International Journal of Phytomedicine 7 (2015) 246-253
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

In vitro Antioxidant and Anti-inflammatory Studies on Bark, Wood and Fruits of *Terminalia catappa* L.

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Abstract

The present study was carried out to study the *in vitro* antioxidant and anti-inflammatory activities of bark, wood and fruits of *Terminalia catappa*. *In vitro* antioxidant studies were performed using DPPH assay and lipid peroxidation assay. *In vitro* anti-inflammatory studies were also performed in the aqueous extracts using protease inhibition, membrane stabilization and protein denaturation inhibition assays. Aqueous extract of bark has shown maximum antioxidant and anti-inflammatory activities when compared to that of wood and fruits. The results of the present study clearly revealed the dose dependent activities of all the parts selected. The present study showed *in vitro* antioxidant and anti-inflammatory activities which scientifically proved the ethnomedicinal claim of the selected plant drugs.

Keywords: Terminalia catappa L.; Antioxidant; Anti-inflammatory.

Introduction

Oxygen is an element indispensable for life and necessary for the generation of energy. Free radicals are released as a consequence of incomplete reduction of oxygen during ATP production in the mitochondria. The free radicals include reactive oxygen species (ROS) as well as reactive nitrogen species (RNS), which play both toxic and beneficial compounds. At low concentration, ROS and RNS exert beneficial effects on cellular responses and immune functions, but at high concentration, they generate oxidative stress, a deleterious process that can cause cellular damage [1]. Oxidative stress plays a major role in the development of chronic inflammatory and degenerative ailments such as cancer, arthritis, auto-immune disorders, cardiovascular and neurodegenerative diseases. Human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced *in situ* or externally supplied through foods. Endogenous and exogenous antioxidants can act as "free radical scavengers" by preventing and repairing the damages caused by ROS and RNS, and therefore can enhance the immunity, lower the risk of cancer and other degenerative diseases [2].

Inflammatory abnormalities underlie a vast variety of human diseases. The immune system is often involved in inflammatory disorders and is demonstrated in both allergic reactions and myopathies. Non-immune diseases with inflammation include atherosclerosis and ischemic conditions [3]. Anti-inflammatory drugs are divided into two classes: Non steroidal anti-inflammatory drugs (NSAIDs) and Corticosteroids. NSAIDs are known analgesics, anti pyretics and anti-inflammatory drugs. Their analgesic and anti-inflammatory effects are mainly due to inhibition of prostaglandin synthesis in the inflammed tissues and therefore it is on a peripheral level. Aspirin is the oldest NSAID and is very effective. It is used at far lower doses to inhibit clotting of blood and prevent strokes and heart attacks. However, up to 50% of patients are unable to tolerate the adverse effects i.e. nausea, vomiting, epigastric pain and tinnitus that are caused by high doses of Aspirin that often administered to produce effective anti-inflammatory activity.

Due to the serious side effects produced by the synthetic drugs a trend from the usage of synthetic drugs to herbal medicine is observed among mankind which can be called 'Return to Nature'. Antioxidant and anti-inflammatory principles present in the natural resources are providing enormous scope in herbal medicine. Therefore, in the recent years, research interest is centered on phytochemicals that are derived from herbal sources in view of their therapeutic benefits [4]. Phytochemicals are commonly available with less toxic effect and serving as medicinal components have been suggested to reduce threat of adverse effects of ROS [5]. Hence, the present work is focused on evaluating the antioxidant and anti-inflammatory properties of phytochemical extract obtained from Indian almond tree (*Terminalia catappa* L.).

Terminalia catappa L., a large spreading tree belongs to the family Combretaceae, is distributed throughout the tropics in coastal environments. The dried leaves are used as an alternative to antibiotics to control fish pathogens [6]. The leaves are also reported to possess antioxidant and anti-clastogenic properties [7]. Various extracts of leaves and bark of *T. catappa* have been reported to exhibit antibacterial [8], anti-inflammatory [9], antioxidant and anti-tumor [10], anti-HIV [11] and hepato-protective [12] and anti-diabetic properties [13] besides being aphrodisiac [14]. The moderate consumption of the seed kernel is useful in treating sexual dysfunction among men, primarily for premature

ejaculation [14]. The ethanol extract of the leaves of *T. catappa* inhibits osmotically-induced hemolysis of human erythrocytes in a dose-dependent manner [12]. Punicalagin and punicalin isolated from the leaves are used to treat dermatitis and hepatitis as both have strong antioxidative activity [9].

