STUDIES ON THE ANTIOXIDATIVE AND FREE RADICAL SCAVENGING ACTIVITIES OF MYROBALAN (*TERMINALIA CHEBULA* RETZ) THROUGH VARIOUS IN VITRO MODELS

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Summary

Terminalia chebula Retz. (Combretaceae), a native plant in India and traditionally been used to treat various ailments in Asia. To understand the mechanisms of pharmacological actions, the *in vitro* antioxidant activity of aqueous extract of *T. chebula* was investigated for activities of scavenging superoxide anion radicals, hydroxyl radicals, nitric oxide radicals, and hydrogen peroxide, metal chelation and reducing power. The extract was also studied for lipid peroxidation assay using young and aged rat brain mitochondria. The total phenolic content was assayed as gallic acid equivalents. In all the testing, a significant correlation existed between concentrations of the extract and percentage inhibition of free radicals, metal chelation, reducing power or inhibition of lipid peroxidation. The antioxidant property may be related to the antioxidant vitamins, phenolic acids, tannins and micronutrients present in the extract. These results indicate that the antioxidant potential of *T. chebula* and is effective in degenerative diseases.

Keywords: Antioxidants; Lipid peroxidation; Radical scavenging; *Terminalia chebula*

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Introduction

Reactive oxygen species (ROS) in the forms of superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2) are generated by normal metabolic processes or from exogenous factors and agents. These ROS are capable of damaging a wide range of essential biomolecules [1] such as carbohydrates, proteins, lipids and DNA [2], thus accelerating aging, cancer, cardiovascular diseases, neurodegenerative diseases and inflammation [3,4]. Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. They exert their effects by scavenging or preventing the generation of ROS [1]. Flavonoids, tannins and other phenolic constituents from plant origin are potential antioxidants [5] and they plays an essential role in the prevention of neurodegenerative diseases, including Parkinson's and Alzheimer's diseases [6] as well as problems caused by cell and cutaneous aging [7].

Chebulic myrobalan (*Terminalia chebula* Retz.) belonged to the family Combretaceae known, as 'Kadukkai' in Tamil is a native plant in India and found in the deciduous forests. It is a carminative, deobstruent, astringent and expectorant agent [8] and used in Indian system of medicines such as Ayurveda and Siddha for treating liver diseases, urinary disorders, and heart diseases, ulcer, diabetes, arthritis, neuropathy, memory loss, etc. [9]. It is a well-known ayurvedic rasayana and also possess adaptogenic property [10]. *T. chebula* is one of the ingredients in popular ayurvedic formulation of Triphala [11]. The important active principle constituents of *T. chebula* are chebulagic, chebulinic acid, corilagin [12], beta-sitosterol, gallic acid, terchebulin, caffeic acids, carbohydrates, etc. [13]. It is highly nutritious and could be an important source of dietary supplement in vitamin C, energy, protein, amino acids and mineral nutrients [14]. Pharmacological actions of *T. chebula* indicate that cardioprotective [15], antioxidant activity [16], anticancer [17], antidiabetes [18], antimutagenic [19] and hypolipidemic [20].

The aim of this study was to investigate the in vitro antioxidant activity of the aqueous extract of *T. chebula* included superoxide anion radical scavenging, hydroxyl radical scavenging, nitric oxide scavenging and hydrogen peroxide scavenging activities, metal chelating activity, reducing power and lipid peroxidation inhibition assay using young and aged rat brain mitochondria.

Methods

Chemicals

Nitro blue tetrazolium (NBT), ethylene diamine tetra acetic acid (EDTA), sodium nitroprusside (SNP), trichloro acetic acid (TCA), thio barbituric acid (TBA), potassium hexa cyano ferrate [$K_3Fe(CN)_6$], and L-ascorbic acid were purchased from Sisco Research Laboratories Pvt. Ltd., India. All other chemicals and solvents used were of analytical grade available commercially.

