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Original Research Article

In Vitro Antioxidant and Antibacterial Activity of Methanol Leaf Extract and Fractions of Terminalia Catappa L

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Abstract

Medicinal plants have played a key role in world health. Plants and their products have been used since time immemorial as principal ingredients of various traditional medicines. It has been the mainstay of many traditional cultures for their low cost and wide accessibility in addition to their preventive and therapeutic value. The aim of this research was to evaluate the antioxidant and antibacterial activity of *T. catappa* methanolic leaf extract and its fractions. The antioxidant activity was determined using DPPH radical scavenging activity using varied concentrations (31.25-500µg/ml) of different extracts and fractions then 100µl of purified water at absorbance of 515nm using ELISA plate reader. Ascorbic acid was used as a standard. The antibacterial activity was evaluated by determining the MIC and MBC of different fractions at different concentration. The results of the study indicated the presence of major phyto-compounds like alkaloids, steroids, phenolics and saponins. It also showed that the ethyl acetate and n-butanol fractions of the leaf of *T. Catappa* possess the highest significant scavenging activity against $DPPH^{\bullet}$ (64.02% at 250µg/ml and 62.43% at 500 µg/ml respectively). Moreover, total phenolics concentration equivalent to gallic acid was found to be up to 70 mg/g for the ethyl acetate fraction, which correlated with antioxidant activity. The result of the antibacterial study showed evidence that, the methanol leaf extract of *T. Catappa* possess antibacterial activity and N. butanol portion has the highest activity. In conclusion this study showed that the methanol extract and fractions of the leaf of *T. Catappa* have demonstrated significant antioxidant and antibacterial activities but further study is recommended to ascertain the compounds responsible for their antioxidant activity.

Key words: *Terminalia catappa*, fractions, antioxidant, antibacterial, MIC, MBC.

INTRODUCTION

Plants and their products have been used since time immemorial as principal ingredients of various traditional medicines and have played a key role in global health improvement. Plants have also been the mainstay of many traditional cultures for their low cost and wide accessibility in addition to their preventive and therapeutic value. Currently, interest in the medicinal use of plants has increased dramatically. The World Health Organization (WHO) reported that approximately 75-80% of the World's population uses plant medicines either in part or entirely [1].

Recently, antioxidants have attracted considerable attention in relation to radicals and oxidative stress, cancer prophylaxis and therapy, and longevity. Phenols and polyphenols are the target analytes in many such cases; they may be detected by enzymes like tyrosinase or other phenol oxidases, or even by plant tissues containing these enzymes [2]. The recommendations based on epidemiological studies are such that fruits, vegetables and less processed staple foods ensure the best protection against the development of diseases caused by oxidative stress, such as cancer, coronary heart disease, obesity, type 2 diabetes, hypertension and cataract. The explanation consists in the beneficial health effect, due to antioxidants present in fruits and vegetables. There are numerous antioxidants in dietary plants: carotenoids, phenolic compounds, benzoic acid derivatives, flavonoids, proanthocyanidins, stilbenes, coumarins, lignans, and lignins[3].

Bacterial infection is a proliferation of a harmful strain of bacteria on or inside the body. Bacteria can infect any area of the body. Pneumoma, meningitis and food poisoning are just a few illnesses that may be caused by harmful bacteria.

MATERIALS AND METHOD

Chemicals and reagents

Distilled water, methanol, ethanol, Fehling's solution, 10% sulphuric acid, Dragendroffs reagent, Wagner's reagent, Mayer's reagent, Molisch's reagent, Ferric chloride, Folin-Ciocalteu phenol reagent, Sodium carbonate, Sodium hydroxide, Conc. Hcl, Conc. H_2SO_4, 2,2 –diphenyl-1-picrylhydrazy (DPPH), Gallic acid.

