EVALUATION OF *IN VITRO* ANTIOXIDANT AND FREE RADICAL SCAVENGING EFFECTS OF *TERMINALIA ARJUNA* LEAF

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Suumary

Terminalia arjuna Roxb (Combretaceae) is commonly known as Arjuna, is a large tree grown throughout the Indian peninsula. Present study evaluated the *in vitro* antioxidant and free radical scavenging potential of petroleum ether, chloroform and methanol extracts of *T. arjuna* leaf. The different antioxidant assays, including DPPH radical scavenging, total reductive activity, super oxide anion radical, nitric oxide scavenging, lipid peroxidation and total phenolic content were estimated. Ascorbic acid was used as the reference. All of the extracts exhibited potent *in vitro* antioxidant activity that increased with extract concentration. The different concentrations of all the extracts showed effective free radical scavenging, reducing power, super oxide anion scavenging, nitric oxide scavenging, lipid peroxidation and total phenolic content depending on concentration. Present findings showed marked *in vitro* antioxidant activity of different extracts of *Terminalia arjuna* leaf.

Key words: Free radical scavenging, *in vitro* antioxidant, *Terminalia arjuna*.

Introduction

Antioxidants protect living organisms from damage caused by uncontrolled production of reactive oxygen species (ROS) and the concomitant lipid peroxidation, protein damage and DNA strand breaking. Current interest is focused on the potential role of antioxidants and antioxidant enzymes in the treatment and prevention of atherosclerosis, heart failure, neurodegenerative disorders, aging, cancer, diabetes mellitus and several others diseases (1).

Antioxidants are added to a variety of foods to prevent free radical induced lipid peroxidation, which is responsible for the development of off-flavors and the undesirable chemical compounds in food (2). These ROS cause destructive and irreversible damage to the components of a cell, such as lipids, proteins DNA and other macromolecules. Although normal cells possess antioxidant defense systems against ROS in the cells induces diseases such as cancer and aging (3).

ROS are formed and degraded by all aerobic organisms. ROS can readily react with most biomolecules including proteins, lipids, lipoproteins and DNA. Exogenous chemical and endogenous metabolic processes in the human body or in the food system might produce highly reactive oxygen species, which are capable of oxidizing biological molecules, resulting in tissue damage and cell death. When the mechanism of antioxidant protection becomes unbalanced by exogenous and endogenous factors, it results in inflammation, diabetes, genotoxicity, cancer and accelerating aging (4).

Antioxidant supplements or foods containing antioxidants may be used to help the human body reduce oxidative damage. The most commonly used antioxidants are BHA, BHT, propyl gallate and tert-butyl-hydroquinone (5). However, they have been suspected of being responsible for liver damage and carcinogenesis in laboratory animals. Therefore, the development and use of more effective antioxidants is desired.

Traditional medicine worldwide is being reevaluated by extensive research on different plant species and their therapeutic principles. Plants produce antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity.

Terminalia arjuna Roxb (Combretaceae) is commonly known as Arjuna, is a large tree, often with buttressed trunk, smooth grey bark and about 20-25 m in height. Leaves are usually sub-opposite, oblong or elliptic-long, pale dark green above and pale-brown beneath, 10-20 cm long and hard. The flowers are yellowish-white. The fruits are 2.5-5.0 cm ovoid or ovoid-oblong, fibrous-woody, glabrous. It is common on the banks of rivers, streams and dry watercourses in Sub-Himalayan-tract and in Central and South India and the West Bengal. The bark of the plant is known to contain a crystalline compound arjunine, a lactone, arjunetin, essential oil and reducing sugar. Beside this it also contains 34% calcium carbonate, 9% of other salts of calcium, 13% tannin and aluminum, magnesium, organic acids, coloring matter and other substances (6). The fruits of the plant are used as tonic (7). Externally, its leaves are used as a cover on sores and ulcer. The bark is anti dysenteric, antipyretic, astringent, cardiotonic, lithotriptic and tonic (8) and powder of the bark acts as diuretic in cirrhosis of liver and gives relief in symptomatic hypertension. A decoction of this thick bark made with milk is given every morning on an empty stomach or its powder with milk and gur as a cardiotonic (9). The powder of the bark is also given with honey in fractures and contusions with echymosis. Beside this, the extract of the bark as astringent is used for cleaning sores, ulcers and cancers etc. An ointment made from this bark by mixing with honey is used to cure acne. The ashes of the bark are prescribed in scorpion-stings (10).

