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Studies on Antioxidant Activity, Total Phenolic and Total Flavonoid Contents of Terminalia Catappa Leaves Using In-vitro Models

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ABSTRACT

Plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines. A regular and widespread use of herbs throughout the world has increased serious concern over their quality, safety and efficacy. Thus, a proper scientific evidence or assessment has become the criteria for acceptance of herbal health claims. In the present study, we examined the antioxidant effects of *Terminalia Catappa* plant extract. Dried and powered of *T. Catappa* was extracted with hexane, chloroform, methanol, ethanol and water. Total phenolic and flavonoid contents of different solvent extracts were determined. Of the different solvent extracts, methanol extract had the highest phenol and flavonoid content of 121.9±3.1mg/g and 59.6±1.5mg/g respectively. Antioxidant assays were carried out by using different *in vitro* models such as total reducing power, total antioxidant activity, lipid peroxidation inhibitory activity, DPPH radical scavenging activity and superoxide radical scavenging activity. methanol extract showed the highest total antioxidant activity of 41.4±0.45 µM Fe(II)/g. The EC_{50} values of ethanol extract for lipid peroxidation inhibitory activity and DPPH radical scavenging activity was found to be 0.1 and 0.5 mg/ml respectively. The anti-oxidant activities of other solvent extracts were poor when compared to the ethanol extract. These results suggest that, the active antioxidant compounds are better extracted in ethanol and there is a direct correlation between the total polyphenols extracted and its anti-oxidant activity. The *invitro* anti-oxidant activity of *T. Catappa* justifies the ethno medical use of this plant.

Keywords: Medicinal plant; *Terminalia Catappa*; solvent extracts; anti-oxidant activity.

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INTRODUCTION

Plants have been a source of medicine in the past centuries and today scientists and the general public recognize their value as a source of new or complimentary medicinal products. Recently, wide array of research investigations highlight the potential health beneficial principles from phytal sources. Beyond this pharmaceutical approach to plants, there is a wide tendency to utilize herbal product to supplement the diet, mainly with the intention of improving the quality of life and preventing the diseases of elderly people[1].

The WHO estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their medicine[2]. India has been identified as a major resourceful area in the traditional and alternative medicines globally. Multi-factorial health beneficial activity of these plant extracts has been attributed to multi-potent anti-oxidant, anti-microbial, anti-cancer, anti-ulcerative and anti-diabetic properties. Generally, anti-oxidants have been identified as major health beneficial compounds reported from varieties of medicinal plants and are sources for alternative medicines[3]. Free radicals or reactive oxygen species (ROS) are formed in our body as a result of biological oxidation. The over production of free radicals such as hydroxyl radical, super oxide anion radical, hydrogen peroxide can cause damage to the body and contribute to oxidative stress[4].

Oxidative damage of proteins, DNA and lipid is associated with chronic degenerative diseases including cancer, coronary artery disease, hypertension, diabetes etc[5] and compounds that can scavenge free radicals have great potential in ameliorating these disease processes[6]. Most of the reactive oxygen species are scavenged by endogenous defense systems such as catalase, superoxide dismutase and peroxidase-glutathione system[7]. But these systems may not be completely efficient requiring them to depend on exogenous anti-oxidants from natural sources. Medicinal plants constitute one of the main sources of new pharmaceuticals and health care products. A whole range of plant derived dietary supplements, phytochemicals and pro-vitamins that assist in maintaining good health and combating disease are now being described as functional ingredients and neutraceuticals. The role of medicinal plants in disease prevention or control has been attributed to antioxidant properties of their constituents[8].

The protective effect of plant products are due to the presence of several components such as enzymes, proteins, vitamins[9], carotenoids[10], flavonoids[11] and other phenolic compounds[12]. Since synthetic anti-oxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have restricted use in foods, the search for natural anti-oxidants has greatly increased in the recent years. The researchers have focused on natural anti-oxidants and numerous crude extracts

and pure natural compounds have been recognized to have beneficial effects against free radicals in biological systems as anti-oxidants[13].