In view of these therapeutic potentials of this plant, only the leaf material was extensively studied and other parts such as bark, wood and fruits are not investigated in detail. Hence, the present study has been taken up with an objective of assessing the *in vitro* anti-inflammatory and antioxidant potentials of bark, fruits and wood of *Terminalia catappa*.

Materials and methods

Collection of Plant Materials

The bark, fruits and wood of *Terminalia catappa* L. were collected from Mannargudi, Tamil Nadu. Plant materials were identified and authenticated in the department of CARISM, SASTRA University, Thirumalaisamudram, Tamil Nadu. The collected materials were cleaned, shade dried and coarsely powdered.

Preparation of the Extract

Plant powder (50 g) was taken with 250 ml water and incubated for 36 h. Then, it was filtered and the filtrate was allowed to evaporate at 56°C until semi solid consistency is obtained. Then the aqueous extract was then re-dissolved in water at 1 mg/ml ratio and used for evaluating *in vitro* antioxidant & anti-inflammatory potentials.

Anti-inflammmatory Activity

HRBC Membrane Stabilization

Human red blood cells (HRBC) suspension was prepared according to the previously described method [15]. The blood was collected from healthy human volunteers who have not taken any NSAIDs for 2 weeks prior to the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of normal saline. The volume of blood was measured and re-constituted as 10% v/v suspension with normal saline. Hypotonicity–induced haemolysis was used for membrane stabilization assay [16]. The reaction mixture (4.5 ml) consisted of 2 ml hypotonic saline (0.25% NaCl), 1 ml of 0.15 M phosphate buffer (pH 7.4), 1 ml extract (1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml) in normal saline and 0.5 ml of 10% human RBC in normal saline. In blank 1 ml of isotonic saline was used instead of extract while control was devoid of red blood cells. The mixtures were incubated at 50°C for 30 min. The tubes were cooled under running tap water for 20 min followed by centrifugation at 1500 rpm for 10 min. Absorbance of the supernatant was read at 560 nm. Membrane stabilization was calculated by using the formula (Abs of blank – Abs of extract) / Abs of control x 100. The control represents 100% lysis and the result was compared with the standard acetyl salicylic acid (250 µg /ml).

Inhibition of Protein Denaturation

The reaction mixture (0.5 ml) consisted of 450 µl of 5% aqueous bovine serum albumin solution and 50 µl of test solution (1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml), pH was adjusted at 6.3 using 1N HCl [17]. The samples were incubated at 37°C for 20 min followed by heating at 57°C for 3 min. The mixture was then brought to room temperature and 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured at 660 nm. Distilled water (50 µl) was used in place of extracts for blank, while control lacks bovine serum albumin. Inhibition of protein denaturation was calculated by using the formula (Abs of blank - Abs of extract) / Abs of control x 100 and expressed in percentage basis. The control represents 100% protein denaturation. Results were compared with the standard acetyl salicylic acid (250 µg/ml) treated samples.

Protease Inhibitory Activity

The reaction mixture (2.0 ml) consisted of 0.06 mg trypsin, 1.0 ml of 25 mM Tris-HCl buffer (pH 7.4) and 1.0 ml (1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125, 250,500 and 1000 µg/ml) of the test solution [18]. The reaction mixture was incubated at 37°C for 5 min followed by the addition of 1.0 ml of 0.8% (w/v) casein. This was then incubated for an additional 20 min followed by addition of 2 ml of 70% (v/v) perchloric acid to terminate the reaction. The cloudy suspension was centrifuged and absorbance of the supernatant was read at 280 nm against buffer as blank. The percent inhibition was calculated by using the formula (Abs of test - Abs of extract) / Abs of control x 100.

Antioxidant Activity

DPPH Radical Scavenging Activity

Antioxidant activity can be measured using DPPH radical scavenging assay [19]. This test provides information on the ability of compounds present in the extract to donate a hydrogen atom which results in antioxidant ability. Antioxidants react with DPPH, a stable free radical and reduce it to DPPH-H form. The degree of discolouration indicates the scavenging potential of the antioxidant compound. The extracts were dissolved in ethanol and 5% DMSO and various concentrations (1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml) of the extracts were used for the study. BHT (3.6 mg/ml) was used as a positive control. Assay mixture contained 500 µl of the extract, 125 µl of DPPH (100 mM) and 375 µl solvent (5% DMSO). This mixture was incubated for 30 min at 25° C in dark condition. The decrease in absorbance was measured at 517 nm spectrophotometrically and the percentage of radical scavenging activity was calculated using the formula (Abs. control - Abs. extract) / Abs. control x 100.