Present study evaluated the *in vitro* antioxidant and free radical scavenging activity of petroleum ether, chloroform and methanol extracts of *T. arjuna* leaf. An important objective of this research was to compare *in vitro* antioxidative potential of petroleum ether, chloroform and methanol extract of *Terminalia arjuna* leaf.

Materials and methods

Plant material: The mature leaves of *Terminalia arjuna* were collected during January 2008 from Nadia, West Bengal, India. The plant material was taxonomically identified by Dr. Lakhmi Narashimhan, Scientist, Botanical Survey of India, Central National Herbarium, Howrah, West Bengal, India. The voucher specimen [CNH/I-I/(216)/2008/Tech.II/216] was maintained in our laboratory for future reference. The plant material was shade-dried with occasional shifting and then powdered with mechanical grinder, passing through sieve no. 40, and stored in an air-tight container.

Preparation of plant extract: The dried powdered material (350 g) was defatted with petroleum ether (60-80 °C), the percentage extractive value was 5.98 % w/w. The defatted powder material thus obtained was further extracted with chloroform and methanol for 72 h in a percolator. The solvent was distilled off in reduced pressure and resulting semisolid mass was vacuum dried using rotary flash evaporator to yield a solid residue and the percentage extractive values were accordingly 15.96 % w/w and 21.45 % w/w respectively. The preliminary phytochemical analysis was performed to identify the phytoconstituents present in the extracts (11).

Chemicals: Ammonium thiocyanate was purchased from E Merck (Mumbai, India) and L-ascorbic acid (vitamin C) from Sigma Chemical Co Ltd. (Mumbai, India). Ferrous chloride, ferric chloride, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), nitroblue tetrazolium (NBT), thiobarbituric acid (TBA), tricholoacetic acid (TCA), pyrocatechol, phenazine methosulfate (PMS), and potassium ferric cyanide were purchased from Sigma Chemical Co Ltd. (St. Louis, MO, USA). All other chemicals and reagent were of analytical grade obtained commercially.

Free radical scavenging activity measured by 1, 1-diphenyl-2-picryl-hydrazil: The free radical scavenging activity of all of the extracts were measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) using the reported method (12). Briefly, an 0.1 mM solution of DPPH in methanol was prepared, and 1 ml of this solution was added to 3 ml of the all of the extracts solution respectively in petroleum ether, chloroform and methanol at different concentrations (2, 4, 6, 8, 10, 15 µg/ml). The mixture were shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm using a UV-Vis spectrophotometer (Genesys 10 UV: Thermo Electron Corporation). Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = $[(A_0 – A_1) / A_0) \times 100]$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance of presence of all of the extract samples and standard. The results are stated in Table 1.

Total reduction capability by Fe^{3+}-Fe^{2+} transformation: The total reducing powers of all the extracts were determined according to the reported method (13). Briefly, different concentrations of all the extracts (10, 20, 40, 80, 160, 320 µg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml. 0.2 M. pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5ML., 1%). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (2.5ml, 10%) was added to the mixture, which was centrifuged for 10 min at 1000 g (Remi T8A, Mumbai, India). The upper layer of solution (2.5 ml) was mixed with distilled water (2.5ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm using a UV-Vis spectrophotometer (Genesys 10UV: Thermo Electron Corporation). Higher absorbance of the reaction mixture indicated greater reducing power. The results are presented in Table 2.

Super oxide anion radical scavenging activity in PMS-NADH system: Measurements of super oxide anion scavenging activity of all the extracts were based on the method described by previous workers (14) with slight modification. One milliliter of nitroblue tetrazolium (NBT) solution (156 µM NBT in 100mM phosphate buffer, pH 7.4), and various concentrations (10, 20, 40, 80, 160, 320 µg/ml) of sample solutions of all the extracts respectively in petroleum-ether, chloroform and methanol were mixed. The reaction started by adding 100 µl phenazine methosulfate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The mixtures were incubated at 25°C for 5 min, and the absorbance at 560nm was measured against blank samples. A decreased in the absorbance of reaction sample indicated increased super oxide anion scavenging activity and ascorbic acid was used as standard drug. The results are presented in Table 3.

