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## Antioxidant and antimutagenic activities of bark extract of *Terminalia arjuna*

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## ABSTRACT

**Objective:** The alcoholic extract of stem bark of *Terminalia arjuna* (ALTA) was screened for antioxidant and antimutagenic (anticlastogenic) activity. **Methods:** Antioxidant property was determined by 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay, superoxide radical scavenging activity, lipid peroxidation assay and total polyphenolic content was determined by Folin-Ciocalteu's reagent. Antimutagenic activity was evaluated using micronucleus test in mice. **Results:** The ALTA has shown potent antioxidant activity with EC<sub>50</sub> of 2.491±0.160, 50.110±0.150 & 71.000±0.250 in DPPH assay, superoxide radical scavenging activity and lipid peroxidation assay, which is comparable with ascorbic acid with EC<sub>50</sub> of 2.471±0.140, 40.500±0.390 and 63.000±0.360 respectively. In micronucleus test, ALTA (100 & 200 mg/kg, p.o.) showed significant reduction in percentage of micronucleus in both polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) and also shown significant reduction in P/N ratio. **Conclusions:** These results suggested that ALTA possess significant antioxidant and antimutagenic activity.

### 1. Introduction

Free radicals are the highly reactive species capable of wide spread, indiscriminate oxidation and peroxidation of proteins, lipids and DNA which can lead to significant cellular damage, the involvement of free radicals in pathological process such as aging, behavioural and psychiatric disorders, cancer, atherosclerosis and rheumatoid arthritis is well recognized[1]. Antioxidant reacts with reactive oxygen species (ROS) to quench the radicals and to produce less aggressive chemicals species likely to cause tissue damage. Much attention has been focused on the use of antioxidants because of their protective effect against damage from reactive oxygen species, on this basis the beneficial effect of antioxidants are being increased[2]. It is now widely recognized that the antioxidants are also useful in treatment of cancer. The changes in the base

pair sequence of genetic material (either DNA or RNA) is called mutations, mutations can be caused by copying errors in the genetic material during cell division and/or by exposure to ultraviolet and/or ionizing radiation, chemical mutagens, viruses or it can also occur deliberately under cellular control during processes such as meiosis or DNA replication[3–5]. One of the best ways to minimize the effect of a mutagen is by the use of anticlastogens; they act by interfering with DNA repair and/or with mutagen metabolites and/or with free radicals.

Naturally occurring substances from plant origin and dietary components have been widely studied for the antimutagenic activity, which includes phenolics, pigments, allylsulphides, glucosinolates, anthocyanins, phytosterols, protease inhibitors, phytoestrogens, carotenoids, flavonoids, tea-polyphenols, vitamins, cucuminoids, tannins, coumarins, chlorophyllin, porphyrins and alkylresorcinols from cereal grains[6]. The anticlastogenic phytochemicals also play an important role in prevention of cancer. Hence there is a need to establish the relations between antioxidants and anticlastogenic agents[7].

*Terminalia arjuna* (Combretaceae) (*T. arjuna*) is an

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evergreen large deciduous tree; the plant has been reported to be used in ayurvedic system of medicine for derangement of all the three humours kafa, pitta, vayu and all types of cardiac failure[8], dropsy, diuretics, antiinfective[9], antiasthmatic, treatment of rheumatoid arthritis and treatment of cancer[10]. Keeping in mind the great medicinal value of *T. arjuna* and its high content of polyphenols, flavones and flavonoids, present study was planned to investigate the antimutagenic effect of ALTA.

## 2. Materials and methods

### 2.1. Drugs and chemicals

Analytical grade petroleum ether, 95% ethanol, distilled water, methanol (AR Grade, Rankem, S.A.S nagar), cyclophosphamide (Sigma, Germany), bovine albumin fraction-V (Otto kemi, Mumbai), geimsa's stain (Loba Chemi, Mumbai), May-Gruenwald's stain (Loba Chemi, Mumbai) and sodium azide (Loba Chemi, Mumbai) were used for the study.