Lipid Peroxidation Inhibition Assay

Lipid perpxidation was estimated as evidenced by the formation of thiobarbituric acid reactive sustances (TBARs). Lipid peroxides in plasma was assayed by the method of Yagi [20]. The colour formation with thiobarbituric acid (TBA) was used as the index. Plasma was deproteinised with phosphotungstic acid and the precipitate was treated with thiobarbituric acid at 90ºC for 1 hour. The pink colour formed gives a measure of the thiobarbituric acid reactive substances (TBARs). To 0.5 ml of plasma, 4 ml of 0.083 N sulphuric acid and 0.5 ml of 10% phosphotungstic acid were added, mixed and allowed to stand for 5 min. Then the mixture was centrifuged at 3000 x g for 10 min. The supernatant was discarded and the sediment was mixed with 2 ml of sulphuric acid and 0.3 ml of 10% phosphotungstic acid. The mixture was shaken well and centrifuged at 3000 x g for 10 min. The sediment was suspended in 4 ml of distilled water and 1 ml of TBA reagent was added. The reaction mixture was heated at 95ºC for 60 min. After cooling, 5 ml of n-butanol was added and mixture was shaken vigorously and centrifuged at 3000 x g for 15 min. The colour extracted in the butanol layer was read at 530 nm. The blank contains 4 ml of distilled water. The formula (Abs of test / Abs of standard x concentration in mol x total volume / volume taken) is used to calculate the lipid peroxide levels and the lipid peroxide levels were expressed as n mols/ml plasma.

Results and discussion

The genus *Terminalia* harbours a number of species with phyto pharmaceutical significance such as *T. chebula, T. arjuna* and *T. bellerica*. *T. chebula* and *T. bellerica* along with *Emblica officinalis* constitute triphala - an *Ayurvedic* medicine with much therapeutic and rejuvenating potential. All the parts such as leaves, fruits, seeds, wood and bark of the selected species *Terminalia catappa* are found to be useful in treating various human ailments. But still, intensive research needs to be carried out to provide scientific evidence. Little or no evidences are available validating the pharmacological activities of bark, wood and fruits. Hence in the present work, attempts were taken to evaluate the anti inflammatory and antioxidant potentials of *T. catappa* bark, fruits and wood.

Anti-inflammatory Activity

Inflammation is a bodily response to injury, infection or destruction characterized by heat, redness, pain, swelling and disturbed physiological function. Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical or microbial agents. It is the body response to inactivate or destroy the invading organisms, to remove the irritants and set the stage for tissue repair. It is triggered by the release of chemical mediators from injured tissue and migrating cells. The commonly used drug for the management of inflammatory conditions are non-steroidal anti-inflammatory drugs, which produce several adverse effects especially gastric irritation leading to formation of gastric ulcers [21]. Natural products have contributed significantly towards the development of modern medicine. Recently traditional medicine worldwide is being re-evaluated by extensive researches on different plant species and their active therapeutic principles. The rich wealth of plant kingdom can represent a novel source of newer compounds with significant anti-inflammatory activities. The major merits of herbal medicine seem to be their perceived efficacy, low incidences of serious adverse effects and low cost.

There are certain problems in using animals in experimental pharmacological research, such as ethical issues and the lack of rationale for their use when other suitable methods are available or could be investigated [22]. Hence, in the present study, *in vitro* methods such as HRBC membrane stabilization, proteinase inhibitory activity and protein denaturation were selected for the assessment of anti-inflammatory property of *T. catappa*.

HRBC Membrane Stabilization

Erythrocytes have been used as a model by a number of scientists to investigate the interaction of drugs with membranes [23, 24]. Anesthetics, tranquilizers and NSAIDs stabilize erythrocytes against hypotonicity induced (stress) hemolysis. Therefore, they prevent the release of haemoglobin as a result of their membrane stabilizing activity [25]. The human red blood cells (HRBC) model is selected to assess the anti-inflammatory activity of *T. catappa*.

In the present study, 10 different concentrations of the aqueous extract of *T. catappa* bark, fruits and wood have been evaluated for their HRBC membrane stabilization activity. High concentration (1000 µg/ml) of the bark extract was found to stabilize the HRBC membrane up to 86.45% (Table 1), which is comparable to the activity of the standard analgesic Aspirin (80.68%). All the extracts exhibited membrane stabilization activity in a dose-dependent manner. The aqueous extracts of bark and fruit exhibited membrane stabilization activity in a satisfactory manner even at a concentration of 15 µg/ml.