Nitric oxide radical scavenging assay: *In vitro* nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which was measured by Griess reaction (15). Reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate-buffered saline (PBS) and various concentrations (10, 20, 40, 80, 160, 320 µg/ml) of the plant extract were incubated at 25°C for 150 min. At the end of incubation, 0.5ml of the reaction mixture was removed and 0.5 ml of Griess reagent (1 % sulfanilamide, 2 % H_3PO_4, and 0.1 % naphthylethylene diamine dihydrochloride) was added. The absorbance of chromophore formed was measured at 546 nm. The percentage inhibition of nitrite oxide generated was measured by comparing the absorbance values of control and test compounds. The results are reported in Table 4.

Determination of inhibition of lipid peroxidation: Rat liver homogenate was used as a source of polyunsaturated fatty acids for determining the extent of lipid peroxidation (16). Rat was sacrificed by cervical dislocation. The liver was collected and was homogenized with 40 mM Tris-HCl buffer (pH 7.0) and centrifuged at 3000 g for 10 min. Clear supernatant was taken and to it 100 µl of each of 0.15 M KCl, 15 mM $FeSO_4$ and 6 mM ascorbic acid were added and was incubated at 37°C for 1 hr. 1.0 ml of TCA (10 %) was added to the mixture and the sample was centrifuged at 3000 g for 20 min at 4°C to remove insoluble protein. 2.0 ml of the supernatant was removed and 1.0 ml TBA (0.8 %) was added to this fraction followed by heating at 90°C for 20 min in a water bath. After cooling the colored TBA-MDA complex was extracted with organic solvent (2.0 ml n-butanol) and absorbance was measured at 532 nm. The results are stated in Table 5.

Determination of total phenolic compounds: The total phenolic compounds in the leaves of *T. arjuna* at different concentrations were determined with Folin-Ciocalteu reagent using pyrocatechol as a standard phenolic compound (17). Briefly, 1 mg of all the extracts solution (1000 µg of extract) was placed in a 100 ml Erlenmeyer flask diluted with distilled water (46 ml). Folin-Ciocalteu reagent (1 ml) was added and the contents of the flask were mixed thoroughly. After 3 min, 3 ml of Na_2CO_3 (2 %) was added, then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in a spectrophotometer (Genesys 10 UV; Thermo Electron Corporation). The amount of total phenolic compounds in the fruits of *Dregea volubilis* was determined in micrograms of pyrocatechol equivalent, using the equation obtained from the standard pyrocatechol graph.

Absorbance = 0.001 × pyrocatechol (µg) + 0.0033

Statistical analysis: Experimental results were expressed as mean ± standard error of mean (SEM) of three parallel measurements.

Results and discussion

The qualitative chemical analysis of the leaves of *Terminalia arjuna* showed positive results for the presence of alkaloids, flavonoids, tannins, saponin glycosides, triterpenoids, steroids. Antioxidant methods and modifications have been proposed to evaluate antioxidant characteristics and to explain how antioxidants function. Of these, antioxidant activity, reducing power, free radical scavenging, superoxide anion radical scavenging, and nitric oxide radical inhibition activities and estimation of phenolic contents are most commonly used for the evaluation of the total antioxidant behavior of extracts.

Effect on scavenging DPPH radical: The stable DPPH radical model is a widely used, relatively quick method for the evaluation of free radical scavenging activity. The effects of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen-donating ability. DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The absorption maximum of a stable DPPH radical in methanol was 517 nm. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progressed, results in the scavenging of the radicals by hydrogen donation. It is visually noticeable that as a change in color from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the antioxidant activity of antioxidants (17). It has been reported that oxidative stress, which occurs when free radical formation exceeds the body's ability to protect itself, forms the biological basis of chronic conditions such as arteriocslerosis (16).

Based on the data obtained from this study, all the extracts were effective free radical inhibitor or scavenger, as well as a primary antioxidant that reacts with free radicals, which may limit free radical damage occurring in the human body. Figure 1 illustrates a significant decrease in the concentration of DPPH radicals due to the scavenging ability of the extracts and standard. Free radical scavenging activity also increased with increasing concentration in the range of 2-15 µg/ml.

Table 1. DPPH scavenging power of the different extracts of *T. arjuna* and Vitamin C.