Terminalia Catappa Miers. is a widely used shrub in folk and Ayurvedic systems of medicine. It is a large, glabrous, deciduous climbing shrub belonging to the family menispermaceae. It is distributed throughout tropical Indian subcontinent and China. It is reported to possess anti-spasmodic, anti-inflammatory, anti-allergic, anti-diabetic, anti-oxidant properties[14]. The objective of the present study was to determine the anti-oxidant activity of *T. Catappa* leaves in different solvent extracts using standard methods. The findings from this work may add to the overall value of the medicinal potential of the shrub, since most of the studies have focused on antioxidant activities of root and stem of *T. Catappa.*

MATERIALS AND METHOD

Plant material

Fresh and healthy leaves of *T. Catappa* were collected from local growers. The leaves were washed thoroughly in distilled water and the surface water was removed by air drying under shade. The leaves were subsequently dried in a hot air oven at 40 0C for 48h, powdered and used for extraction.

Preparation of aqueous extract

Fifty grams of powdered leaves of *T. Catappa* was macerated with 100 ml sterile distilled water in a blender for 10 min. The macerate was first filtered through double layered muslin cloth and centrifuged at 4000 g for 30 min. The supernatant was filtered through Whatman No.1 filter paper and heat sterilized at 120 0C for 30 min. The extract was preserved aseptically in a brown bottle at 4 0C until further use.

Preparation of solvent extract

Fifty grams of shade dried powered leaf material was extracted successively with chloroform, hexane, methanol and ethanol until the plant material became colorless. It was then filtered with sterile Whatman filter paper into a clean conical flask and the filtrate was transferred into the sample holder of the rotary flash evaporator. The extracts so obtained was weighed and preserved at 4 0C in airtight bottles until further use.

Total phenolic content

Total soluble phenolic content was estimated by Folin-Ciocalteau reagent method[15] using gallic acid as a standard phenolic compound. One ml of stock solutions of different solvent extracts was prepared (1g/ml) from which different aliquots were pipetted out into test tubes. The volume was

made up to 3 ml with distilled water to which freshly prepared Folin-Ciocalteau reagent was added. After 3 min, 2 ml of 20% sodium carbonate solution was added to each tube and mixed thoroughly. The tubes were placed in boiling water for one minute, cooled and the absorbance was measured at 650 nm in a spectrophotometer against a reagent blank. The concentrations of the total phenolic compounds in the extracts were obtained by extrapolating the absorbance of gallic acid on standard gallic acid graph. The experiment was repeated thrice and concentration of total phenols was expressed as mg /g of dry extract.

Total flavonoid content

The total soluble flavonoid content was estimated by aluminium chloride colorimetric method for both aqueous and solvent extracts[16].0.5ml of stock solution (1g/ml) of the extract, 1.5 ml methanol, 0.1ml potassium acetate (1M) was added to reaction test tubes and volume was made up to 5 ml with distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. Total flavonoid content was calculated by extrapolating the absorbance of reaction mixture on standard curve of rutin. The experiment was repeated thrice and the total flavonoid content was expressed as equivalent to rutin in mg/ g of the extracts.

ANTIOXIDANT ACTIVITY ASSAYS

Total reducing power

Various extracts (0.1 - 0.9 mg/ml) were mixed with phosphate buffer (500 µl, 20 mM, pH 6.6) and 1% potassium ferricyanide (500 µl), and incubated at 50 0C for 20 min; 500 µl of 10% trichloro acetic acid were added, and the mixture was centrifuged at 2500 rpm for 10 min. The supernatant was mixed with distilled water (1.5 ml) and 0.1% ferric chloride (300 µl) and the absorbance was read at 700 nm. The experiment was repeated thrice. Increase in the absorbance of the reactions mixture indicated increase in the reducing power.

Ferrous reducing antioxidant power assay (Total antioxidant activity assay)

The stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM 2, 4, 6-tripyridyl-s-tri-azine solution in 40 mM HCl, and 20 mM FeCl3.6H2O solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ and 2.5 ml FeCl3.6H2O. The temperature of the solution was raised to 37 0C before using. Plant extracts (150 µl) were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark condition. Readings of colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The experiment was repeated thrice. Results were expressed in µM Fe (II)/g dry mass and compared with that of BHT.

