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In Vitro Anti-hemolytic Activity of *Terminalia arjuna* (Roxb.) Wt. & Arn. Bark Powder Aqueous Extract

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ABSTRACT

Bark powder aqueous extract of Terminalia arjuna (AETA) has been used for quantification of phytochemical constituents viz., total phenolic, flavonoid and tannin contents (1120 mg/g gallic acid equivalents, 104 mg/g rutin equivalents, and 0.296 mg/g respectively). High-performance liquid chromatography analysis of the extract revealed the presence of specific phenolic compounds like β-sitosterol, catechin, rutin, and tannic acid. Antioxidant activity of the extract was determined by employing in vitro assays such as hydrogen peroxide, nitric oxide, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging and total reductive ability. The results were encouraging and comparable to standard antioxidants like Butylated hydroxyanisole/ascorbic acid. Plant extract exhibited a strong anti-hemolytic activity against free radical (H_2O_2) induced erythrocyte damage, which is a significant observation paving way for expanding its applications in treating many free radical induced pathophysiological conditions in humans.

Key words: *Terminalia arjuna, Phytochemicals, Radical scavenging, Anti-hemolysis.*

1. INTRODUCTION

Unhealthy dietary habits and lifestyle patterns (smoking and alcohol consumption), exposure to physical (ultraviolet [UV] light, ionizing radiations) or chemical agents (drugs, pollutants, pesticides) along with deficiencies in the physiological antioxidant defenses may result in pathological stress to the cells and tissues, which can have multiple effects [1]. Superoxide, hydroxyl, nitric oxide (NO), hydrogen peroxide, hypochlorous acid and peroxynitrite - collective termed as reactive oxygen/nitrogen species are deposited in the biological systems either by endogenous metabolic processes or by exogenous physical/chemical factors [2,3].

Free radical induced damage on biological membranes is best understood with erythrocytes as a promising model [4]. Due to their high membrane polyunsaturated fatty acid content and high cytoplasmic hemoglobin and oxygen contents, erythrocytes become easily prone for oxidative stress resulting in hemolysis causing the release of hemoglobin which is known to be a strong trigger in initiating lipid peroxidation and subsequent chain of events which all account for their structural and functional loss [5,6]. Imbalance in the proportions of free radicals generated and the inherent antioxidant repertoire of the system has been demonstrated to account for shortened lifespan of normal red cells during many pathological conditions like diabetes, infections, hypoxia and also due to chronic alcohol consumption and smoking; as all of them are characterized by an enhances free radical system deposition [7].

Herbal medicines by virtue of their multi-targeted and less toxic features have attracted attention of the society for the prevention of many diseases. The increasing scientific evidences in support of the rich phytoconstituent profile that the herbal product possesses and its resultant therapeutic potentials have created remarkable confidence, which is evidenced by a convincing rise in the number of people depending on them as an alternative medicine [8]. The present work is an attempt to look at the protective effect of *Terminalia arjuna* against free radical-induced oxidative damage of erythrocytes.

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T. arjuna (family: Combretaceae) is one of the most widely distributed avenue tree in India, and its parts (majorly bark) are used in several ayurvedic formulations [9]. It is a rich source of several therapeutic principles such as phenolic acids, glycosides and antioxidants like flavonoids and tannins [10]. Stem bark powder of *T. arjuna* (aqueous extract of *T. arjuna* [AETA]) has been used as a cardiotonic to treat several diseases related to cardiovascular system [11] and many studies have been conducted to establish its antioxidant [12], antilipidemic [13], antimicrobial [14], anti-diabetic [15], hepato and renal protective [16,17] and osteopotentiating [18] properties. The present study was conducted to evaluate the anti-hemolytic property of AETA.

2. MATERIALS AND METHODS

2.1. Plant Material

Shade dried bark of *T. arjuna* was procured from a local ayurvedic dealer and authenticated by Dr. Shiddamallayya N, National Ayurveda Dietetics Research Institute, Jayanagar, Bangalore and voucher specimens (Drug Authentication/SMPU/NADRI/BNG/2012-13/1129) were deposited in the institutes herbarium.

2.2. Chemicals

Standard polyphenols: Gallic acid, tannic acid, catechin, β sitosterol, rutin, quercetin and betain were of high performance liquid chromatography (HPLC) grade with 99% purity. 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide and butylated hydroxyanisole (BHA) were procured from Sigma Chemicals. All the other chemicals were of analytical grade and were procured from Merck and SDFCL.

