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Research Article

ANTIOXIDANT AND ANTHELMINTIC ACTIVITY OF TERMINALIA ARJUNA ROXB. STEM BARK EXTRACTS

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ABSTRACT

In the present investigation, four complementary assays, total reductive power, total antioxidant activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and metal chelating ability for ferrous ions were used to screen the antioxidant property of *Terminalia arjuna* bark extracts. The extracts showed significant antioxidant activity in correlation to the phytoconstituents present in the extracts. The phytochemical analysis revealed that the major bioactive components of the plants are terpenoides, polyphenols, saponins, tannins and glycosides. Live earthworms *Pheretima posthuma* were used for screening anthelmintic activity of bark extract. All extracts showed effective anthelmintic activity at 20 mg ml^{-1} concentration.

Keywords: *Terminalia arjuna*, Antioxidant, Anthelmintic, DPPH, *Pheretima posthuma*

INTRODUCTION

The usage of herbs to treat variety of ailments is universal and exists in every human culture on Earth. Herbal medicine is still the mainstay of about 75–80% of the world population, mainly in the developing countries for primary health care because of better cultural acceptability, better compatibility with the human body and lesser side effects [1]. However, the last few years have seen a tremendous increase in their use in the developed world. In recent decades, many studies have been conducted with the aim of identifying natural origin substances, which can be used in the development of insecticides, especially for vectors of human and animal pathogens.

India always stands as a golden mark of well-recorded and well-practiced knowledge of traditional herbal medicine. Herbal plants play an important role in the development of potent therapeutic agents. Over the years, various medicinal plants and their extracts have been reported to be effective in the treatment of diseases [2]. Plants are rich sources of anthelmintic and antioxidant agents such as flavonoids [3], alkaloids [4], terpenoids [5] and other related polyphenols[6]. These plant phytoconstituents have potential to treat number of diseases. *Terminalia arjuna* Roxb. (Combretaceae) commonly known as *arjuna* is one of the medicinally important evergreen tree [7] which possesses antimicrobial, cytotoxic, antidiabetic [8], antidiarrheal, antidysentric [9] and hepatoprotective [10] activities.

Metabolic pathway of the body results in continuously exposures to several degradative stresses, like Reactive Oxygen Species (ROS) and free radicals. These reactive ions extensively cause the oxidative damage to the biomolecules [11] and contribute to the pathogenesis of oxidative stress related diseases like cancer, ageing and heart failure, etc., [12,13]. On the other hand, antioxidants are considered as possible protective agents against oxidative damage of human body. Consequently, there is a growing interest in the substances exhibiting antioxidant properties that are supplied to human and animal organisms. Hence natural antioxidants have become one of the major areas of scientific research [14, 15].

Helminth infection is a medical and public health problem of high magnitude, both in humans and domestic animals, causing considerable suffering and poor growth. In addition, helminths of livestock pose a serious economic loss, in particular in areas where extensive grazing is practiced [16]. Chemical control of helminths coupled with improved management has been the important worm control strategy throughout the world. However, increasing problems of development of resistance in helminthes [17] against anthelmintic have led to the proposal of screening of medicinal plants for their anthelmintic activity. Therefore, the present study focused on antioxidant and anthelmintic activity of *T.arjuna* bark extracts.

MATERIALS AND METHODS

Chemicals

All the chemicals and solvents used were of analytical grade. Butylated hydroxyl anisole (BHA) was purchased from Merck (Germany). Gallic acid, catechin hydrate, Quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich (Poznan, Poland). Ferric chloride, Ferrozine, Trichloroacetic acid (TCA) and Folin-Ciocalteu reagents were procured from Himedia (India).

Plant material

Healthy stem bark of *T.arjuna* was collected from Bhadra Reserve forest, Shivamogga, Karnataka, India. Plant was identified as *T.arjuna* with the help of faculty of Department of Applied Botany, Kuvempu University, Shankaraghatta, Karnataka, India. Bark was separated from unwanted materials, brushed, shade dried and powdered mechanically.

Estimation of inorganic constituents in plant material

The inorganic components of *T. arjuna* was estimated by slightly modified method described by Narendhirakannan et, al [18]. Briefly, the shade dried and pulverized plant material was placed in a vitreosil crucible overnight in an electric muffle furnace maintained at a temperature between 400 to 420°C. Ashing will destroy all the organic materials present in the samples. The crucible containing pure ash was then taken out from the muffle furnace and kept in a desiccator. Then, 2 gm of the ash was digested with the triple acid mixture of nitric acid: sulfuric acid: perchloric acid (11: 6: 3). The digested sample was dissolved in 100 ml distilled water and filtered through Whatman filter paper. The filtrate was used for the assay of trace elements by atomic absorption spectrophotometer (AAS-Varion 200AA) using suitable hollow-cathode lamp.