The erythrocyte membrane is analogous to the lysosomal membrane [26] and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzyme and proteases, which causes further tissue inflammation and damage upon extra-cellular release [27]. The lysosomal enzymes released during inflammation can produce various disorders. The extra-cellular activity of these enzymes is related to acute or chronic inflammation. The NSAIDs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane [28]. The activity of the presently studied *T. catappa* extracts was comparable to the action of NSAIDs.

Table 1: HRBC membrane stabilization activity of the aqueous extract of *Terminalia catappa*

S. No.	Concentration (µg/ml)	Stabilization (%)		
		Bark	Fruits	Wood
1	1.95	12.55 ± 1.55	48.77 ± 0.80	1.71 ± 0.78
2	3.90	17.91 ± 1.30	51.64 ± 0.42	3.11 ± 0.63
3	7.81	22.33 ± 2.94	53.82 ± 0.19	4.81 ± 0.21
4	15.62	63.57 ± 2.23	55.32 ± 0.35	5.37 ± 4.10
5	31.25	62.83 ± 4.01	57.23 ± 0.19	5.67 ± 0.42
6	62.50	67.17 ± 5.47	59.73 ± 0.19	18.01 ± 2.48
7	125	73.33 ± 5.79	61.82 ± 0.19	29.57 ± 1.66
8	250	79.80 ± 7.92	64.19 ± 0.77	44.61 ± 0.21
9	500	84.23 ± 5.83	66.19 ± 0.10	53.91 ± 6.29
10	1000	86.45 ± 12.63	68.37 ± 0.26	59.91 ± 4.03
11	Aspirin (100 µg/ml)	80.68 ± 0.51	80.68 ± 0.51	80.68 ± 0.51

Inhibition of Protein Denaturation

Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure due to an external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of tissue protein is one of the well-documented causes of inflammatory and arthritic diseases. Production of auto antigens in certain arthritic disease may be due to denaturation of proteins *in vivo* [29]. Agents that can prevent protein denaturation could be a useful method in anti-inflammatory drug development research. The ability of a plant extract to inhibit protein denaturation can be studied to assess the anti-inflammatory potential of the selected extracts.

In the present study, the aqueous extract of *T. catappa* bark exhibited high degree of inhibition of protein denaturation (Table 2). The result indicates that the extract at a concentration of 1000 µg/ml inhibited the protein denaturation up to 94.41%, where as the extracts of wood and fruits exhibited 87.15% and 76.72% respectively. All the three extracts inhibited protein denaturation remarkably even at a concentration of 30 µg/ml. The activity was dose-dependent, which has been found to be increased with increase in the concentration of the extract.

Table 2: Protein denaturation inhibitory activity of the aqueous extract of *Terminalia catappa*

S. No.	Concentration (µg/ml)	Inhibition (%)		
		Bark	Fruits	Wood
1	1.95	29.55 ± 1..57	51.49 ± 0.16	0.00
2	3.90	30.34 ± 1.42	53.27 ± 0.16	0.00
3	7.81	32.56 ± 5.50	54.20 ± 0.13	10.25 ± 6.86
4	15.62	41.92 ± 4.79	56.20 ± 0.53	26.20 ± 2.82
5	31.25	59.25 ± 1.56	58.23 ± 0.08	50.20 ± 1.69
6	62.50	63.27 ± 13.48	60.14 ± 0.05	59.60 ± 3.11
7	125	63.63 ± 4.39	64.08 ± 0.17	68.10 ± 13.43
8	250	74.91 ± 6.27	68.44 ± 0.03	75.26 ± 12.94
9	500	88.33 ± 5.94	73.36 ± 0.06	81.66 ± 4.03
10	1000	94.41 ± 16.52	76.72 ± 0.08	87.15 ± 3.60
11	Aspirin (100 µg/ml)	82.21 ± 0.14	82.21 ± 0.14	82.21 ± 0.14

Protease Inhibitory Activity

Neutrophils are known to be a rich source of serine protease and are localized at lysosomes. It was previously reported that

leukocytes protease play an important role in the development of tissue damage during inflammatory reaction and significant level of protection was provided by protease inhibition. The aqueous extracts of *T. catappa* bark, fruits and wood exhibited significant protease inhibition activity at different concentrations as shown in Table 3.

