced leads in the form of chloroform extract as cytotoxic candidate, whereas acetone and methanol extracts were found to be the most potent candidates for the isolation of antioxidant principles.

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Table 1. Antioxidant bands obtained in solvent extracts of Terminalia catappa leaf using DPPH assay

Solvents	*T. catappa* leaf Extracts (Number of antioxidant bands)				
	Hexane	DCM	Chloroform	Acetone	Methanol
EMW(ethyl acetate: methanol: water 40.5:4:4)	1	3	2	Complete streak from Rf 0-100	Complete streak from Rf 0-100
CEF(Chloroform: ethyl acetate: formic acid 5:4:1)	2	6	2	10	4
BEA(Benzene:ethanol: ammonium hydroxide 90:10:1)	4	6	6	3	0

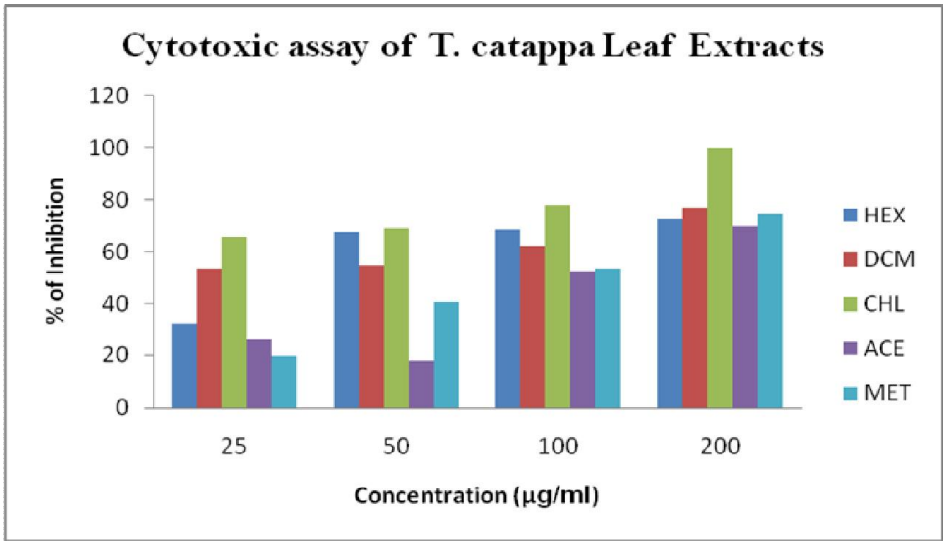

Figure 1. Brine-shrimp motility assay of leaf extracts of *Terminalia catappa*

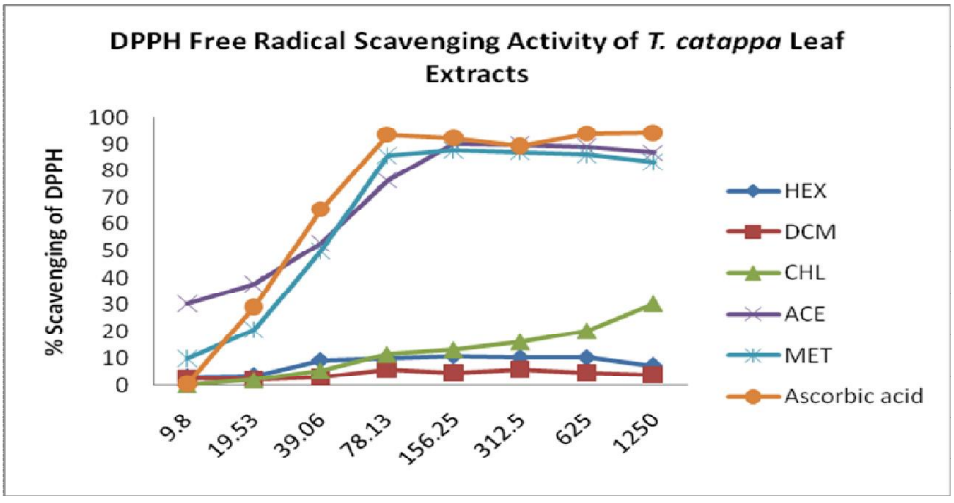

Figure 2. Antioxidant assay (DPPH)

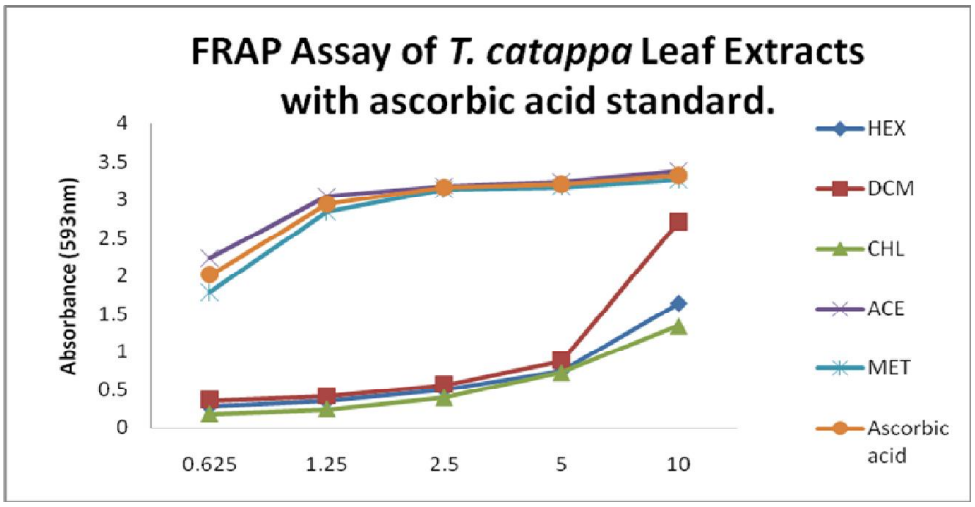

Figure 3. Antioxidant assay (FRAP)