Preparation of plant extract

300 Gm of stem bark powder was defatted using petroleum ether and sequentially extracted with chloroform, double distilled ethanol and double distilled water in soxhlet apparatus and extracts were referred as *T. arjuna* chloroform extract (TACE), *T. arjuna* ethanolic extract (TAEE) and *T. arjuna* aqueous extract (TAAE) respectively. The extracts were filtered and concentrated *in vacuum* using rotary flash evaporator (Buchi Rotavapor R-200) and the obtained crude extracts were stored in desiccator until further studies.

Qualitative phytochemical screening

The crude extracts were qualitatively examined for the presence of various phytochemical constituents using standard tests as described by Kokate, C and Harborne [19, 20].

Quantitative analysis of crude extracts

Determination of total phenolic contents

Total phenolic content in the extracts were estimated by Folin-Ciocalteu method using Gallic acid as a standard [21]. Briefly, 2 ml of each extract at different concentrations (50-150 µg) in ethanol was mixed with 2.5 ml Folin-Ciocalteu reagent (diluted 1:10 v/v) and 2 ml of Na_2CO_3 (7.5 % v/v) solution in the test tubes. The mixture is than allowed to stand for 90 min. at room temperature. Absorbance of content in each test tube was measured against the blank at 750 nm using a spectrophotometer (Shimadzu UV-1609, Japan). Total phenolic content of the extract was expressed in terms of µg equivalent to gallic acid. Estimation was repeated thrice and the results were averaged.

Determination of total flavonoid content

Total flavonoid content in the extracts was determined according to modified method of Zhishen et al., [22] using catechin as a standard. Briefly, 2 ml of extract at different concentrations (50-150 µg) in ethanol was mixed with 0.3 ml each of 5 % $NaNO_2$ and 10% $AlCl_3$, incubated at room temperature for 6 min. Then, 2 ml of 1M NaOH was added and the volume in each test tube was made up to 5 ml by adding distilled water. Absorbance was measured against the blank at 510 nm using a spectrophotometer. Total flavonoid content in the extract was expressed in terms of µg equivalent to quercetin. Tests were repeated thrice and the results were averaged.

Biological evaluation

Further, all extracts were screened for biological activities such as *in vitro* antioxidant (total antioxidant capacity, total reductive capability, DPPH radical scavenging and metal chelating activity) and *in vitro* anthelmintic activity**.**

***In vitro* antioxidant activities**

Total antioxidant capacity

Total antioxidant capacity of extract was determined as described by Prieto [23]. Extracts in 3000 µl of methanol at different concentrations (20–100 µg ml^{-1}) were taken in separate test tubes. To this, 3 ml of reagent mixture containing 4 mM ammonium molybdate, 0.6 M sulfuric acid and 28 mM of sodium phosphate was added. Test tubes were kept for incubation at 95˚C for 90 min. and allowed to cool. Absorbance of the content of each test tube was measured at 695 nm against a blank and antioxidant capacity of each extract is expressed as equivalents of ascorbic acid. Test was performed in triplicate and the results were averaged.

Total reductive capability

Total reductive capacity of extract was determined according to the method of Oyaizu [24]. Briefly, 1 ml of extract solution at different concentrations (20-100 µg ml^{-1}) in ethanol was mixed with 2.5 ml of 0.2 mol/L phosphate buffer having pH 6.5 and 2.5 ml, 1% potassium ferricyanide. Then, the mixture was incubated at 50˚C for 20 min. At the end of the incubation, 2.5 ml, 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer solution was collected and mixed with 2.5 ml distilled water and 0.5 ml, 0.1% ferric chloride. The absorbance was measured at 700 nm against a blank. Test was performed in triplicate, and the results were averaged.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

All the extracts were screened for free radical scavenging activity by DPPH method of Braca[25]. The plant extracts in ethanol at different concentrations (20-100 µg ml^{-1}) were added to each test tube and volume was made up to 4 ml using methanol. To this, 3 ml of 0.004% DPPH in methanol was added and the mixtures were incubated at room temperature under dark condition for 30 min. The scavenging activity on the DPPH radical was determined by measuring the absorbance at 517 nm. Radical scavenging activity was calculated using the formula:

$$\% \text{ of Radical scavenging activity} = ([(A_{control} - A_{test})/A_{control}]) \times 100,$$

Where $A_{control}$ is the absorbance of the control sample (DPPH solution without test sample) and A_{test} is the absorbance of the test sample (DPPH solution + test compound). The DPPH radical scavenging activity of BHA was also assayed for comparison. Test was performed in triplicate, and the results were averaged.