Plant Material and Preparation of crude extract and fractions

The leaves of *Terminalia catappa*were collected in the premises of UDUTH, Sokoto State, Nigeria in March 2017. The plant sample was identified and authenticated by Dr. H.E. Mshelia, of Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Science, Usmanu Danfodiyo University Sokoto. The Leaves were shade dried and powdered. From the powdered leaves, 300g was weighed and extracted in 80% methanol at room temperature by maceration with occassional stiring for 24 hours. The procedured was repeated 2 more times to ensure complete extraction. The resulting liquid extract was then filtered through cotton and Whatman filter paper and concentrated with a rotary evaporator under reduced pressure at 45^0C. From the dried extact, 10g was taken and then subjected to fractionation using hexane, ethyl acetate, n-butanol and then aqueous solvents. The crude extract and its fractions were later subjected to phytochemical analysis using established method.

Preliminary phytochemical screening

The methanol extract and its fractions (hexane, ethyl acetate, n-butanol and aqueous) were subjected to phytochemical analysis of phytoconstituents such as alkaloids, flavanoids, steroids, glycosides, saponins and tannins. This was carried by using standard procedure [4].

Test for Flavonoids

a) Sodium hydroxide test: 1ml of 10% sodium hydroxide solution will added to the extract. A yellow color indicates the presence of flavonoids.

b) Ferric chloride test: The extract will be boiled with water and filtered. To 2mls of the filtrate, 2 drops of ferric chloride solution was added. A blue green or violet color indicates the presence of a phenolic nucleus.

c) Shinoda test: Small quantity of extract dissolved in water. Concentrated hydrochloric was added and magnesium chips. Cherry red precipitate indicate presence of flavonoid

Test for Alkaloids

a) About 0.5g of the extract will be stirred with 5ml of 1% aqueous hydrochloric acid in a water bath and filtered. 3ml of the filtrate was divided into three test tubes;

a) Dragendroffs test: To the first test tube, few drops of freshly prepared Dragendroff's reagent was added and observed for formation of an orange to brownish precipitate.

b) Mayer's test: To the second test tube few drops of Mayer's reagent was added and observed for the formation of white to yellowish or cream precipitate.

c) Wagner's test: To the third test tube, one drop of Wagner's reagent was added and observed for brown-reddish or brown precipitate.

Test for Saponins

a) **Frothing Test**: Small amount of each extract of was put in a test tube and about 10 mls of water was added to it. It was thoroughly shaken for 30 seconds. The test is positive if honeycomb froth persists in the test tube for 10-15 minutes.

Test for Anthraquinones

a) Borntragger's Test: a small amount of each extract of *Tapinanthusglobiferus* was dissolved in chloroform and an equal volume of 10% Ammonium hydroxide was added to each extract sample and shaken well. Appearance of a bright pink colour in the upper layer indicates the presence of Anthraquinones.

Tests for Carbohydrates

a) **Molisch's Test**: a little amount of each extract was put in a test tube and a little amount of Molisch's reagent was added. Few drops of concentrated sulphuric acid were added. Appearance of a reddish colored ring indicates the presence of carbohydrates.

b) **Fehling's Test**: a little amount of extract was put in a test tube and a few drops of Fehling's reagents A and B were added. It was then heated. Presence of brick red precipitate indicates the presence of carbohydrates.

Test for Tannins

a) Lead Sub Acetate Test: a little amount of extract was put in separate test tubes. They were dissolved with chloroform. Then a few drops of lead sub acetate were added. Presence of a heavy precipitate indicates that tannins are present in the plant extract.

b) Ferric Chloride Test: a little amount of extract was put in separate test tubes. A small amount of water was added. Then a few drops of ferric chloride were added. Presence of blue black color indicates that tannins are present.

Tests for Steroids

a) Liebermann-Burchard's Test: a small amount of extract was dissolved in chloroform and equal volume of acetic anhydride and then a few drops of concentrated sulphuric acid were added. The

extract is positive for steroids if the upper layer is blue-green in colour while the lower layer is red.

a) **Salkowski's Test**: A small amount of extract of was taken and dissolved in some chloroform. Few drops of conc. Sulphuric acid was added. Appearance of a brown ring indicates the presence of steroids.

