Free Radicals and Antioxidants 3 (2013) 35–39

Contents lists available at SciVerse ScienceDirect

Free Radicals and Antioxidants

journal homepage: www.elsevier.com/locate/fra

Original article

DNA damage protecting and free radical scavenging properties of *Terminalia arjuna* bark in PC-12 cells and plasmid DNA

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ARTICLE INFO

Article history:
Received 9 March 2013
Accepted 18 April 2013
Available online 25 April 2013

Keywords:
DNA damage protection
PC-12 cells
Antioxidant activity
Free radical scavenging activity
Terminalia arjuna

ABSTRACT

Objective: Terminalia arjuna is commonly known as Arjuna and widely used as cardioprotective agent in Indian traditional medicine. The present study was undertaken to evaluate the protective effect of ethanolic extract of *T. arjuna* bark (TAA) and its fractions, including dichloromethane (TAD), ethyl acetate (TAE), butanol (TAB) and water (TAW) against free radicals, protein oxidation and DNA damage.
Methods: Protective effect of arjuna bark against H_2O_2 induced DNA damage on pBR322 plasmid and rat adrenal PC-12 cells was analyzed by DNA strand breakage assay and single cell gel electrophoresis (Comet assay) respectively. AAPH induced protein oxidation was analyzed with SDS-PAGE. *In vitro* antioxidant activities were carried out by spectrophotometric methods to asses free radical scavenging activities, such as DPPH, hydroxyl, ABTS, nitric oxide, metal chelation, FRAP and reducing power.
Results: The ethanolic extract and its fractions of arjuna bark effectively protected H_2O_2 induced DNA damage and AAPH induced protein oxidation in the following manner: TAE > TAB > TAA > TAD > TAW. The maximum inhibition of DPPH, hydroxyl, ABTS, nitric oxide radicals and metal chelation was observed in TAE fraction (IC_{50} values: 270 ± 2 μg/ml, 175 ± 11 μg/ml, 25 ± 1.2 μg/ml, 405 ± 9 μg/ml, 310 ± 11 μg/ml, 82 ± 4 μg/ml, respectively).
Conclusion: In the present study we report that arjuna bark extracts ameliorate various impairments associated with DNA damage and free radical formation.

1. Introduction

Terminalia arjuna (Roxb.) Wight and Arn, belonging to the family *Combretaceae*, commonly known as "Arjuna". It is an important ayurvedic medicinal plant to treat hypertension, ischemic heart diseases and a well known cardioprotective agent since long.[1] *T. arjuna* bark known for its ethnomedicinal significance[2] and is frequently used in cardiovascular disorders such as heart failure, coronary artery diseases, and to reduce hypercholesterolemia.[3] The bark is also prescribed in biliousness and sores and as an antidote to poison, and it is believed to have an ability to cure hepatic, congenital, ulcers, blood diseases, anemia, asthma and viral diseases.[4] The extract of the bark has been also reported for its activities like antimicrobial, antimutagenic and anthelmintic.[5] In addition, the arjuna bark has also evaluated as for its antifertility and anti-HIV properties.

Several researchers worked on phytochemical investigation of the *T. arjuna* and evaluated a variety of phytochemicals from different parts of the plant. Few triterpenoids were isolated from the bark of the *T. arjuna* are arjunin, arjunic acid, arjunolic acid, arjungenin, terminic acid.[6–8] Some of the glycosides identified from the bark are: arjunetin, arjunoside I, arjunoside II, arjunaphthanoloside, terminoside A.[6–9] Flavonoids detected from its bark are, arjunolone, bicalein, flavones, kempferol, quercetin, and pelorgonidin.[10] Besides, several compounds related to tannins have been isolated from the bark are punicallin, castalagin, casuariin, pyrocatechols, terchebulin, punicalagin, terflavin C and casuarinin.[11] The bark is also rich in essential mineral such as calcium (3133 μg/g), magnesium (4000 μg/g), copper (19 μg/g), zinc (119 μg/g) and silica.[12]

Screening medicinal plants for novel bioactive compounds are the sole remedy since, plant based drugs are biodegradable, safe and have fewer side effects. Thus, the therapeutic potential of the herbs opens up new vista in the future pharmacological research of herbal drug development. Besides, in the last few years, interest in

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2231-2536/$ – see front matter
http://dx.doi.org/10.1016/j.fra.2013.04.001

the antioxidant activity of the plant extracts has increased tremendously which is very important due to the fact that hyper-physiological load of oxidants can make an imbalance state between free radicals and antioxidants in the system. This difference between oxidants and antioxidants leads to oxidative stress, which is the root cause of several disorders like aging, stroke, atherosclerosis, cancer, diabetes and other neurological disorders such as anxiety, depression, Parkinsonism and Alzheimer's diseases. In view of the above facts, the present study designed to evaluate the protective activity against H_2O_2 induced DNA damage, protein oxidation and antioxidant potential of different extracts of *T. arjuna*.

2. Materials and methods

2.1. Plant material and extraction

T. arjuna bark samples were collected from Mysore, India and properly authenticated by the Department of Botany, University of Mysore, Mysore. It was defatted with the diethyl ether for 24 h and the same materials were extracted with ethyl alcohol for 72 h in soxhlet apparatus. The extract was filtered and dried under reduced pressure. The alcoholic extract was further fractionated with several solvents. The extraction procedure and their yield are explained in Fig. 1. Then we obtained total five different extracts viz., alcoholic extract (TAA), dichloromethane extract (TAD), ethyl acetate extract (TAE), butanol extract (TAB), and water extract (TAW).