Metal chelating activity

The chelating activity of ferrous ions by the extract and standard (EDTA) was estimated by the method of Dinis [26]. Briefly, 3ml of extract solution at different concentrations (20-100 µg ml^{-1}) was taken and 0.05 ml of 2 mM $FeCl_2$ was added. The reaction was initiated by adding 0.2 ml 5 mM ferrozine and the mixture was mixed vigorously and incubated at room temperature for 10 min. Absorbance of the solution was measured at 562 nm. The control contains $FeCl_2$ and ferrozine, complex with 3.0 ml of ethanol. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated from the formula:

$$\% \text{ of inhibition} = ([(A_{control} - A_{test})/A_{control}]) \times 100$$

Where $A_{control}$ is the absorbance of the control sample and A_{test} is the absorbance of the test sample. Test was performed in triplicate, and the results were averaged.

***In vitro* Anthelmintic activity**

Test organisms

Live earthworms *P. posthuma* of nearly equal size (6±1cm) were collected from Vermicomposting manufacturing farm. Worm type was identified at the Agriculture Research Station, Shivamogga, Karnataka. The worms were acclimatized to laboratory conditions for one week before experimentation.

Anthelmintic activity

The *in vitro* anthelmintic assay was carried out according to the method of Ajaiyeoba [27] with appropriate modifications. The earthworms were divided into five groups of six each. Albendazole diluted with normal saline solution to obtain 1% (m/V) is served as standard and normal saline served as a control. The extracts were dissolved in normal saline to prepare different concentrations (5-20 mg ml^{-1}) of each extract. The time taken for complete paralysis and death was recorded. The mean paralysis time and mean lethal time were calculated for each extract (each reading was taken in triplicate). The time taken by worms to become motionless was noted as paralysis time. To ascertain death, each worm was frequently subjected to external stimuli that stimulate and induce movement in earth worms, if alive.

Statistical analysis

Data was statistically expressed as mean (SEM, Statistical evaluation was done using one-way analysis of variance (ANOVA)), $P<0.05$. Difference was considered to be significant at $P<0.05$.

RESULTS AND DISCUSSION

In the present study, our primary focus was to examine the *in vitro* antioxidant and anthelmintic, activity of *T.arjuna* bark extracts. The yield and physical appearance of bark extracts have been tabulated in **Table 1**. The analysis for inorganic component in the sample indicated the presence of Cd, Mn, Cu, Ni, Pb, Zn, K and Na. **Table 2** represents the concentration of elements found in plant material.

These elements play a pivotal role in the formation of secondary metabolites which in turn responsible for pharmacological actions of medicinal plants [28].

The crude extracts were prepared from the *T.arjuna* stem bark and the preliminary phytochemical screening of crude extract indicates the presence of flavonoids, glycosides, triterpenoids, saponins, and tannins in the crude ethanolic extract (**Table 3**).

Table 1: Yield and physical appearance of extracts

Extracts	Yield (gm/300 g.)	Color	Physical appearance
TACE	8	White	Amorphous
TAEE	50	Red	Crystalline
TAAE	10	Brown	Amorphous

Table 2: Concentration of inorganic elements in plant sample

Metals	Wave length (nm)	Concentration (ppm)
Cadmium	228.8	0.948
Manganese	279.5	1.233
Cupper	240.7	1.686
Nickel	232.0	0.0795
Lead	217.0	0.0087
Zinc	213.9	0.5406
Sodium	589.0	91.2548
Potassium	766.5	205.465

Table 3: Phytochemical screening of *T.arjuna* stem bark extracts

Test	TACE	TAEE	TAAE
Alkaloids	+	+	+
Steroids	+	+	+
Flavonoids	-	-	+
Glycosides	+	+	+
Tannins and Phenolics	-	+	+
Quinones	+	-	-
Saponins	-	+	+
Terpenoid	-	+	+

+ = Present, - = Absent

Total phenolic and flavonoid contents of all the extracts are shown in **Table 4**. From the observation, it was found that the phenolic and flavonoid contents were high in TAEE compared to other extracts. The total amount of phenols were found to be 174.80±0.82µg/mg, 623.20±1.84 µg/mg and 372.66±0.17µg/mg equivalent to Gallic acid for TACE, TAEE and TAAE respectively and the flavonoid content were 29.33±1.6 µg/mg, 418.06±2.5 µg/mg and 220.00±0.9 µg/mg equivalent to catachin in TACE, TAEE and TAAE respectively.