Name the Extracts	Concentrations (µg/ml)	% of DPPH scavenging activity respectively	Mean ± SEM
Terminalia arjuna (Petroleum ether)	2, 4, 6, 8, 10, 15	26.28, 32.09, 38.69, 49.51, 67.69 80.67	49.15±8.68
Terminalia arjuna (Chloroform)	2, 4, 6, 8, 10, 15	29.31, 38.65, 47.98, 56.97, 69.85 79.55	53.71±7.73
Terminalia arjuna (Methanol)	2, 4, 6, 8, 10,15	45.69, 58.27, 69.35, 78.92, 86.79 95.81	72.47±7.57
Vitamin C	2, 4, 6, 8, 10, 15	25.70, 32.10, 42.50, 92.20 97.23	57.946±15.29

Effects on total reductive activity: For the measurement of the reductive it had been investigated that the Fe^{3+}-Fe^{2+} transformation in the presence extracts. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (18). The antioxidant activity of putative antioxidants has been attributed to various mechanisms, among which are the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity, and radical scavenging (19). Like the antioxidant activity, the reducing power of all the

extracts increased with increasing concentration of samples. Figure 2 shows the reductive capabilities of all the extracts compared with ascorbic acid. All the extracts concentrations tested showed higher activities than the control.

Table 2: Reducing power of the different extracts of *T. arjuna* and Vitamin C.

Name the Extracts	Concentrations (µg/ml)	% of Reductive ability respectively	Mean ± SEM
Terminalia arjuna (Petroleum ether)	10, 20, 40, 80, 160, 320	13.69, 29.61, 35.69, 49.51, 65.35, 78.18	45.38±9.727
Terminalia arjuna (Chloroform)	10, 20, 40, 80, 160, 320	37.29, 42.85, 52.52, 67.57, 84.27, 89.57	62.345±8.857
Terminalia arjuna (Methanol)	10, 20, 40, 80, 160, 320	29.35, 37.51, 49.36, 55.69, 71.14, 85.89	54.82±8.584
Vitamin C	10, 20, 40, 80, 160, 320	15.62, 38.75, 62.78, 79.87, 88.45, 91.45	62.82±1.348

Effects on superoxide anion radical scavenging activity: Superoxide anion radical (O_2^-) are formed by activated phagocytes such as monocytes, macrophages, eosinophils and neutrophills, and the production of O_2^- is an important factor in the killing of bacteria by phagocytes. In the PMS-NADH-NBT system, superoxide anion, derived from dissolved oxygen from the coupling reaction of PMS-NADH, reduces NBT (20). The decrease in absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixtures, Figure 3 showed the percent inhibition of superoxide radical generation by 20, 40, 60, 80, 100 µg/ml of all the extract compared with ascorbic acid. All the concentrations of all the extracts have a marked extent of superoxide radical scavenging activity compared with control.

Table 3: Super oxide scavenging of the different extracts of *T. arjuna* and Vitamin C.

Name the Extracts	Concentrations (µg/ml)	% of Super oxide scavenging activity respectively	Mean ± SEM
Terminalia arjuna (Petroleum ether)	10, 20, 40, 80, 160, 320	21.69, 31.37, 45.68, 56.98, 67.28, 80.59	50.598±9.033
Terminalia arjuna (Chloroform)	10, 20, 40, 80, 160, 320	29.34, 37.32, 49.35, 58.47, 69.79, 78.79	53.84±7.728
Terminalia arjuna (Methanol)	10, 20, 40, 80, 160, 320	37.18, 48.92, 56.15, 69.13, 78.45, 87.35	62.86±7.705
Vitamin C	10, 20, 40, 80, 160, 320	25.62, 35.25, 54.35, 69.23, 78.29, 89.64	58.73±10.90

Effects on nitric oxide (NO^-) scavenging activity: Nitric oxide (NO) and superoxide anion cause ischemic renal injury separately, and these radicals work together to bring about further damage. The toxicity and damage caused by NO and O_2 is multiplies as they react to produce reactive peroxynitrite, which leads to serious toxic reactions with biomolecules such as protein, lipids, and nucleic acids. High concentration of nitric oxide (NO) has deleterious effects, so it is

necessary that the production of NO be tightly regulated (21). When NO is produced by macrophages, the nitric oxide radical can be converted into peroxynitrites, which will cause diverse chemical reactions in a biological system including nitration of tyrosine residue of protein, triggering lipid peroxidation, inactivation of aconites, inhibition of mitochondrial electron transport and oxidation of biological thiol compound (22).