The bark extract exhibited proteinase inhibitory activity up to 95.51%, where as the extracts of fruits and wood exhibited up to 91.26% and 87.79% respectively, which are comparable to that of standard Aspirin (94.74%). The extracts of bark and fruits exhibited proteinase inhibitory activity of more than 70% even at a very low concentration i.e., 1.9 µg/ml. Proteinase have been implicated in arthritic reactions. It was already reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors. Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the anti-inflammatory activity of many plants [30]. Due to the presence of bioactive compounds, the *T. catappa* extracts might have shown anti inflammatory activity.

Table 3: Proteinase inhibitory activity of the aqueous extract of *Terminalia catappa*

S. No.	Concentration (µg/ml)	Inhibition (%)		
		Bark	Fruits	Wood
1	1.95	74.62 ± 0.08	83.38 ± 0.13	8.09 ± 1.83
2	3.90	77.65 ± 1.57	85.01 ± 0.02	12.99 ± 4.80
3	7.81	83.24 ± 0.59	86.17 ± 0.09	22.99 ± 5.09
4	15.62	85.46 ± 0.39	87.30 ± 0.02	33.25 ± 5.86
5	31.25	86.56 ± 2.75	87.78 ± 0.05	42.95 ± 10.97
6	62.50	89.30 ± 1.57	89.38 ± 0.10	51.64 ± 11.38
7	125	87.61 ± 1.57	89.88 ± 0.03	59.99 ± 2.55
8	250	83.36 ± 1.37	90.13 ± 0.05	74.47 ± 5.16
9	500	92.70 ± 5.50	90.72 ± 0.05	79.94 ± 5.16
10	1000	95.51 ± 6.09	91.26 ± 0.05	87.79 ± 1.56
11	Aspirin (100 µg/ml)	94.74 ± 0.21	94.74 ± 0.21	94.74 ± 0.21

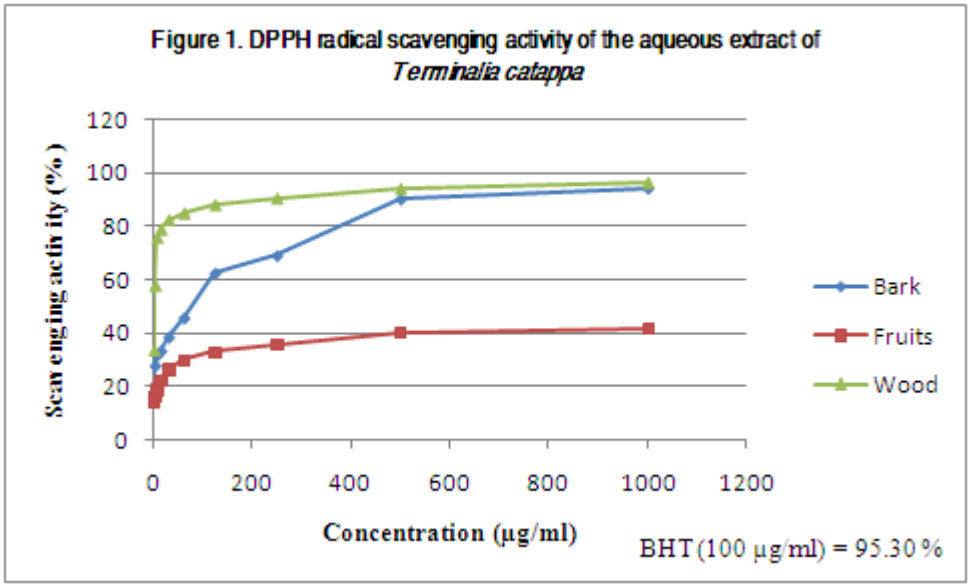
In vitro Antioxidant Activity

The ROS such as superoxide anion radical, hydrogen peroxide, hydroxyl radical, and singlet oxygen are capable of damaging DNA and Proteins at cellular level. Free radicals contribute more than one hundred disorders in human beings including atherosclerosis, hypertension, arthritis, ischemia, gastritis, central nervous system injury, reperfusion injury of many tissues, cancer, Alzheimer's disease, parkinsonism, diabetes mellitus and AIDS [31, 32]. These adverse effects of ROS are controlled by a system of enzymic and non-enzymic antioxidants in biological system. These antioxidants eliminate pro-oxidants and scavenge free radicals [33]. There are considerable evidences that antioxidants could prevent these diseases because they have the capacity to quench free radicals [34]. Apart from endogenous source, external antioxidants have also been reported to prevent the oxidative damage caused by the ROS and protect from oxidative-stress mediated diseases. Although some synthetic antioxidants, such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT), exhibit potent free radical scavenging effects, they have been demonstrated to exert toxicological effects as compared to natural antioxidants [35, 36]. Hence, there is a great emerging interest on natural source of antioxidants. Antioxidant compounds obtained from natural sources such as grains, oilseeds, beans, leaf waxes, bark, roots, spices, fruits and vegetables have been investigated by Chen *et al.* [37].