2.3. AETA Preparation

Chunks of bark were made into smaller pieces by using mortar and pestle, and further powdered using a blender. For 25 g of powder taken in a conical flask, approximately 150 ml of distilled water was added to soak the powder completely, followed by the addition of chloroform (1 ml per 100 ml of extract mixture). The extract obtained by incubating the flask overnight on an orbital shaker was filtered using a muslin cloth, and the residue was subject to two more rounds of extraction. Filtrate pooled out of the three extractions was poured into steel trays and concentrated by keeping on the water bath set at 25-30°C. The deposited extract concentrate was scraped into petri plates, kept in a desiccator for 24-48 h to rid the scrapings of moisture. Dried extract was powdered using a mortar and pestle and stored in air tight containers. Percentage of yield for the above-mentioned procedure was found to be 35.07%.

2.4. Total Phenolic Content (TPC)

TPC of the extracts were determined by Folin–Ciocalteu's method [19]. Accordingly, to 1 mg of dry extract powder, 0.5 ml of the Folin–Ciocalteu reagent (1:1 diluted with distilled water) was added followed by addition of 7.5 ml of distilled water. The mixture was kept at room temperature for 10 min and 1.5 ml of aqueous sodium carbonate solution (20% w/v) was added. The final mixture was incubated in the water bath at 40°C for 20 min, cooled and the absorbance was read at 755 nm against a blank (solution with no extract added). Gallic acid was used as a standard and TPC was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract samples (mg GAE/g of dry extract).

2.5. Total Flavonoid Content (TFC)

TFC of the extracts was determined as employed by Meda et al., 2005 [20]. Briefly, to 2 ml of aqueous extract containing 1 mg/ml of dry matter, 2 ml of $AlCl_3$ (2% prepared in methanol) was added and incubated at room temperature for 10 min. Rutin (25-200 µg dissolved in methanol) was used as a standard flavonoid and TFC in the plant extract was expressed as milligrams of rutin equivalents (RE) per gram of dry extract sample (mg RE/g of dry extract).

2.6. Total Tannin Content (TC)

TC of the extracts was determined by Folin–Dennis method [21]. To 0.5 g of powdered material taken in a flask, 75 ml of distilled water was mixed and gently boiled for 30 min. Later the mixture was centrifuged at 1000 g for 20 min, and the supernatant was collected and made up to 100 ml with distilled water to prepare a stock solution. To 0.1 ml of stock, 7.5 ml of distilled water, 0.5 ml of Folin–Dennis reagent (10 g sodium tungstate, 2 g phosphomolybdic acid dissolved in 75 ml distilled water and 5 ml of phosphoric acid added and refluxed for 2 h and final volume made up to 100 ml), 1 ml of Na_2Co_3 (35% in distilled water and overnight incubated) and 0.9 ml distilled water were added, shaken well and incubated at room temperature for 30 min. Absorbance was read at 700 nm and the amount of tannin was quantified from the standard curve of tannic acid (50 µg/ml working standard).

2.7. Identification and Quantification of Polyphenols using HPLC

AETA (5 mg) was dissolved in 1 ml of methanol and 20 µL of this was injected onto a HPLC system equipped with UV-Visible (UV-Vis) detector (Shimadzu SPD10A UV-Vis, Japan) set at 280 nm. Polyphenols were chromatographically separated on a reverse phase Luna 5 µm C18 (2) (100 Å, LC Column 250 mm × 4.6 mm). A solvent mixture of methanol/phosphate buffer (pH 3) taken in the ratio of 70:30 was used as a mobile phase and with a flow rate of 1 ml/min the isocratic elution of hydrophobic polyphenols was achieved. Quantification of the polyphenols in AETA was achieved by comparing the chromatogram with that of standards (100 µg/ml) such as gallic acid, tannic acid, catechin, β sitosterol, rutin, quercetin and betain.

2.8. *In Vitro Antioxidant Activities*

2.8.1. Hydrogen peroxide scavenging activity

Hydrogen peroxide radical scavenging ability of the extracts were examined according to the method employed by Tuba and Gulcin, 2008 [22]; with minor modifications. To 3.4 ml of a plant extract and the standard antioxidant - ascorbic acid (dissolved at different concentrations of 5-100 µg/ml in phosphate buffer - 50 mM, pH 7.4), 0.6 ml of H_2O_2 was added and incubated at room temperature for 10 min. Decrease in the absorbance of H_2O_2 upon oxidation was monitored at 230 nm spectrophotometrically against suitable blank (phosphate buffer alone). BHA was used as a standard antioxidant and control (phosphate buffer and H_2O_2) was prepared and the percentage inhibition was calculated using the expression:

Percentage inhibition (%) = (OD of control − OD of extract/OD of control) × 100 (1)

The extracts concentration providing 50% of inhibition (EC_{50}) was calculated from the graph of percentage inhibition plotted against extract concentration.