### 2.2. Plant material

#### 2.2.1. Collection and identification of plant material

The dried bark of *T. arjuna* was obtained from the Natural Remedies Ltd. Bangalore and it was authenticated by Dr. H.B. Singh, Head, Raw Material Herbarium & Museum, National Institute of Science Communication and Information Resources, New Delhi.

#### 2.2.2. Extraction of plant material

The shade dried plant material was powdered. The coarse powder was subjected to successive extraction with petroleum ether, alcohol (95%) in soxhlet apparatus (at 60–80 °C) and the marc obtained after alcoholic extraction was macerated with distilled water to obtain an aqueous extract.

#### 2.2.3. Phytochemical investigation

The alcoholic (ALTA) and aqueous extracts (AQTA) of *T. arjuna* bark obtained were subjected to various phytochemical tests for identification of secondary metabolites present in them[11].

#### 2.2.4. Determination of total polyphenols

The total polyphenol content (TPC) was determined by spectrophotometry, using tannic acid as standard, according to the method described by the International Organization for Standardization (ISO) 14502–1. Briefly, 1.0 mL of the diluted sample extract was transferred in duplicate to separate tubes containing 5.0 mL of a 1/10 dilution of Folin-Ciocalteu's reagent in water. Then, 4.0 mL of a sodium carbonate solution (7.5% w/v) was added. The tubes were then allowed to stand at room temperature for 60 min before absorbance at 765 nm was measured against water. The concentration of polyphenols in samples was derived from a

standard curve of tannic acid ranging from 10 to 50  $\mu$ g/mL and expressed in terms percentage[10].

### 2.3. Experimental animals

Wistar rats (200 to 250g) and Swiss albino mice (18–22) purchased from Bioneed, Tumkur for experimental purpose were all acclimatized for 7 days under standard husbandry conditions, *i.e.*; room temperature of (25±1) °C; relative humidity of 45%–55% and a 12:12 h light/ dark cycle.

All the experimental protocols were approved by Institutional Animal Ethical Committee (IAEC) of P.E.S College of Pharmacy, Bangalore (Karnataka) and were conducted in strict compliance according to ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

### 2.4. Acute toxicity studies

Acute oral toxicity of the ALTA was determined by using female, nulliparous and non pregnant mice weighing 18–22 g. The animals were fasted for 3 h prior to the experiment. Up and down procedure OECD guideline No. 425 was adopted for toxicity studies (<http://www.epa.gov/oppfead1/harmonization/>). Animals were administered with single dose of extract and observed for their mortality during 48 h (acute) and 14 days (chronic). LD<sub>50</sub> was calculated as per OECD 425 using AOT 425 stat program.

### 2.5. Antioxidant activity

#### 2.5.1. DPPH assay[12]

A working solution of methanolic DPPH (Sigma, Germany) having an absorbance of 0.9 at 516 nm was used. This was prepared by taking 150  $\mu$  L of stock solution (12.9 mg of DPPH in 10 mL of methanol) in 3 mL of methanol. To 150  $\mu$  L of DPPH solution in methanol, different concentrations of ascorbic acid were added and the total volume was made up to 3 mL with methanol. DPPH diluted to 3 mL was taken as blank. Decrease in absorbance in the presence of ascorbic acid was noted down at 516 nm after 15 min. A standard graph was plotted between concentration vs absorbance and EC<sub>50</sub> values were calculated. The test solutions were treated in the similar manner and the EC<sub>50</sub> values were calculated.

#### 2.5.2. Superoxide radical scavenging activity[13]

It was carried out by using Nitro blue tetrazolium (NBT) reagent, the method is based on generation of superoxide radical (O<sub>2</sub><sup>-</sup>) by auto oxidation of hydroxylamine hydrochloride in presence of NBT, during the reaction the NBT is reduced to nitrite.