Table 4: Qualitative phytochemical analysis of *T.arjuna* stem bark extracts

Extracts	Total phenolic content (µg mg^{-1} of extract)	Total Flavonoid content (µg mg^{-1} of extract)
TACE	174.80±0.82	029.33±1.6
TAEE	623.20±1.84	418.06±2.5
TAAE	372.66±0.17	220.00±0.9

Total antioxidant capacity of extracts was performed by phosphomolybdenum method and expressed in terms of equivalents of ascorbic acid. Among the extracts, TAEE possess significant antioxidant activity, TAAE shown moderate activity whereas TACE shown very least activity (TAEE>TAAE > TACE) (Fig. 1). The enhanced antioxidant property could be attributed to the presence of good phenolic and flavonoid content in extracts. The ethanolic extract of *T.arjuna* had highest antioxidant capacity with a value of 85.73±2.45 mg ascorbic acid/g extract and the lowest capacity was found in the chloroform extract with a value of 61.13±1.56 mg ascorbic acid/g extract, the aqueous extracts showed 73.56±1.98 mg ascorbic acid/g extract.

The reductive ability of compounds was determined according to the method of Oyaizu, (1986). The reductive activity of extracts was expressed in equivalents of BHA. The results obtained imply that the ethanol extract of bark exhibit the maximum reducing capacity and is found to be 86.12±1.01 mg BHA/g extract, chloroform extract have least value of 60.01±0.98 mg BHA/g extract and the aqueous extract showed 68.11±2.08±1.64 mg BHA/g extract. The high reducing ability of ethanol extract may be due to the presence of high concentrations of polyphenols in the extract. Among the extracts, TAEE and TAAE emerged as good reducing agents whereas TACE shown minimal activity (Fig. 2).

The extracts were screened for free radical scavenging evaluation by using DPPH as a radical. All the extracts exhibited promising scavenging activity comparing with the standard ascorbic acid in dose dependent manner (Fig. 3). The results of free radical scavenging ability clearly indicate that percentage inhibition was observed in the range of 29–96% for ethanol extract (IC_{50}- 44.74±1. 38 µg/mL), 22-82 % (IC_{50}-54.49±2.45µg/mL) for aqueous extract and 19-61 % (IC_{50}-83.81±2.63µg/mL) for chloroform extract at100 µg/mL concentration while ascorbic acid showed 33-97 % (IC_{50}-41.21±2.01µg/mL) of inhibition. The results imply that there was no significant difference between chelating capacity of ethanol extract and standard ascorbic acid at all concentrations.

The plant extracts were also screened for metal chelating activity using EDTA as a standard. All extracts possess dose dependent activity. At the concentration of 100µg/mL, they showed 45.67% (IC_{50}-108.52±1.05µg/mL), 75.16% (IC_{50}-58.21±1.78µg/mL) and 64.74 % (IC_{50}-71.41±1.44µg/mL) chelating ability for chloroform, ethanol and aqueous extract respectively. Among them, TAEE shown significant activity, while TAAE and TACE shown moderate activity (Fig. 4).

The crude extracts were screened for anthelmintic activity against *P.posthuma* owing to its resemblance in all the way with the intestinal parasites. In the study, the effectiveness of drug was judged on the basis of loss of spontaneous movement or death of trematodes. Anthelmintic activities of all the extracts are represented in **Table 5**. Among all the extracts, TAEE activity was more significant than TACE and TAAE activity.

Table 5: Anthelmintic activities of *T. arjuna* stem bark extracts

Extracts/samples	Time taken for	
	Paralysis (Min.)	Death (Min.)
TACE (20 mg)	70±1.54	138±1.86
TAEE (20 mg)	60±1.32	115±1.94
TAAE (20 mg)	65±2.12	128±1.64
Albendazole (1 %)	52±1.42	108±1.30

Each value represents mean±SE, Where, n= 3.

The ethanol extract showed more significant effect on paralyzing the worms, in terms of paralysis time, at every concentration compared to that of water and chloroform extracts. Similar observations were made in the anthelmintic activity as well. The ethanol extract exhibited an increased paralytic as well as helminthiatic effect over albendazole at the given experimental concentrations. This may be due to the increased level of polyphenols in ethanol followed by aqueous and chloroform extracts.