2.8.2. NO

NO radical scavenging activities of plant extracts were assessed by using the method employed by Royer et al., 2011 [23]. Accordingly, 200 µL of sodium nitroprusside (SNP) was added to 800 µL of the extract taken at various concentrations i.e. 0.1-1 mg/ml dissolved in phosphate-buffered saline (PBS) (25 mM, pH 7.4) and the mixture was incubated at 37°C for 2.5 h under normal light exposure and further the samples were kept in dark at room temperature for 20 min. Thereafter, 300 µl of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylene diamine hydrochloride in 2% H_3PO_4) was added to all the tubes and incubated for 40 min. Color intensity of the chromophore formed as a result of diazotization of nitrite with sulfanilamide and subsequent coupling with naphthyl ethylene diamine hydrochloride was measured calorimetrically at 546 nm against suitable blank (2 ml H_2O and 0.6 ml Griess reagent). Control (200 µL SNP, 800 µL H_2O and 300 µL Griess reagent) was run and the percentage inhibition was calculated using equation 1 mentioned above and compared with ascorbic acid which is used as a standard antioxidant. EC_{50} values for the plant extracts were estimated as explained above.

2.8.3 DPPH free radical scavenging activity

Antioxidant activities of the plant extracts were assessed based on DPPH free radical scavenging activity using Blois method [24] with minor modifications. To 2 ml of extract taken at various concentrations (5-100 mg/ml) 1 ml of DPPH solution (125 µM, dissolved in methanol) was added, shaken well and incubated for 30 min at 37°C in dark and the decline in absorbance of DPPH was read at 517 nm against suitable blank (2 ml methanol and 1 ml of DPPH). Ascorbic acid prepared at similar concentration ranges like AETA extracts was used as a standard positive control. Percentage inhibition was calculated by comparing tests with control (3 ml of DPPH) using equation 1.

2.8.4 Reductive ability

The reductive ability of the extracts was evaluated according to the method described by Fu et al., 2010 [25]. To 1 ml of the plant extract (0.02-0.64 mg/ml), 1 ml of 0.2M phosphate buffer (pH 6.6) and 1.5 ml of 1% potassium ferricyanide were added and then the mixture was incubated at 50°C for 20 min. The reaction was later stopped by adding 1 ml of 10% trichloroacetic acid and the mixture was centrifuged for 10 min at 3000 rpm. To 2.5 ml of the supernatant, 2 ml of distilled water and 0.5 ml of freshly prepared 0.1% $FeCl_3$ were added, and the absorbance was measured at 700 nm. Ascorbic acid was used as a standard antioxidant.

2.8.5. Preparation of erythrocyte suspension

Blood samples from healthy male/female (non-smoker and non-alcoholic) volunteers were collected into heparinized vaccuettes through venepuncture after taking informed consent. After gently swirling, the tubes were centrifuged at 1500 g for 10 min at 4°C and the plasma and buffy coat were removed. The resulting erythrocytes were washed thrice with 10 volumes of PBS (PBS – 10 mM having NaCl - 150 mM, NaH_2PO_4 - 1.9 mM and Na_2HPO_4 - 8.1 mM, pH 7.4) and centrifuged at 1500 g for 5 min. The buffy coat was carefully removed after each centrifugation and the erythrocyte suspension stock of 10% v/v was prepared in PBS, stored at 4°C and used within 6 h.

2.8.6. In vitro hemolysis assay

To look at the protective effect of AETA on the free radical induced oxidative damage on human erythrocytes, *in vitro* hemolysis assay was performed as adapted by Girish et al., 2012 [1]. To 200 µl of the 10% (v/v) red blood cell suspension, 50 µl of respective extracts (5-25 µg GAE prepared in PBS) and 100 µl of H_2O_2 (200 µM prepared in PBS, pH 7.4) were added and the reaction mixture was incubated at 37°C for 30 min and centrifuged at 2000 g for 10 min. To 200 µl of the supernatant, 800 µl of PBS was added, and the absorbance was monitored at 410 nm. Control was prepared by incubating erythrocyte suspension directly with H_2O_2 to obtain complete hemolysis and absorbance of the supernatant was measured as mentioned. Sample controls were run at every individual concentration of the plant extract by incubating the erythrocyte suspension with plant extract. BHA was used as a standard antioxidant, and the percentage of hemolysis was calculated by taking

hemolysis caused by 200 μM H_2O_2 as 100%. EC_{50} values for both the plant extracts were calculated from the curve and compared with the standard antioxidant.