Preparation of aqueous extract of *T. chebula*

The fruits of *T. chebula* ripen from November to March and fall soon after ripening. The fully ripe fruits were collected from Kolli hills, Tamilnadu, India during the month of January 2005 from the ground as soon as they have fallen and shade dried. Hundred gm of dried fruit skins were hammered in to small pieces followed by extraction with 800 ml distilled water for 24 h in water bath at 40^{o}C and repeated for two times. The extracts were then combined, concentrated and finally lyophilized to dry. The final yield of the water extracts was 43.7 g. The extract was re-dissolved in distilled water for further experiments.

Evaluation of antioxidant activity

Superoxide anion scavenging activity assay

The scavenging activity of the *T. chebula* towards superoxide anion radicals was measured by the method of Liu *et al*. [21]. Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 ml of Tris-HCl buffer (100mM, pH 7.4) containing 0.75 ml of NBT (300 μM) solution, 0.75 ml of NADH (936 μM) solution and 0.3 ml of different concentrations of the extract. The reaction was initiated by adding 0.75 ml of PMS (120 μM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured with a spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation:

% Inhibition = $[(A_0-A_1) / A_0 \times 100]$,

where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Hydroxyl radical scavenging activity assay

The scavenging activity for hydroxyl radicals was measured with Fenton reaction [22]. Reaction mixture contained 60 μl of 1.0mM $FeCl_2$, 90 μl of 1mM 1,10-phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 1.5 ml of extract at various concentrations and adding 150 μl of 0.17 M H_2O_2 started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560nm was measured with a spectrophotometer. The hydroxyl radicals scavenging activity was calculated according to the following equations:

% Inhibition = $[(A_0-A_1) / A_0 \times 100]$,

where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Nitric oxide scavenging activity assay

Nitric oxide radical scavenging activity was determined according to the method reported by Garrat [23]. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. 2 ml of 10mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract at various concentrations and the mixture incubated at 25°C for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 ml naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm was measured with a spectrophotometer. The nitric oxide radicals scavenging activity was calculated according to the following equations:

% Inhibition = $[(A_0\text{-}A_1) / A_0 \times 100]$,

where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Hydrogen peroxide scavenging activity assay

Hydrogen peroxide scavenging activity of the extract was estimated by replacement titration [24]. Aliquot of 1.0 ml of 0.1 mM H_2O_2 and 1.0 ml of various concentrations of extracts were mixed, followed by 2 drops of 3% ammonium molybdate, 10 ml of 2 M H_2SO_4 and 7.0 ml of 1.8 M KI. The mixed solution was titrated with 5.09 mM NaS_2O_3 until yellow color disappeared. Percentage of scavenging of hydrogen peroxide was calculated as

% Inhibition = $(V_0 - V_1) / V_0 \times 100$

where V_0 was volume of NaS_2O_3 solution used to titrate the control sample in the presence of hydrogen peroxide (without extract), V_1 was the volume of NaS_2O_3 solution used in the presence of the extracts.

Fe^{2+} chelating activity assay

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of Dinis *et al*. [25]. To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of $FeCl_2$ (2 mM) was added. After 30 s, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}–Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated as

% Chelating rate = $(A_0 - A_1) / A_0 \times 100$

where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Reducing power assay

The Fe^{3+} reducing power of the extract was determined by the method of Oyaizu [26] with slight modifications. The extract (0.75 ml) at various concentrations was mixed with 0.75 ml of phosphate buffer (0.2 M, pH 6.6) and 0.75 ml of potassium hexacyanoferrate [$K_3Fe(CN)_6$] (1%, w/v), followed by incubating at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75 ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 3000 r/min for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride ($FeCl_3$) solution (0.1%, w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