In brief, aliquots of 0.1 to 1.0 mL to ascorbic acid solution were taken in a test tube, to which 1 mL of sodium carbonate, 0.4 mL of NBT and 0.2 mL of EDTA were added and zero minute reading was taken at 560 nm. The reaction was initiated by the addition of 0.4 mL of hydroxylamine

hydrochloride to the above solution. Reaction mixture was incubated at 25 °C for 5 mins; the reduction of NBT was measured at 560 nm. A parallel control was also treated in the similar manner. All the extracts were treated in the similar manner, absorbance was recorded and EC<sub>50</sub> values were calculated.

### 2.5.3. Ferric chloride induced lipid peroxidation<sup>[14]</sup>

Male albino rats (200 to 250 g) were sacrificed by cervical dislocation, the skin over the abdomen was cut open and the liver was perfused with ice cold 0.15 M KCl via portal vein. After perfusion, the liver was isolated and 20% (w/v) homogenate in 0.15 M KCl was prepared using tissue homogenizer under ice cold (0–4 °C) conditions. The homogenate was centrifuged at 1 500 g for 5 min and clear supernatant was used for further study.

Different concentrations of test extracts were taken in test tubes, to which 1 mL of 0.15 M KCl and 0.5 mL of cell free homogenate were added, peroxidation was initiated by adding 100 µL of 1 mM ferric chloride. The mixture was incubated for 30 min at 37 °C. After incubation, the reaction was stopped by adding 2 mL of ice cold 0.25 N HCl containing 15% trichloroacetic acid (TCA), 0.38% thiobarbituric acid (TBA) and 0.5% of 0.05% butylated hydroxyl toluene (BHT). The reaction mixture was heated for 60 min at 80 °C. The sample was cooled and centrifuged at 5 000 g for 15 min and absorbance of the supernatant was measured at 532 nm.

An identical experiment (control induced) was performed in absence of test compounds to determine the amount of lipid peroxidation obtained in the presence of inducing agents without any test compounds. A blank was performed with all the reagents except ferric chloride and test extracts. % Anti lipid peroxidation effect (% ALP) was calculated using the equation:

$$\% \text{ ALP} = 1 - \frac{(\text{Sample absorbance} - \text{Blank absorbance})}{\text{Absorbance of control}}$$

## 2.6. Antimutagenic activity

### 2.6.1. Micronucleus test in mice<sup>[15]</sup>

Swiss albino mice of weight range 18–22 g were divided into eleven groups (G1 to G11), each group consisting of six animals; the G1 – normal control group without any treatment, G2 – vehicle control received 2% acacia; G3, G4 and G5 were positive control groups challenged with cyclophosphamide (75 mg/kg, i.p.) and bone marrow samples were collected from these animals 24, 48 and 72 h after cyclophosphamide injection respectively. The animals in the groups G6 to G8 and G9 to G11 received ALTA 100 mg/kg and 200 mg/kg, p.o. respectively for 7 consecutive days. On 7th day after the ALTA treatment all the animals in the group G6 to G11, were treated with cyclophosphamide and the bone marrow samples from G6, G7 and G8 were collected after 24, 48 and 72 h of cyclophosphamide administration, similarly from G9, G10 and G11 group animals, the bone

marrow samples were collected after 24, 48 and 72 h after the cyclophosphamide administration respectively; in brief, bone marrow was aspirated from femur into 1 mL of 5% bovine albumin in phosphate buffered saline (pH 7.2)<sup>[15–17]</sup>. The cell suspension was centrifuged (1 000 rpm for 5 min) and the smears were prepared from the pellet on chemically clean glass slides and stained with may–gruenwald and giemsa stain. The smears were analyzed under the oil immersion objective (100×) for the presence of micronuclei (MN) in polychromatic erythrocytes and normochromatic erythrocytes (NCE). P/N (polychromatic erythrocytes/normochromatic erythrocytes) ration was determined by counting a total of 500 erythrocytes per animal and total of 2 000 erythrocytes were examined for the presence of micronuclei per each animal sample<sup>[18]</sup>.

## 2.7. Statistical analysis

Values were expressed as mean±SEM from 6 animals. Statistical difference in mean will be analyzed using one way ANOVA followed by Turkey's multiple comparison tests  $P < 0.05$  were considered statically significant.