Test for Cardiac Glycosides

a) **Kella – Kelliani Test**: a small amount of extract of was dissolved in glacial acetic acid. Few drops of ferric chloride were added; shaken andfew drops of concentrated sulphuric acid were added. Appearance of a purple-brown ring at the interface indicates the presence of cardiac glycosides.

IN VITRO ANTIOXIDANT STUDIES

Rapid screening of antioxidant by dot-blot and DPPH staining

Each sample of the methanol extract and its fractions were dissolved in ethanol then spotted using a capillary tube onto a TLC layer and allowed to dry. The silica place bearing the dry spots was stained with the DPPH solution. Stained silica layer revealed a purple background with yellow spot at the location where radical-scavenger capacity was present. The intensity of the yellow colour depends upon the amount and nature of radical scavenger present in the sample [5].

Determination of 2,2 –diphenyl-1-picrylhydrazyl DPPH radical scavenging activity

The free radical scavenging activity of the extract and fraction were measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH (2, 2-diphenyl-1-picrylhydrazyl). The stock solutions for the extract and fractio were prepared as 50mg/5ml of ethanol each and 10mg/5ml for the ascorbic acid. DPPH stock solution was prepared by dissolving 20mg DPPH in 500ml of ethanol and 90µl of this solution was mixed with 10µl each of the extract and fractions in ethanol at different concentration (31.25-500µg/ml) then 100µl of purified water. The reaction mixture was shaken well, the plates were left in the foil and incubated for 30 minutes in a dark cupboard. Then the absorbance was taken at 515nm on the ELISA plate reader. Ascorbic acid was used as a standard. All determinations were performed in triplicate. The blank was prepared as above without any sample. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

Percentage (%) inhibition of DPPH activity = $[(A_B - A_A) / A_B] \times 100$, Where A_A and A_B are the absorbance values of the test and of the blank sample, respectively. A percent inhibition versus concentration curve was plotted and the concentration of sample required for 50% inhibition was determined and represented as IC_{50} value for each of the test solutions [6].

Estimation of Total Phenolic Content

The total phenolic content present in the methanolic leaf extract and fractions was determined by the spectrophotometric method. 1ml each of the sample (1mg/ml) was mixed with 5ml of Folin-Ciocalteu's phenol reagent. After 5 minutes, 4ml of sodium carbonaate (Na_2CO_3) solution was added to the mixture followed by the addition of 2ml of distilled water and mixed thoroughly. The mixture was kept in the dark for 30 minutes at 40^0C for colour development. Absorbance was measured at 765nm using Hewlett-Packard UV-VS spectrophometer. The TPC was determined from extrapolation of calibration curve which was made by preparing gallic acid solution as standard. The TPC was expressed as milligrams of gallic acid equivalents (GAE) per g of dried sample [7].

EVALUATION OF ANTIBACTERIAL ACTIVITY

Preparation of plant extract for antibacterial activity

From each of the four extract, four concentrations were prepared, 35mg,30mg, 25mg and 20mg. the hexane portion was dissolved with hexane, the ethyl acetate portion was dissolved with ethyl acetate and n-butanol and methanol extract were dissolved with distilled water.

Media preparation

16 plates were prepared each containing 20ml of nutrient agar, and the plates were inoculated with the aid of sterile swabs. The swab is dipped into the inoculums and excess removed by rotating it several times with firm pressure on the side wall of the test tube. The swab is then steak on the entire surface of each plate, streaking is done successively in three different directions to obtain even inoculums.

The inoculums are dilute overnight cultures of *E. coli* P. aeruginosa, Staph. Aureus and Bacillus specie. The strength of dilution been 1:5000 for gram negative and 1:1000 for gram positive using normal saline.