Lipid peroxidation inhibition assay using young and aged rat brain mitochondria

Young (3-4 months, 120-150 g) and aged (22-24months, 380-410 g) Wistar albino rats were anaesthetized with Thiopentone sodium (50mg / kg); brain was excised and washed with 0.95 NaCl solution. Tissue homogenates were prepared in ice-cold 3 mM Tris buffer containing 250 mM sucrose and 0.1 mM EDTA (pH 7.4). Centrifugation and their protein content characterized the mitochondrial fraction. The inhibition of lipid peroxidation assay was determined according to the method of Okhawa *et al*. [27] with minor modifications. 0.25 ml of mitochondria was mixed with 1.25 ml Tris-HCl buffer (pH 7.2), 1.0 ml 15 mM $FeSO_4$ solution and 0.5ml of extract at various concentrations. The mixture was incubated at 37^{o}C for 1 h, 0.1 ml of this reaction mixture was taken in a tube containing 1.5 ml 10% TCA. After 10 min tubes were centrifuged and supernatant was separated and mixed with 1.5 ml of 0.67% TBA in 50% acetic acid. The mixture was heated in a hot water bath at 85^{o}C for 30 min to complete the reaction. The intensity of pink coloured complex formed was measured at 535nm. The values of MDA were expressed as nmol/mg of protein.

Estimation of total phenol content

The total soluble phenolic content (g/100 g extract) present in the water extract of *T. chebula* was analyzed using the Folin-Ciocalteu reagent method [28]. Extract solution (0.1 ml containing 1000 μg) was transferred to a 100-ml Erlenmeyer flask, and then the final volume was adjusted to 46 ml by the addition of distilled water. Afterward, 1 ml of Folin-Ciocalteu Reagent (FCR) was added into this mixture, and after 3 min, 3 ml of Na_2CO_3 (2%) was added. Subsequently, the mixture was shaken on a shaker for 2 h at room temperature, and then absorbance was measured at 760 nm. The concentration of total phenolic content in the *T. chebula* determined as gallic acid equivalents.

Statistical analysis

Tests were carried out in triplicate for 3–5 separate experiments. The amount of extract needed to inhibit free radicals concentration by 50%, IC_{50}, was graphically estimated using a linear regression algorithm.

Results and Discussion

Superoxide anion radical scavenging activity

Superoxide is biologically important since it is very harmful to the cellular components in a biological system [29]. Superoxide anion plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins, and DNA [30]. In this assay, superoxide anions were generated in a non-enzymatic PMS-NADH system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of

NBT which is measured spectrophotometrically at 560nm. The superoxide anion radical scavenging activities of the extract from *T. chebula* was shown in Table 1. The superoxide scavenging activity of *T. chebula* was increased markedly with the increase of concentrations. The half inhibition concentration (IC_{50}) of *T. chebula* was 0.031 mg ml^{-1}. These results suggested that *T. chebula* had notably superior superoxide radical scavenging effects.

Hydroxyl radical scavenging activity

Hydroxyl radical is the most reactive oxygen species among all reactive oxygen species owing to its strong ability to react with various biomolecules. Hydroxyl radical reacts with several biological materials oxidatively by hydrogen withdrawal, double-bond addition, electron transfer and radical formation, and initiates autoxidation, polymerization, and fragmentation [31]. Table 1 showed the *T. chebula* exhibited concentration dependent scavenging activities against hydroxyl radicals generated in a Fenton reaction system. The IC_{50} of *T. chebula* was 0.097 mg ml^{-1}. The potential scavenging abilities of phenolic substances might be due to the active hydrogen donor ability of hydroxy substitution. Similarly, high molecular weight and the proximily of many aromatic rings and hydroxyl groups are more important for the free radical scavenging by tannins than their specific functional groups [32]. Therefore, higher hydroxyl scavenging activity shown in the extract can be used to minimize the adverse effects from the hydroxyl radical.

Table 1. Radical scavenging activity of aqueous extract of *T. chebula* at different concentrations.

Concentration (μg ml^{-1})	Superoxide radical scavenging %	Hydroxyl radical scavenging %	Nitric oxide radical scavenging %
10	36.21 ± 2.22	47.27 ± 2.22	14.25 ± 0.88
50	61.53 ± 2.18	58.18 ± 2.39	18.68 ± 1.22
100	95.61 ± 2.83	67.27 ± 3.06	24.67 ± 0.96
250	-	76.36 ± 3.04	32.55 ± 1.35
500	-	87.27 ± 2.58	41.22 ± 1.28
750	-	96.36 ± 2.69	49.76 ± 1.69
1000	-	-	59.43 ± 2.04
IC_{50} (mg ml^{-1})	0.031	0.097	0.744

Values are means ± SD (n=3).