## 3. Results

### 3.1. Phytochemical investigation

The phytochemical studies of *T. arjuna* revealed presence of carbohydrates, cardiac glycosides, phytosterols, fixed oils, amino acids, saponins, phenolic compounds and tannins in the ALTA and carbohydrates, cardiac glycoside, proteins, amino acids, phenolic compounds and tannins in the AQTA.

### 3.2. Determination of total polyphenols

The estimation of polyphenolic content in the extracts revealed that, the ALTA and AQTA found to contain 90.33 % and 20.5% of polyphenolic compounds respectively. Based on very high polyphenolic content, only ALTA was selected for further studies.

### 3.3. Acute toxicity studies

The acute oral toxicity study of ALTA produced mortality at 5 000 mg/kg and 2 000 mg/kg and no mortality observed at 550 mg/kg; the LD<sub>50</sub> was calculated by using AOT-425 stat program and the calculated LD<sub>50</sub> was found to be 1 098 mg/kg.

### 3.4. Antioxidant activity

The extracts containing varying quantities of total polyphenols and the monomeric polyphenols were comparatively studied for their antioxidant potentialities. Three different *in vitro* methods namely DPPH assay, super oxide radical scavenging activity and lipid peroxidation assay were employed, ascorbic acid was used as a standard.

**Table 1**EC<sub>50</sub> values of *T. arjuna* extracts in antioxidant studies.

Sl. No.	Extract/Std	EC <sub>50</sub> valves		
		DPPH assay	Super oxide scavenging activity	Lipid peroxidation assay
1	Ascorbic acid	2.471±0.140	40.50±0.39	63.00±0.36
2	Alcoholic extract	2.491±0.160	50.11±0.15	71.00±0.25
3	Aqueous extract	4.910±0.790	59.94±0.16	299.00±0.32

All the values are expressed as Mean± SEM, *n* = 6.**Table 2**

Antimutagenic effect of ALTA after 24 h of clastogenic challenge.

Sl.No.	Group	%MNPCE	%MNNCE	P/N ratio
1	Normal control	1.500±0.028	1.920±0.042	0.780±0.005
2	Vehicle control (Acacia 2% in water)	1.460±0.035	1.870±0.038	0.780±0.024
3	ALTA (100 mg/kg, p.o.)	1.480±0.023	1.810±0.041	0.810±0.023
4	ALTA (200 mg/kg, p.o.)	1.390±0.023	1.680±0.039	0.820±0.028
5	Cyclophosphamide (75 mg/kg, i.p.)	3.330±0.024*	9.550±0.059*	0.349±0.004*
6	ALTA (100 mg/kg, p.o.) +Cyclophosphamide (75 mg/kg, i.p.)	1.510±0.020 <sup>a</sup>	4.720±0.029 <sup>a</sup>	0.320±0.005 <sup>b</sup>
7	ALTA (200 mg/kg, p.o.) +Cyclophosphamide (75 mg/kg, i.p.)	1.440±0.031 <sup>a</sup>	5.070±0.032 <sup>a</sup>	0.284±0.006 <sup>b</sup>

All the values are expressed as Mean± SEM, *n* = 6. \**P* < 0.001 vs normal control group, <sup>a</sup> *P* < 0.001, <sup>b</sup> *P* < 0.05 vs cyclophosphamide after 24 hours of clastogenic challenge.**Table 3**

Antimutagenic effect of ALTA after 48 h of clastogenic challenge.

Sl.No.	Group	%MNPCE	%MNNCE	P/N ratio
1	Normal control	1.500±0.028	1.920±0.042	0.780±0.005
2	Vehicle control (Acacia 2% in water)	1.460±0.035	1.870±0.038	0.780±0.024
3	ALTA (100 mg/kg, p.o.)	1.480±0.023	1.810±0.041	0.810±0.023
4	ALTA (200 mg/kg, p.o.)	1.390±0.030	1.680±0.039	0.820±0.028
5	Cyclophosphamide (75 mg/kg, i.p.)	7.500±0.070*	10.800±0.061*	0.694±0.009
6	ALTA (100 mg/kg, p.o.) +Cyclophosphamide (75 mg/kg, i.p.)	1.740±0.037 <sup>a</sup>	5.020±0.041 <sup>a</sup>	0.346±0.010 <sup>a</sup>
7	ALTA (200 mg/kg, p.o.) +Cyclophosphamide (75 mg/kg, i.p.)	1.580±0.032 <sup>a</sup>	5.230±0.023 <sup>a</sup>	0.302±0.002 <sup>a</sup>