Four of the sixteen plates were inoculated with E. coli, another four with pseudomonas aeruginosa, another four with bacillus specie and the last four with staphylococcus aureus, after inoculation the plates were allowed to dry for 15 minutes at room temperature.

Determination of zone of inhibition

The 16 plates were divided into four, such that each group contains 4 plates inoculated with E. coli, P. aeruginosa, Aureus and B. specie respectively. Four holes were bored in each of the plates, the agar plugs were removed using a sterile ampoule file and the bottom was sealed to the first group of four plates 1ml of hexane fraction was placed in each hole. In hole no one, 35mg/ml was placed, in the second hole 30mg/ml was placed in the third hole 25mg/ml was placed and the last hole 20mg/ml. For the second group ethyl

acetate fraction was used against the four organisms with the four different concentrations, for the third group N- butanol fraction was used and for the fourth group the methanol extract was used as done to group with the four different concentrations against the four different organisms. They were then left at room temperature for 2 hours and incubated at 37 degree for 24 hours. The size of the inhibition zones was recorded.

Determination of minimum inhibitory concentration (MIC)

A set of forty sterile test tube each containing 2ml of nutrient broth and the first of which contains double strength were prepared. They were divided into four. To the first group 1ml of methanol fraction was added (35mg) to the first tube, after mixing 1ml was taken from the tube to the second one, 1ml was transferred to the next test tube until the last test tube. The same was done for the second, third, and fourth group of 10 test tubes with ethyl acetate fraction, n-butanol and methanol extract respectively.

To the first group, 1 ml of standard solution of overnight culture of *S. aureus* was added to each tube, to the second group of 10 test tubes 1ml bacillus culture was added to each tube, to the third group 1ml of E. coli culture was added to each tube, to the fourth group 1ml of *Pseudomonas aeruginosa* was added to each tube. They were then incubated at 37 degree for 24 hours. The minimum inhibitory concentration was determined

Determination of minimum bactericidal concentration (MBC)

Agar plates containing 20ml nutrient agar were prepared. Each of the four plates were divided into 10 portions and labeled 1-10. Each portion was inoculated with corresponding solution of 10 test tubes in minimum inhibitory concentration. They were then incubated at 37 degree for 24 hours and growth was observed.

RESULTS

PRELIMINARY PHYTOCHEMICAL SCREENING

Table-1: Qualitative analysis of methanol extract and fractions of Terminalia catappa leaf

EXTRACTS	PHYTOCHEMICAL COMPOUNDS							
	Alkaloids	**Flavonoids**	**Tannins**	**Saponins**	**Cardiac glycosides**	**Steroids**	**Carbohydrates**	**Anthraquinones**
Methanol	+	+	+	+	+	-	-	-
Hexane	-	-	-	-	-	+	-	-
Ethyl acetate	-	+	+	+	+	+	-	-
n-butanol	+	+	+	+	+	-	-	-
Aqueous	+	+	+	+	+	-	-	-

Key: (-) = Not detected (+) = Present
Samples were analyzed in triplicate

Rapid screening of antioxidant by dot-blot and DPPH staining

Antioxidant capacity of *T. Catappa* methanol extract and fractions was eye-detected semi-quantitatively by a rapid DPPH staining TLC method based on the inhibition of the accumulation of oxidized products. The generation of free radicals was inhibited by the addition of antioxidants and scavenging of the free radicals shifted the end point. In the DPPH staining, the methanol extract showed the highest strong dot-blot staining while the hexane fraction showed no free radical scavenging activity as seen in the figure below.