Lipid peroxidation inhibitory activity

Egg lecithin (3 mg/ml phosphate buffer, pH 7.4) was sonicated in an ultrasonic sonicator for 10 min to ensure proper liposome formation. Test samples (100 µl) of different concentrations (0.1 - 0.9 mg/ml) were added to liposome mixture (1 ml); the control was without test sample. Lipid peroxidation was induced by adding ferric chloride (10 µl, 400 mM) and L-ascorbic acid (10 µl, 200 mM). After incubation for 1 h at 370 C the reaction was stopped by adding hydrochloric acid (2 ml, 0.25 N) containing trichloroacetic acid (150 mg/ml) and thiobarbutyric acid (3.75 mg/ml). The reaction mixture was subsequently boiled for 15 min, cooled, centrifuged at 1000 rpm for 15 min and the absorbance of the supernatant was measured at 532 nm and compared with that of BHA. Percentage radical scavenging was calculated using the following formula:

% Inhibition = [(A control – (A sample – A sample blank / A control] x 100

DPPH radical scavenging activity

0.1 ml of test sample at different concentration (0.1 - 0.9 mg/ml) was mixed with 0.9 ml of Tris-HCl buffer (pH 7.4); then 1 ml of DPPH (500 µM in ethanol) was added. The mixture was shaken vigorously and left to stand for 30 min. The absorbance of the resulting solution was measured at 517 nm in a spectrophotometer and compared with that of BHA. The experiment was repeated thrice. The percentage of DPPH scavenging was calculated using the following formula:

% scavenging = [(A control – (A sample – A sample blank / A control] x 100

Superoxide radical scavenging activity

The 1 ml reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 µM), NBT (50 µM), PMS (15 µM) and various concentrations of sample solution. After incubation for 5 min at ambient temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated. The experiment was repeated thrice. The results were compared with that of quercetin. The % inhibition of superoxide anion generation was calculated using the following formula:

% Scavenging = [(A control – (A sample – A sample blank / A control] x 100

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

Table 1: Polyphenol and Flavonoid content of *Terminalia Catappa* leaves in different solvent extracts

Plant extracts	Total phenolics (mg gallic acid equivalent/g)	Total flavonoid (mg rutin equivalent/g)
Methanol extract	121.9±3.1^{c}	59.6±1.5^{c}
n-Hexane fraction	54.9±2.8^{a}	15.8±0.9^{a}
Chloroform fraction	78.0±1.1^{b}	26.0±1.9^{b}
Ethyl acetate fraction	80.0±5.2^{b}	60.9±2.2^{c}
n-Butanol fraction	80.9±2.9^{b}	55.0±2.5^{c}
Residual aqueous fraction	49.9±4.1^{a}	14.9±0.4^{a}

Table 2: Radical scavenging activities of T. catappa fractions at different concentrations

Plant extracts/ chemical	DPPH radical	Superoxide radical	Phosphomolybdate Assay	Hydroxyl radical	Hydrogen peroxide	ABTS radical
Methanol extract	189±4^{e}	55.0±0.3^{d}	236±1^{e}	76.0±2^{d}	130±5^{e}	179±3^{e}
n-Hexane fraction	395±5^{f}	370±4^{f}	247±3^{e}	281±2^{e}	200±2^{e}	50.0±1^{c}
Chloroform fraction	162±3^{e}	68.0±2^{d}	135±4^{e}	8.0±1^{a}	122±5^{e}	57.0±2^{d}
Ethylacetate fraction	62.0±2^{d}	145±1^{e}	196±4^{e}	27.0±1^{b}	68.0±2^{d}	34.0±3^{b}
n-Butanol fraction	41.0±1^{c}	92.0±1^{d}	10.7±2^{a}	62.0±2^{d}	254±3^{e}	10.0±0.9^{a}
Residual aqueous fraction	264±4^{e}	269±3^{e}	237±3^{e}	345±4^{f}	431±5^{f}	13.0±0.2^{a}
Ascorbic acid	10.0±3^{a}	34.0±3^{b}	8.0±0.7^{a}	6.0±2^{a}	9.0±0.3^{a}	8.0±1^{a}
Rutin	29.0±1^{b}	-	-	-	10.0±0.3^{a}	-

Each value in the table is represented as mean ± SD (n = 3). Values in the same column followed by a different letter ($^{a-f}$) are significantly different ($p< 0.05$). -, not determined.

Total phenolic and flavonoid content

Results obtained in the present study revealed that the level of polyphenols in the methanol extract was 121.9±3.1mg/g which was higher when compared to chloroform, hexane and aqueous extracts of *T. Catappa*. Ethanol extract of the leaves had a flavonoid content of 59.6±1.5mg/g. The flavonoid content of other extracts tested was lower than the ethanol extract. Aqueous extract had the least polyphenol and flavonoid content (Table 1).