All the values are expressed as Mean± SEM, *n* = 6. \**P* < 0.001 vs normal control group. <sup>a</sup> *P* < 0.001 vs cyclophosphamide.**Table 4**

Antimutagenic effect of ALTA after 72 h of clastogenic challenge.

Sl. No.	Group	%MNPCE	%MNNCE	P/N ratio
1	Normal control	1.500±0.028	1.920±0.042	0.780±0.005
2	Vehicle control (Acacia 2% in water)	1.460±0.035	1.870±0.038	0.780±0.024
3	ALTA (100 mg/kg, p.o.)	1.480±0.023	1.810±0.041	0.810±0.023
4	ALTA (200 mg/kg, p.o.)	1.390±0.030	1.680±0.039	0.820±0.028
5	Cyclophosphamide (75 mg/kg, i.p.)	9.260±0.095*	11.450±0.087*	0.812±0.002
6	ALTA (100 mg/kg, p.o.) +Cyclophosphamide (75 mg/kg, i.p.)	2.102±0.031 <sup>a</sup>	3.118±0.022 <sup>a</sup>	0.674±0.010 <sup>a</sup>
7	ALTA (200 mg/kg, p.o.) +Cyclophosphamide (75 mg/kg, i.p.)	1.932±0.042 <sup>a</sup>	3.296±0.029 <sup>a</sup>	0.586±0.008 <sup>a</sup>

All the values are expressed as Mean± SEM, *n* = 6. \**P* < 0.001 vs normal control group. <sup>a</sup> *P* < 0.001 vs cyclophosphamide.

In DPPH assay, the ALTA significantly decreased the absorbance produced by the DPPH and it was found to possess more significant antioxidant activity, however the activity is lesser than ascorbic acid. Where as the AQTA has moderately decreased the absorbance due to DPPH and which is not significant when compare to ALTA and ascorbic acid. In superoxide free radical scavenging activity, the ascorbic acid, ALTA and AQTA has offered good free radical scavenging activity by decreasing the absorbance due to NBT. In lipid peroxidation assay, the ALTA and ascorbic acid

has significant inhibited of lipid peroxidation by decreasing the absorbance of the supernatant, where as it is not significant with AQTA when compare to ALTA and ascorbic acid. The results of antioxidant studies are shown in Table 1.

### 3.5. Antimutagenic activity

The probability of spontaneous mutation was evaluated for untreated normal control group, vehicle control group treated with 2% acacia and ALTA treated groups, by

collecting the bone marrow samples of the respective groups without cyclophosphamide treatment and the samples were evaluated for frequency of PCE's, NCE's and concurrently looked for frequency of MNPCE's and MNCE's respectively and the P/N ratio was calculated.

Similar to untreated group and vehicle treated groups, the positive control group animals treated with cyclophosphamide (75 mg/kg, i.p.) were evaluated for the frequency of MNPCE's, MNCE's and P/N ratio after the 24 h, 48 h and 72 h of cyclophosphamide administration; the observations showed that, there was a significant increase in the frequency of MNPCE's and MNCE's, also there is increase in P/N ratio, at 24 h, 48 h and 72 h after cyclophosphamide administration compare to normal control group.

The antimutagenic activity of ALTA was evaluated by its inhibitory effect on cyclophosphamide induced mutagenesis. The ALTA at 100 and 200 mg/kg, p.o., has significantly decreased the frequency of MNPCEs and MNCEs, also there is a significant decrease in P/N ratio, compare to corresponding positive control group treated with cyclophosphamide. The results are given in Table 2.