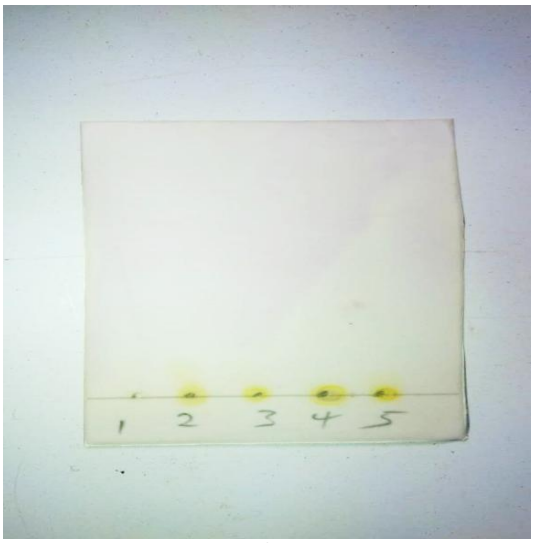

Fig-1: Dot blots Assay of *Terminalia catappa* methanol extract and fractions

Key
1. Hexane
2. Ethylacetate

3. Butanol
4. Aqueous
5. Methanol

Scavenging Activity Against DPPH Radical

The colour changed from purple to yellow after reduction, which was quantified by its absorbance at wavelength 515nm. The tables below show the radical-scavenging activity of the methanol extract and the different fractions of the leaf of *T. Catappa* using ascorbic acid as standard.

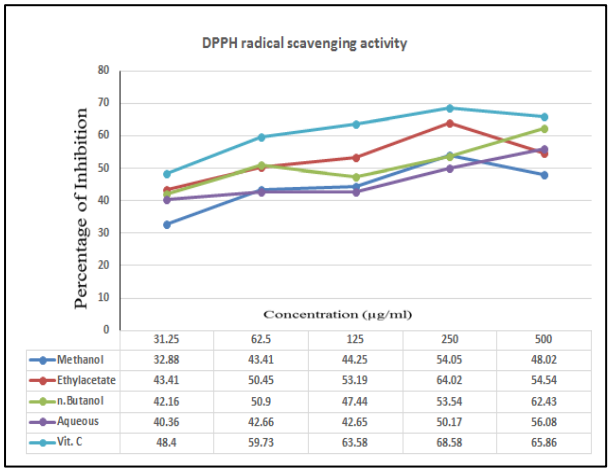

Fig-2: In-vitro percentage inhibition of DPPH radical by methanol leaf extract and fractions of T. catappa

Table-2: Evaluation of IC_{50} value of extracts

S/No.	Sample	IC_{50} value (µg/ml)
1.	Methanol	187.75
2.	Ethyl acetate	59.79
3.	N-Butanol	167.21
4.	Aqueous	246.13
5.	Ascorbic Acid	34.46

Total Phenolic Content

The phenolic content was evaluated and expressed in GAE as milligrams per gram of extract (mg GAE/g extract). The values of the total phenolic content of the extract and fractions of *Terminalia catappa*are shown in figure 2.

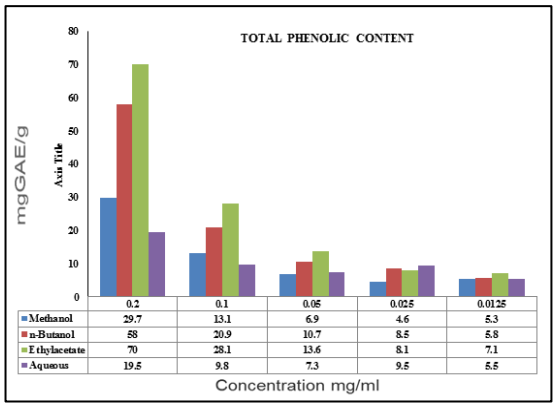

Fig-4: Total Phenolic Content of *Terminalia catappa* methanol extract and fractions

ANTIBACTERIAL ACTIVITY

Zone of inhibition assay

Table-4: Zone of inhibition of different concentrations of *T catappa* methanol extract and fractions