Suppression of NO released may be partially attributed to direct NO scavenging, as all concentrations of all the extracts decreased the amount of nitrite generated from the decomposition of sodium nitroprusside at physiologic pH was found to be inhibited by all the extracts. The concentration of all the extracts necessary for 50 % inhibition was found to be 62.26 µg/ml. The extracts showed effective of inhibition of nitric oxide production by activated peritoneal macrophages with *in vitro* conditions.

Table 4: Nitric oxide scavenging power of the different extracts of *T. arjuna* and Vitamin C.

Name the Extracts	Concentrations (µg/ml)	% of Super oxide scavenging activity respectively	Mean ± SEM
Terminalia arjuna (Petroleum ether)	10, 20, 40, 80, 160, 320	16.65, 26.69, 36.58, 59.68, 66.99, 79.38	47.66±10.084
Terminalia arjuna (Chloroform)	10, 20, 40, 80, 160, 320	14.63, 29.69, 38.65, 49.98, 61.99, 78.69	45.605±9.387
Terminalia arjuna (Methanol)	10, 20, 40, 80, 160, 320	23.69, 35.18, 49.65, 58.19, 71.18, 82.13	53.366±8.932
Vitamin C	10, 20, 40, 80, 160, 320	28.40, 45.82, 64.47, 76.87, 86.50, 95.32	66.23±10.362

Effects on lipid peroxidation: All the extracts retarded the peroxidation of linoleic acid (LH). The peroxidation of LH is well-known to be a chain reaction in which the chains are carried by linoleylperoxyl radicals, LOO., and the products are linoleyl hydroperoxides. The retardation of LH peroxidation by the extracts had been found to be due to rapid chain termination via a very fast cross-reaction between HOO. and LOO. radicals. This antioxidant mechanism is completely different from the mechanism of antioxidant action of vitamin E. Since vitamin E becomes a prooxidant at high concentrations, the addition all of the extracts to edible lipids may provide an alternative or supplementary strategy for obtaining large increases in their oxidative stability and shelf life, something that cannot be achieved by simply adding more and more vitamin C.

The antioxidant activity of rat liver peroxidation due to the presence of phytoconstituents such as flavonoids and biflavones has been reported. Therefore, the current study suggests the antioxidant activity of all the extracts may be attributed to the reduction of free radicals, chelation of metal ions or combination thereof presence of phytoconstituents.

Effects of total phenolic compounds: Phenolic constituents are very important in plants because of their scavenging ability due to their hydroxyl groups. A number of studies have focused on the biological activities of phenolic compounds, which are potential antioxidants and free scavengers. In addition, it has been reported that phenolic compounds are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation (23). 1 mg of each concentration of all the extracts contained 95.03 µg of pyrocatechol equivalents of phenols. These phenolic compounds may contribute directly to the antioxidative action. It has been

suggested that up to 1 g of polyphenolic compounds (from a diet rich in fruits and vegetables) ingested daily has inhibitory effects on mutagenesis and carcinogenesis in humans.

Table 5: Inhibition of lipid peroxidation of different extracts of *T. arjuna* and Vitamin C.

Name the Extracts	Concentrations (µg/ml)	% of inhibition of lipid peroxidation respectively	Mean ± SEM
Terminalia arjuna (Petroleum ether)	10, 20, 40, 80, 160, 320	13.78, 27.98, 38.67, 49.23, 60.57, 79.45	44.946±9.577
Terminalia arjuna (Chloroform)	10, 20, 40, 80, 160, 320	19.65, 31.43, 43.96, 58.45, 69.65, 81.92	50.843±9.617
Terminalia arjuna (Methanol)	10, 20, 40, 80, 160, 320	29.63, 36.27, 45.68, 59.25, 79.82, 89.36	56.66±9.797
Vitamin C	10, 20, 40, 80, 160, 320	27.54, 38.58, 48.98, 60.43, 75.19, 82.34	55.51±8.647

Conclusion

Based on the results of this study, it is clear that all of the test extracts exhibited powerful *in vitro* antioxidant and free radical scavenging capacity against various antioxidant systems. From the present results, it can be concluded that the antioxidant activity of all the extracts was concentration dependent with inhibition of lipid peroxidation. From the above assays, the possible mechanism of antioxidant activity of all the extracts included reductive ability, hydrogen-donating ability, and scavenging of superoxide, nitric oxide and free radicals, which may be due to the presence of phytoconstituents such as flavonoids and polyphenols present in the petroleum ether, chloroform and methanol extracts of *Terminalia arjuna* leaf.

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