#### 4. Discussion

The present study was under taken to evaluate the antioxidant and antimutagenic activity of bark extracts of *T. arjuna* which have been not studied so far, The antioxidant potential of the extract was determined by three methods namely DPPH assay, super oxide radical scavenging activity and lipid peroxidation assay; antimutagenic activity was studied by using micronucleus test.

The chemicals that damage the genetic material either by inducing chromosomal abnormalities (numerical and structural abnormalities) and/or by damaging DNA, leading to mutation are described as genotoxic or clastogens. The mutations in somatic cells leading to pathological conditions such as carcinogenesis, atherosclerosis and other heart diseases, these are the leading causes of death in the human population<sup>[19, 20]</sup>. The mutagenic or clastogenic potential of a drug can be studied by using animal models like *in vivo* micronucleus test and chromosomal aberration test *etc.* The drugs that inhibit the clastogenic potential of a chemical or drug are called as antimutagens.

ROS are the by products of normal cell metabolism during enzymatic electron-transporting processes, such as mitochondrial respiration and metabolism of xenobiotics by the microsomal system of monooxygenases. ROS can also be generated as the main products of membrane-bound NADPH oxidase in phagocytes. Under normal physiological conditions, when the functioning of antioxidant systems is adequate, ROS are probably of low hazard for an organism. However, there is an excessive increase in ROS generation under the influence of some exogenous or endogenous factors, and also when there is an insufficiency

of antioxidant systems, which can result in development of oxidative stress, Oxidative stress is expressed as a disturbance of the stable equilibrium between pro-oxidant and antioxidant processes in a direction where pro-oxidant processes prevail. This disturbance leads to various types of damage at the molecular and cell level<sup>[21–23]</sup>; in such cases antioxidants can acts as stabilizers of homeostasis.

It is well known that antioxidants are almost universal antimutagenic agents<sup>[24, 25]</sup>. A reason for this effect is the genotoxicity of reactive oxygen species.

In present study both ALTA and AQTA showed the significant antioxidant activity in all the experimental antioxidant models. However, the ALTA was found to possess more potent antioxidant activity compare to AQTA, may be because it's high polyphenolic content. Based on the percentage of polyphenolic content and antioxidant potential, the ALTA was selected for evaluating the antimutagenic potential.

The antimutagenic potential of ALTA was determined by cyclophosphamide induced mutagenicity. Cyclophosphamide is a well known anticancer agent belongs to the class of alkylating agents, it undergoes biotransformation to give active metabolites namely aldophosphamide and phosphoramidate mustard; the initial metabolic step is activated by CYP2B6 (and, to a much lower extent by CYP3A4) and involves hydroxylation of oxazaphosphorine ring to generate carbinolamine<sup>[26]</sup>. The carbinolamine undergoes nonenzymatic hydrolysis to give aldophosphamide either in the blood stream or inside the cell, inside the cell the aldophosphamide will undergoes decomposition in to well known mutagens namely acrolein and phosphoramidate mustard<sup>[27]</sup>. Lethal adverse effects associated with cyclophosphamide usage includes immunosuppression, nephrotoxicity, carcinogenicity and mutagenicity, through its metabolites either by depleting the antioxidant levels, acting as pro-oxidant or/and by directly damaging the DNA of the host.

The mutagenicity of cyclophosphamide is associated with the formation of the ultimate cytotoxic metabolite phosphoramidate mustard through the intermediate agents hydroxycyclophosphamide and deschloroethylcyclophosphamide, which is capable of inducing DNA crosslinks and strand lesions<sup>[27]</sup>.

The antimutagenic effect of ALTA observed on micronucleus test was highly significant at 100 and 200 mg/kg, p.o. but there was no significant difference between 100 and 200 mg/kg in there antimutagenic activity. The possible mechanism of action might be associated with detoxification of the toxic metabolites and/or scavenging of super oxide free radicals and/ or by altering the activation and detoxification of xenobiotics.

Therefore, from the present study, it is concluded that alcoholic extract of *T. arjuna* can prove to be a very good antioxidant and effective chemopreventive against cyclophosphamide induced mutagenesis.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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