2.9. Statistical Analysis

All the experiments were done in triplicates and the data expressed as mean±standard deviation and were analyzed using XLSTAT software (Addinsoft Version 2013.4.04) and $p<0.05$ was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Phytochemical Evaluation of AETA

Plants are the known sources of polyphenols like phenolic acids, flavonoids, steroids and tannins which confer them with many therapeutic values [26-30]. The concentrations of these polyphenols vary depending on many environmental factors and the extraction procedures employed. AETA in the present study also was found to be very rich in different polyphenolic components, data are presented in Table 1.

3.2. HPLC Analysis and Quantification of Polyphenols in Extracts

To further characterize and quantify the individual polyphenols present in the extracts, HPLC method with columns specific for separating the hydrophobic compounds and UV detection at 280 nm was performed. Figure 1 depicts the HPLC chromatogram for AETA.

Table 1: Quantification of phytochemical constituents in AETA.

TPC	TFC	Total tannic acid content
1120 mg/g GAE	104 mg RE/g	0.296 mg/g

AETA=Aqueous extract of *Terminalia arjuna*, TPC=Total phenolic content, TFC=Total flavonoid content, GAE=Gallic acid equivalents, RE=Rutin equivalents

3.3. In Vitro Antioxidant Activity

Free radical is a species that contains one or more unpaired electrons with a capability of independent existence. Free radicals such as trichloromethyl, superoxide, hydroxyl, peroxyl and NO are known to be produced metabolically in living organisms. In addition, some non-radical derivatives of oxygen molecules (hydrogen peroxide, hypochlorous acid), can be generated in foods and biological systems. All of this reactive oxygen can cause deleterious effects to the general functioning of the system and thus the tests of the ability of a substance to scavenge radical species may be relevant in the evaluation of antioxidant activity [31]. To look at the antioxidant potential of aqueous extracts of the plant, *in vitro* radical scavenging activities were performed which showed promising results owing to the rich polyphenol profiles both the plants possessed.

Therapeutic efficacy of AETA was determined in terms of *in vitro* free radical scavenging activity (using H_2O_2 and DPPH), inhibition of NO production, total reductive ability. Details are furnished in Figures 2-5. Results of this study demonstrated potent free radical scavenging activity of AETA.

3.4. Antihemolytic Activity of AETA

AETA was screened for its antihemolytic efficacy. This study demonstrated the antihemolytic potential of AETA. Data depicted in Figure 6 indicated that AETA inhibits/prevents H_2O_2 induced hemolysis.

4. CONCLUSION

Role of free radicals in blood cell function has been extensively worked and as understood the free radicals are primarily released *in vivo* by the neutrophils engaged in killing microorganisms as a part of immune responses [32,3]. The present study clearly demonstrates the rich repertoire of flavonoids and polyphenolic compounds in AETA.

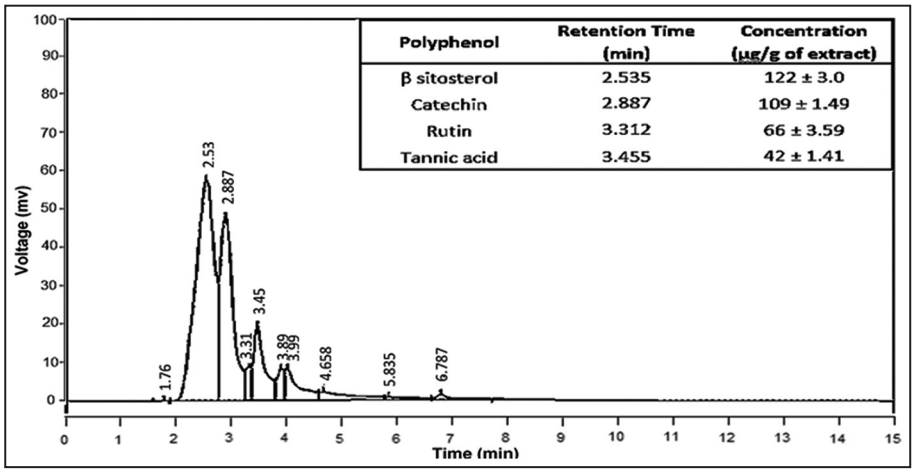

Figure 1: High performance liquid chromatography analysis analysis of bark powder aqueous extract of *Terminalia arjuna* and concentrations of individual phytoprinciples.