Sample	Concentration (mg/mL)	Organisms [(zones of inhibitions (mm)]			
		P. Aeruginosa	*E. coli*	*Bacillus* specie	*S. aureus*
Methanol	35	0.9± 0.3	0.9± 0.2	1.1± 0.4	0.8± 0.1
Hexane	20	-	-	_	_
Ethyl acetate	30	0.5± 0.1	0.6± 0.2	0.5± 0.2	0.6± 0.1
n-butanol	25	1.0± 0.3	1.1± 0.3	1.2± 0.4	1.1± 0.3
Ciprofloxacin (+ve control)	40	1.3 ± 0.2*	1.2. ± 0.2	1.8 ± 0.6	1.8 ± 0.3
Tetracycline (+ve control)	40	1.4 ± 0.6*	1.3 ± 0.2	-	-
H_20 (-ve control)	-	-	-	-	-

Minimum inhibitory cocentration

Table-8: Minimum inhibitory concentration

Test tube S/No	Bacterial specie			
	E. coli	*Pseudomonas*	*Bacillus*	*Staph aureus*
1	_	_	_	_
2	_	_	_	_
3	+	_	_	_
4	+	+	_	_
5	+	+	_	_
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	+	+	+	+
10	+	+	+	+

Minimum bactericidal cocentration

Table-9: Minimum bactericidal concentration

Test tube no	Bacterial Specie			
	E. coli	***Pseudomonas***	***Bacillus***	***Staph aureus***
1	–	–	–	–
2	+	–	–	–
3	+	+	–	–
4	+	+	–	–
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	+	+	+	+
10	+	+	+	+

DISCUSSION

The present study reveals the presence of phytochemical compounds such as alkaloids, flavonoids, cardiac glycosides steroids, saponins and tannins. These constituents are known to possess diverse pharmacological properties including antioxidant and antimicrobial activity [8]. Therefore, the antioxidant and antimicrobial activity of *Terminalia catappa* may be due to the synergetic effect of two or more of the constituents found in the plant extract. The constituents could be a source of useful natural antioxidants that can protect against oxidative stress and also, play a vital role in the protection against injuries from lipid peroxidation similar to what was reported by [9].

It is also known that; antioxidants exert their mode of action by suppressing the formation of reactive oxygen species either by inhibition of enzymes or by chelating trace elements. The DPPH radical has been used widely in the model system to investigate the scavenging activities of several natural compounds such as phenolic compounds, anthocyanins, or crude extracts of plants. The results of the DPPH assay in this study showed that, ethyl acetate fraction has the highest percentage inhibition of 64.02 percent at a concentration of 250µg/mL just slightly less than the highest percentage inhibition of the standard ascorbic acid used with an inhibition percentage of 68.58 at the same concentration. This also reflects from the IC_{50} value which is negatively related to the antioxidant activity with the ethyl acetate fraction having an IC_{50} value of 59.79µg/mL which is the least of all the test samples thus, the lower the IC_{50} value, the higher the antioxidant activity of the tested sample and this study is in line with that of[10]. We can therefore, infer that; the activity of the extract and fractions may be probably due to the presence of substance with an available hydroxyl group as the extracts are able to reduce the stable radical DPPH to the yellow colored diphenyl picrylhydrazine[8]. This suggested that *T. Catappa* leaves contain compounds such as polyphenolics that can donate electron/hydrogen easily. To further confirm this, total phenolic concentration equivalents of Gallic acid was estimated. Gallic acid being the most important polyphenol in natural products was used to determine the phenolic of tested sample from which it was found that the ethyl acetate and n-butanol fractions has the highest equivalence of 70mgGAE/g and 58mgGAE/g respectively as shown in figure 5. These observations clearly indicated a cross linkage between phenolic content and the antioxidant activity which is in accordance with [11].

CONCLUSION

In this study, it has shown that, the ethyl acetate and n-butanol fractions of *T. catappa* has appreciably strong antioxidant activity compared to the ascorbic acid and while the methanol extract and the aqueous fraction has moderate antioxidant activity and the fractions also show significant antibacterial activity. Phenolics are well established to show antioxidant activity and contribute to human health. This study showed that, methanol extract and fractions of the leaf of *T. Catappa* have demonstrated significant antioxidant and antibacterial activities.

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