Nitric oxide radical scavenging activity

Nitric oxide (NO) is a gaseous free radical, which has important roles in physiological and pathological conditions. Marcocci *et al*. [33] reported that scavengers of nitric oxide compete with oxygen, leading to a reduction in the production of nitric oxide. *T. chebula* aqueous extract inhibited nitric oxide in dose dependent manner (Table 1) with the IC_{50} being 0.744 mg ml^{-1}. The reactivities of $NO^{\cdot}$ and $O2^{\cdot -}$ were found to be relatively low, but their metabolite $ONOO^{-}$ (peroxynitrite) is extremely reactive and directly induces toxic reactions, including SH-group oxidation, protein tyrosine nitration, lipid peroxidation and DNA modifications [34,35]. Therefore, the strong NO scavenging effect of aqueous extract was showed that *T. chebula* might contain $NO^{\cdot}$ scavengers. Thus, the NO scavenging effect observed in *T. chebula* can be used to minimize or retard the damage from $NO^{\cdot}$ radicals.

Hydrogen peroxide scavenging activity

Hydrogen peroxide, a reactive nonradical compound, is very important as it can penetrate biological membranes. Although H_2O_2 itself is not very reactive, it may convert into more reactive species such as singlet oxygen and hydroxyl radicals [36]. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects [37]. Also it can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. As shown in Fig. 1, *T. chebula* extract demonstrated hydrogen peroxide scavenging activity in a concentration dependent manner with an IC_{50} of 0.659 mg ml^{-1}. It is biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate.

The ferrous ion chelating activity

Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is

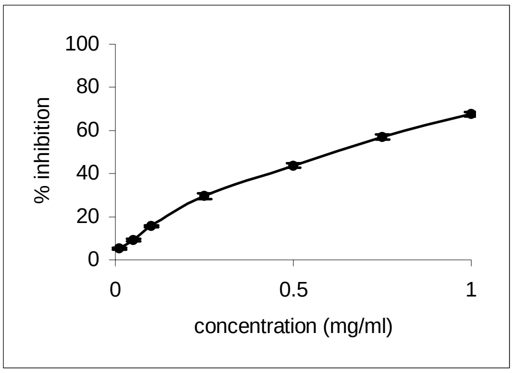

Figure.1. H_2O_2 scavenging activity of *T. chebula* aqueous extract at different concentrations. Each value represents means ± SD (n=3).

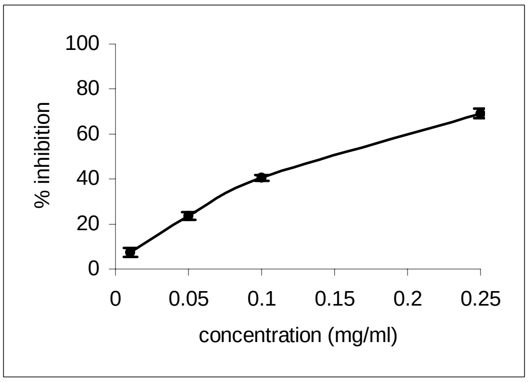

Figure.2. Ferrous ion chelating activity of *T. chebula* aqueous extract at different concentrations. Each value represents means ± SD (n=3).

decreased. Thus, the chelating effect of the coexisting chelator can be determined by measuring the rate of color reduction. The formation of the ferrozine– Fe^{2+} complex is interrupted in the presence of aqueous extract of *T. chebula*, indicating that have chelating activity with an IC_{50} of 0.163 mg ml^{-1} (Fig. 2). Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals [38]. Metal chelating activity can contribute in reducing the concentration of the catalyzing transition metal in lipid peroxidation. Furthermore, chelating agents that forms bonds with a metal are effective as secondary antioxidants because they reduce the redox potential, and thereby stabilize the oxidized form of the metal ion [39]. Thus, *T. chebula* demonstrate a marked capacity for iron binding, suggesting their ability as a peroxidation protector that relates to the iron chelating capacity. Flavanoids are phenolic compounds; their scavenging potential and metal chelating ability are dependent upon their unique phenolic structure and the number of position of the hydroxyl groups [40].