The antioxidant and Anthelmintic Activity of *T. arjuna* may be due to the presence of phytoconstituents, arjunic acid, arjunetin, arjunlitin, arjunolic acid, β-sitosterol, tomentosic acid, arjunin, terminoside, arjunophthanoloside, casuarianin, arjunglucoside, arjunasides, terminarjunoside and arjungenin [29]. Several studies on these compounds have demonstrated neuroprotective effect, antifeedant, antidiabetic, antioxidant, antimicrobial, anticancer and anti-inflammatory properties [30- 33]. Polyphenols and flavonoids in the bark extract are well known for their antioxidant properties, they may scavenge the free radicals generated in the normal function of body [34,35] and these might have crucial roles in the observed results of antioxidant and anthelmintic activity of the bark.

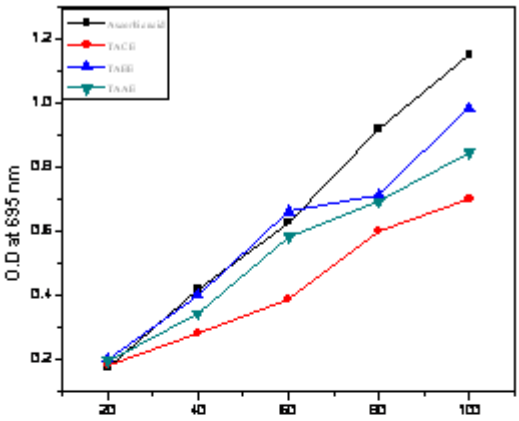

Figure 1. Total antioxidant activity of T. arjuna

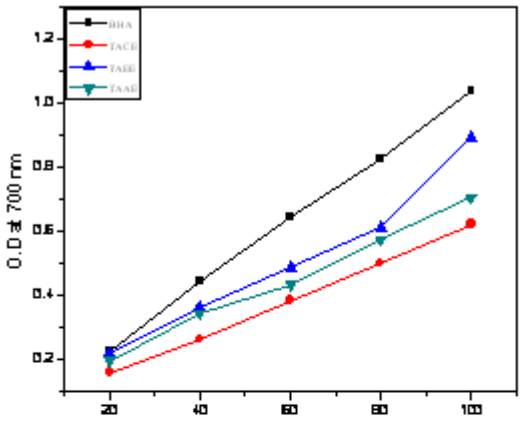

Figure 2. Reductive capability of T. arjuna

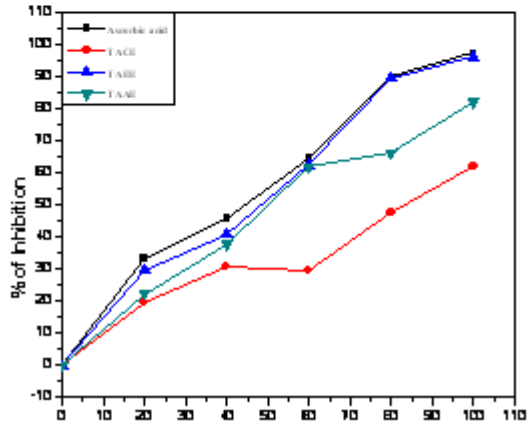

Figure 3. DPPH radical scavenging activity of T. arjuna

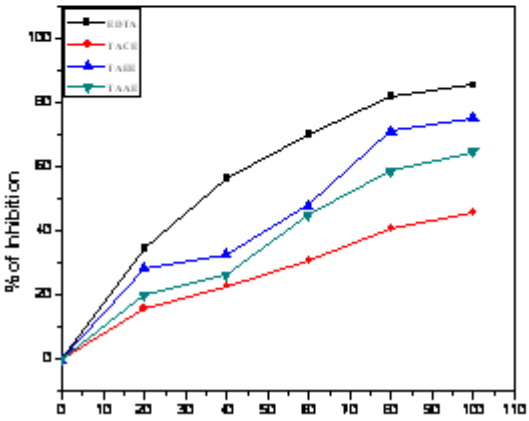

Figure 4. Metal chelating ability of T. arjuna

CONCLUSION

In conclusion, the extracts TACE, TAEE and TAAE of *T.arjuna* emerged as potentially active antioxidants and also tend to be possesses good anthelmintic activity. Hence, this plant can be used as a source of pharmaceutical agent. The current analysis has also opened a path for further research especially with reference to the development of potent herbal formulation and purification of the active components present in these plant extracts.

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