Scavenging activities of t. catappa fractions at different concentrations:

DPPH radical scavenging activity:

The scavenging effects of samples on DPPH radical and were in the following order: TCB > TCE > TCC > TCM >TCA > TCH. The EC_{50} values of scavenging DPPH radicals for the TLB and TLE were 41.0±1 and 62.0±2 µg/ml, respectively (Table 2). Though the antioxidant potential of fractions was found to be low ($P < 0.05$) than those of ascorbic acid and rutin, the study revealed that TCB and TCE have prominent antioxidant activity; the presence of phenolic compounds

(containing phenolic hydroxyls) are mainly found in these two fractions and could be attributable to the observed high anti-radical properties of these fractions.

Superoxide radical scavenging activity

The superoxide radical scavenging effect of different fractions was compared with the same doses of ascorbic acid ranging from 25 - 500 µg/ml. The EC_{50} values in superoxide scavenging activities were in the order of TCM > TCC > TCB > TCE > TCA > TCH (Table 2). When compared to ascorbic acid; the super-oxide scavenging activity of the extract was found to be low ($P < 0.05$). In spite of this TCM and TCC (EC_{50} 55.0 ±0.3 and 68.0±2 µg/ml respectively) behave as powerful superoxide anion scavengers that may include therapeutic use against oxidative stress.

Phosphomolybdate assay

The phosphomolybdate method is quantitative, since the total antioxidant capacity (TAC) is expressed as ascorbic acid equivalents. The antioxidant capacity of various solvent fractions of T. catappa was found to decrease in this order: TLB > TLC > TLE > TLM > TLA > TLH fraction (Table 2). All results showed antioxidant activity in dose dependent manner at concentration 25 to 250 µg/ml. The EC_{50} value of antioxidant capacity for the TLB (10.7±2 µg/ml) was most pronounced ($P < 0.05$) than TLC (135±4 µg/ml) and TLE (196±4 µg/ml) (Table 4). Strong antioxidant activity of TLB statistically similar to ascorbic acid indicates strong antioxidants in this fraction and these could be attributable to the presence of phenolic compounds.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of T. catappa extract and its derived fractions can be ranked as TCC > TCE > TCB > TCM > TCH and TCA (Table 2). All fractions showed antioxidant activity in dose dependent manner at concentration 25 - 500 µg/ml. In the present investigation, the EC_{50} value scavenging DPPH radicals for the TCB and TCE were 41.0±1 and 62.0±2 µg/ml, respectively (Table 2). Though the antioxidant potential of fractions was found to be low ($P < 0.05$) than those of ascorbic acid and rutin, the study revealed that TCB and TCE have prominent antioxidant activity; the presence of phenolic compounds (containing phenolic hydroxyls) are mainly found in these two fractions and could be attributable to the observed high anti-radical properties of these fractions.

Hydrogen Peroxide scavenging activity

The hydroxyl radical scavenging activity of T. catappa extract and its derived fractions can be ranked as TCC > TCE > TCB > TCM > TCH and TCA (Table 2). All fractions showed antioxidant activity in dose dependent manner at concentration 25 - 500 µg/ml. In the present investigation, the EC_{50} value of hydroxyl radical scavenging activity for the TCC and TCE was 8.0±1 and 27.0±1

µg/ml while for TLB was 62.0±2 µg/ml (Table 2). The markedly strong ($P < 0.05$) antioxidant response of TCC and TCE in comparison with ascorbic acid might be helpful in characterizing the significant sources of natural antioxidant reaction. strong H_2O_2 scavenging activity (EC_{50} 68.0±2 µg/ml) whereas that of the standard, ascorbic acid exhibited 9.0±0.3 µg /ml. The scavenging activities of TCC and TCM were (EC_{50} 122±5 µg/ml and 130±5 µg/ml, respectively) (Table 2). EC_{50} values of the fractions in scavenging hydrogen peroxide were significantly differ-ent ($P < 0.05$) from the EC_{50} values obtained for ascorbic acid. The scavenging activity for hydrogen peroxide of various solvent extracts from T. catappa was in the order of TLE > TLC > TLM > TLH > TLB > TLA respectively.

ABTS radical scavenging activity

All the fractions of T.catappa scavenged ABTS radical in a concentration-dependent way (25 - 250 µg/ml). Present results showed that the ABTS radical scavenging ability of samples can be ranked as TCB > TCA > TCE > TCH > TCC > TCM. TCB and TCA exhibited prominent ABTS radical scavenging activities.