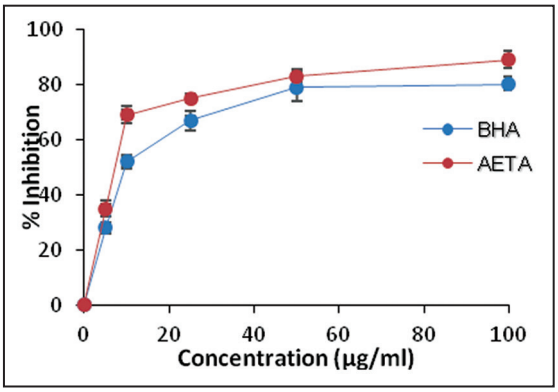

Figure 2: Hydrogen peroxide scavenging activity of aqueous extract of *Terminalia arjuna.*

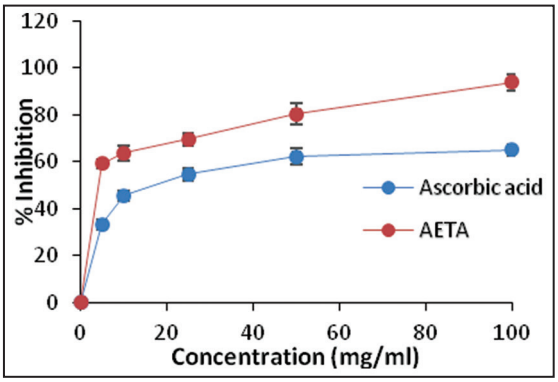

Figure 3: 1,1-diphenyl-2-picrylhydrazyl free radical scavenging activity of aqueous extract of *Terminalia arjuna.*

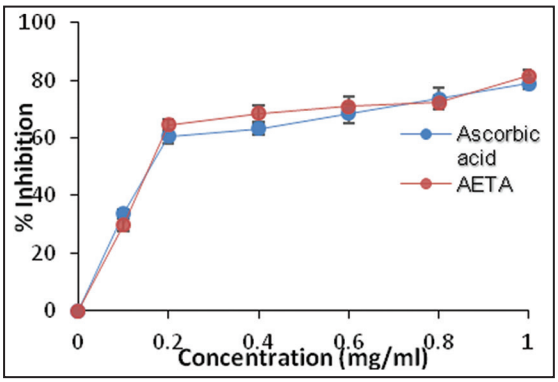

Figure 4: Inhibition of nitrous oxide production by aqueous extract of *Terminalia arjuna.*

These phytoprinciples in the native extract might have shown a synergistic, multi-modal therapeutic action by scavenging the free radicals generated *in vitro* and thus protect the erythrocytes against free radical-induced hemolysis, thus confirming the anti-hemolytic ability to AETA. The results of the present study also encourages for further investigations on the therapeutic efficacy of *T. arjuna* against oxidative stress-induced damage of not only erythrocytes but also other tissues and organs using animal and human cell line models.

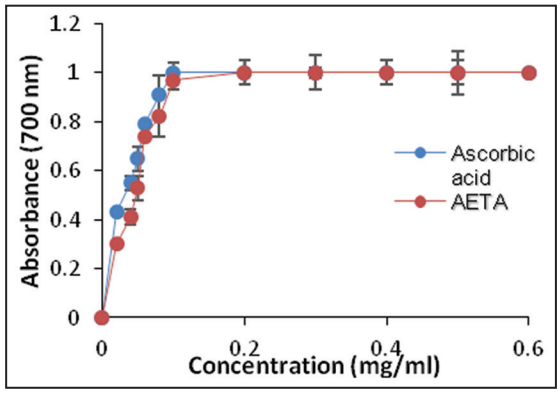

Figure 5: Reducing ability of aqueous extract of *Terminalia arjuna.*

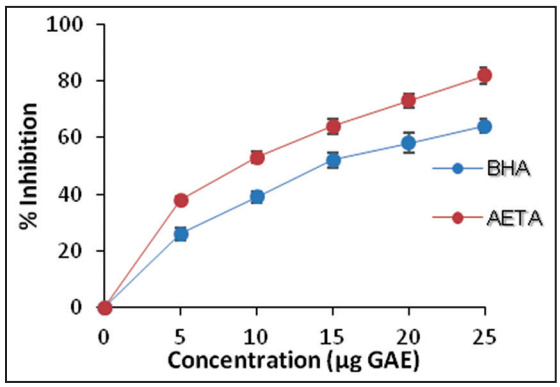

Figure 6: Inhibition of lysis of red blood cells in presence of aqueous extract of *Terminalia arjuna.*

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