DPPH Radical Scavenging Activity

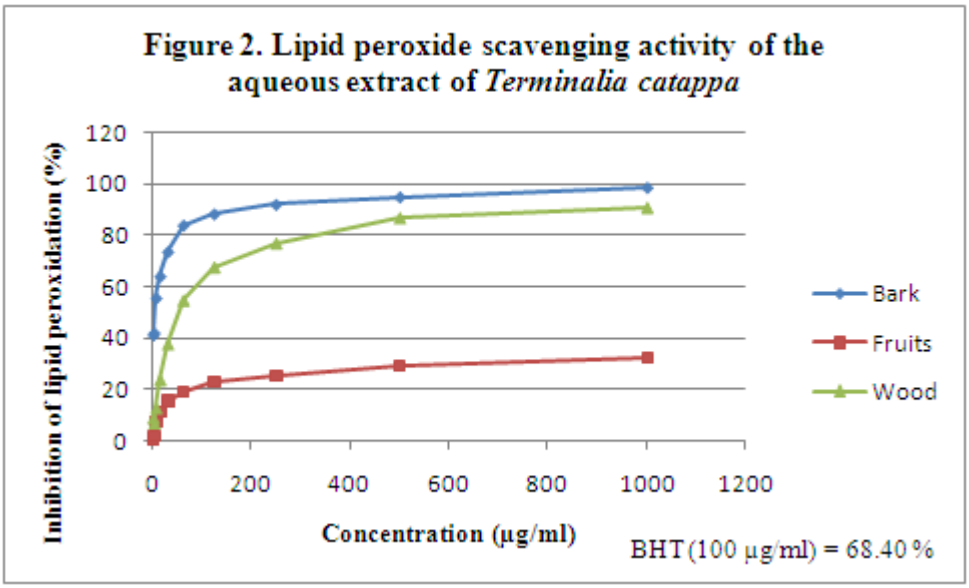
In the present study, the radical scavenging activity of different parts of *T. catappa* extracts was tested using methanolic solution of a stable free radical, DPPH (2, 2-diphenyl-1-picrylhydrazyl). Unlike laboratory generated free radical such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition brought about by various additives. A freshly prepared DPPH solution exhibits a deep purple colour, which get fades/disappears when an antioxidant is present in the medium. Thus, antioxidant molecule can quench DPPH free radicals (conceivably by providing hydrogen atom or by electron transfer)

and convert them to a colourless product (substituted analogous hydrazine) resulting in a decrease in absorbance at 518 nm [38]. Figure 1 reveals the free radical scavenging activity of different parts of *T. catappa* extracts and the results are expressed as percentage. The aqueous extract of wood exhibited radical scavenging activity of 96.5% where as bark exhibited 94.14% activity at 1000 µg/ml. Radical scavenging activity was found to be low for fruits (41.57%) as compared to bark and wood. Such radical scavengers may protect tissues from ROS and thereby prevent oxidative-damage related diseases [39].



Lipid Peroxidation Inhibition Activity

Free radical induced lipid peroxidation has been associated with a number of diseases [40]. Lipid peroxidation of cell membrane has been associated with a number of pathologic phenomena such as cancer, diabetes mellitus and inflammatory disorders. In the present study, the aqeuous extract of *T. catappa* bark, fruits and wood have been screened for their antioxidant activity using lipid peroxide scavenging assay. The bark extract exhibited maximum activity of 98.25% at 1000 µg/ml while wood extract exhibited 90.55%, but the activity was found to be low in fruit extracts of *T. catappa* (Figure 2).



Conclusion

Present paper deals with *in vitro* studies on anti-inflammatory and antioxidant potentials of *Terminalia catappa* bark, fruits and wood. Among the three parts investigated the bark of *T. catappa* exhibited higher anti-inflammatory and antioxidant activities. Results obtained from the present study provide scientific evidences for the use of this plant in folk medicine. Further, the present study suggests that parts of *T. catappa* could serve as a lead in the development of a novel herbal anti-inflammatory and antioxidant agent.

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