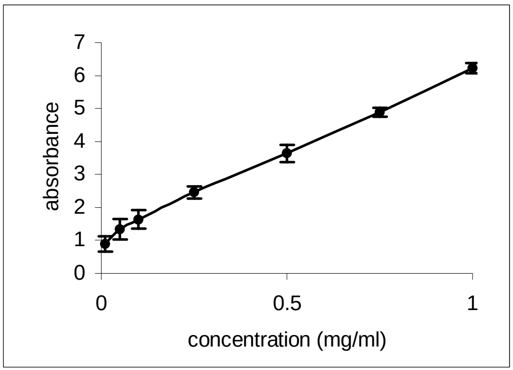

Figure.3. Reducing power of *T. chebula* aqueous extract at different concentrations. Each value represents means ± SD (n=3). High absorbance at 700 nm indicates high reducing power.

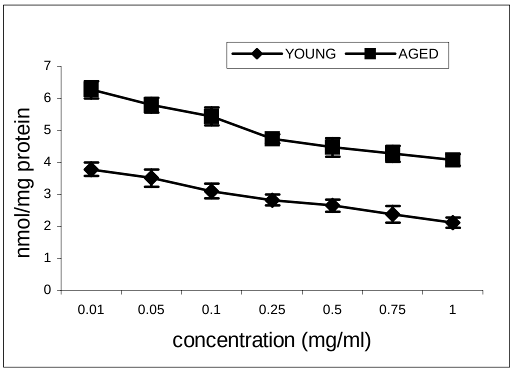

Figure.4. Activity of aqueous extract of *T. chebula* at different concentrations on ferrous sulphate induced lipid peroxidation in young and aged rat brain mitochondria. Each value represents means ± SD (n=3).

Reducing power activity

For the measurements of the reducing ability, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of *T. chebula*. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [41,42]. Fig. 3 depicts the reductive effect of *T. chebula*. Similar to the antioxidant activity, the reducing power of *T. chebula* increased with increasing dosage. All the doses showed significantly higher activities than the control exhibited greater reducing power, indicating that *T. chebula* consist of hydrophilic polyphenolic compounds that cause the greater reducing power.

Lipid peroxidation inhibition assay using young and aged rat brain mitochondria

Initiation of the lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex or through $^{\cdot}OH$ radical by Fenton’s reaction. Fig. 4 shows that the *T. chebula* extract inhibited $FeSO_4$ induced lipid peroxidation in young and aged rat brain mitochondria as a dose dependent manner. The inhibition could be caused by absence of ferryl-perferryl complex or by scavenging the $^{\cdot}OH$ radical or the superoxide radicals or by changing the Fe^{3+}/Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself. Iron catalyses the generation of hydroxyl radicals from hydrogen peroxide and superoxide radicals. The hydroxyl radical is highly reactive and can damage biological molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids, lipid hydroperoxides is produced [43]. Lipid hydroperoxide can be decomposed to produce alkoxy and peroxy radical they eventually yield numerous carbonyl products such as malondialdehyde (MDA). The carbonyl products are responsible for DNA damage, generation of cancer and aging related diseases [27]. Thus the decrease in the MDA level in young and aged with the increase in the concentration of the extract indicates the role of the extract as an antioxidant as well as adaptogen.

Total phenolic contents

The total soluble phenolic contents of water extract of *T. chebula* was 52.68 ± 3.69 g gallic acid equivalents. Phenolics are the secondary metabolite and they have much attention as potential natural antioxidant for their ability to act as both efficient radical scavengers and metal chelator. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donators and single oxygen quenchers [44].

Using different free radical scavenging systems, it can be said that the *T. chebula* aqueous extract have significant antioxidant activity. Although we have not isolated the compounds responsible for the antioxidant activity, we speculate that it may be related to the flavonoids, vitamins, phenolic acids or tannins in the *T. chebula* extract. Their antioxidant properties likely contributed to their usages in aging and the age related diseases. Overall, the plant extracts, as sources of antioxidants, are important in health promoting and disease prevention.

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