Reducing power activity

It was found that the reducing power increased with concentration of each sample. The ranking order for reducing power was TCM > TCB > TCC > TCA >TCE > TCH. Significantly higher reducing power (1.47 ±0.14 at 250 µg/ml) was evident in TCM fraction.

CONCLUSION

The antioxidant activity of terminalia catappa can be determined accurately, conveniently, and rapidly using DPPH testing. The trend in antioxidant activity obtained by using the DPPH method is comparable to trends found using other methods reported in the literature. This method can be used successfully for solid samples without prior extraction and concentration, which saves time. The reaction time of four hours and a temperature of 35°C facilitates the extraction and reaction of antioxidant compounds with DPPH. Antioxidant activity measured using DPPH accounts partially for the bound and insoluble antioxidants. Relative antioxidant content provides an indication of importance of terminalia catappa. Antioxidant activity and nutritional labeling data including vitamins, fibers, minerals will aid in the interpretation of clinical results obtained as various food products are tested in biological models for chronic disease. It is reasonable to expect that high antioxidant foods have greater potential to reduce free radicals in the body than do low antioxidant foods. Thus it is important to know the antioxidant content of terminalia catappa, in addition to knowing the basic nutritional information such as the protein, fiber, fat, mineral and vitamin

contents.

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REFERENCES

1. Adedapo AA, Jimoh OF, Koduru S, Afolayan JA, Masika JP. Antibacterial and antioxidant properties of the methanol extracts of the leaves and stems of *Calpurnia aurea*. BMC Complementary and Alternative Medicine 2008; 8:53-60.
2. Angolo AC, Sant Ana AE, Pletseh M, Coelho LC. Antioxidant activity of leaf extracts from *Bauhinia monandra.* Bioresource Technology; 95:229-233.
3. Ara N, Nur H. *In vitro* antioxidant activity of methanolic leaves and flower extracts of *Lippia alba*. Research Journal of Medicine and Medical Science 2009;4(1):107-110.
4. Behera BC, Verma N, Sonone A, Makhaija U. Determination of antioxidant potential of lichen *Usnea ghattensis in vitro*. LWT Food Science and Technology 2006; 39:80-85.
5. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical Biochemistry 1996; 239:70-76.
6. Brand-Williams W, Cuvelier ME, Berset C. Use of free radical method to evaluate antioxidant activity. Lebanon Wissen Technology 1995; 28:25-30.
7. Daniel M. Medicinal plants: Chemistry and properties. Science Publishers, USA. 2005:1-9.
8. Demiray S, Pintado ME, Castro PML. Evaluation of phenolic profiles and antioxidant activities of Turkish medicinal plants: *Tilia argentea, Crataegi folium* leaves and *Polygonum bistorta* roots. World Academy of Science, Engineering and Technology 2009; 54:312-317.
9. Di Malteo V, Esposito E. Biochemical and therapeutic effects of antioxidants in the treatment of Alzheimers disease, Parkinson disease and amyotrophic lateral sclerosis. Current Drug Target- CNS and Neurological Disorders 2003; 2:95-107.
10. Diplock AT. Free radical damage and control. In: Rice Evans CA and Burden RH, ed. Antioxidant and free radical scavengers. Elsevier, New York, USA. 1994.
11. Duh PD, Yen GH. Antioxidative activity of three herbal water extracts. Food Chemistry 1997;60:639-645.
12. Edge R, Mc Greevy DJ, Truscott TG. The carotenoids as antioxidants: A review. Journal of Photochemistry and Photobiology B: Biology 1997;41:189-200.

13. Fontana M, Mosca L, Rosei MA. Interaction of enkephalines with oxyradicals. Biochemical Pharmacology 2001;61:1253-1257.
14. Halliwell B. Ascorbic acid in the prevention and treatment of cancer. Alternative Medicine Reviews 1996; 3:174-186.
15. Hasan SMR, Hossain M, Akter R, Jamila M, Mazumder EH, Rahman S. DPPH free radical scavenging activity of some Bangladeshi medicinal plants. J Medicinal Plant Research 2009;3(11):875-879.
16. Hazra B, Biswas S, Mandal N. Antioxidant and free radical scavenging activity of *Spondias pinnata*. BMC Complementary and Alternative Medicine 2008; 8:63-75.