2.2. DNA damage protection assay

2.2.1. Determination of oxidative plasmid DNA strand breakage

Conversion of plasmid DNA from super coiled form to open circular and further linear form is an indication of DNA damage. Plasmid DNA protection assay was performed by using pBR322 plasmid DNA as described by Lee et al.[13] Briefly, 10 μl of different fractions of arjuna extract in different concentrations separately (50–200 μg/ml, respectively) and plasmid DNA (0.5 μg) were incubated at 37 °C, followed by UV exposure for 20 min and addition of 10 mM H_2O_2. The final volume was made up to 20 μl and electrophoresed on 1% agarose gel. Easy win 32 software (Herolab, Germany) was used to analyze band intensities.

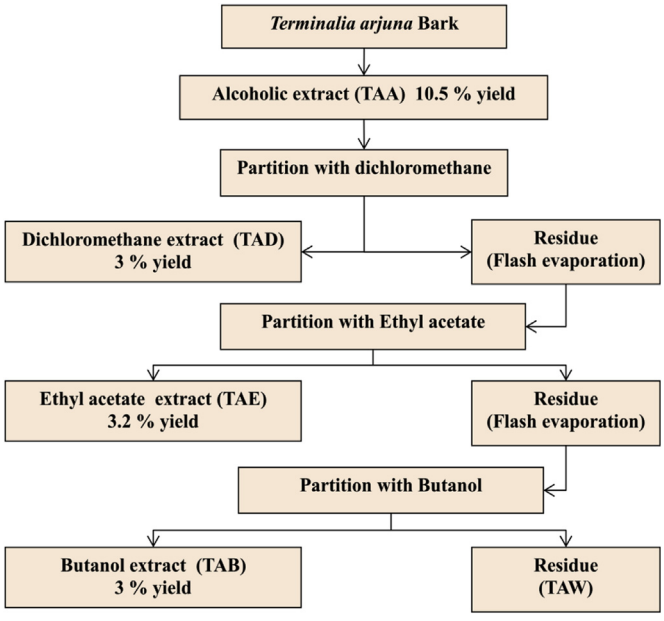

Fig. 1. Sequential extraction of *Terminalia arjuna* bark.

2.2.2. Single cell gel electrophoresis (Comet assay)

Alkaline comet assay[14] was performed to measuring the DNA damage and evaluate the apoptosis induced by H_2O_2. Rat adrenal PC-12 cell line was obtained from National centre for cell science (NCCS), Pune, India. To examine possible toxic effects of H_2O_2, the control cells were treated with H_2O_2 at a concentration of 100 μM for 24 h, and different extracts of *T. arjuna* viz., TAA, TAD, TAE and TAW at a concentration of 100 μg/ml for 24 h. Similarly, after treatments, the comet slides were prepared with 1 ml aliquots containing 1×10^5 harvested cells. The photographs were taken with fluorescence microscope (Olympus, Japan) equipped with digital camera. The tail length was measured with the help of Image Pro® plus software and expressed as percent inhibition of tail length.

2.3. Determination of protein oxidation

Oxidation of protein was assayed by Kwon et al[15] with slight modifications. Oxidation of BSA (5 μg) in phosphate buffer was initiated by 20 mM AAPH and inhibited by different extracts of *T. arjuna* (100 μg/ml). After incubation for 2 h at 37 °C, 0.02% BHT was added to prevent the formation of further peroxyl radical. The samples were then analyzed with normal SDS-PAGE.

2.4. Quantification of total polyphenols and flavonoids

Total polyphenols were determined by the Folin–Ciocalteu procedure.[16] The absorbance of blue-colored mixtures recorded after 40 min at 725 nm against blank. The amount of total polyphenols was calculated from the calibration curve of gallic acid standard solutions and expressed as mg/g GAE of dry extract. The quantification of flavonoids were measured as described by Ordon et al.[17] Briefly, 0.5 ml of Al_2Cl_3 (2%) ethanolic solution was added to the equal amount of sample. The absorbance was measured at 420 nm after incubation for 1 h. Flavonoid concentration of the extract was expressed as mg/g equivalent of rutin.

2.5. Determination of in vitro antioxidant activities

2.5.1. DPPH radical scavenging activity

The free radical scavenging activity of different extracts of arjuna bark were determined *in vitro* by DPPH (1, 1 diphenyl 2, picryl hydrazyl) assay.[18] DPPH in methanol (0.1 mM) was prepared and 3.0 ml of this solution was added to 40 μl of extract (different concentration of various fractions of arjuna extracts). The absorbance of incubated samples was measured at 515 nm after incubation for 30 min at room temperature. Percent inhibition was calculated as follows: DPPH Scavenged (%) = [A cont−A sample]/A cont × 100, where A cont is the absorbance of the control and A sample is the absorbance of the arjuna fractions.

2.5.2. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was analyzed by Halliwell and Gutteridge method with few modifications.[19] The reaction was initiated by the addition of EDTA (0.1 ml; 1 Mm), $FeCl_3$ (0.01 ml; 10 mM), H_2O_2 (0.1 ml; 10 mM), deoxyribose (0.36 ml; 10 mM), phosphate buffer (0.33 ml; 50 mM, pH 7.4), ascorbic acid (0.1 ml) and 1.0 ml of arjuna extract (50–300 μg/ml) separately for each fraction. The sample mixture was incubated at 37 °C for 1 h and followed by addition of equal amounts of trichloroacetic acid (10%) and thiobarbituric acid (0.05%) to develop the pink chromogen, which was measured at 532 nm and the activity of the

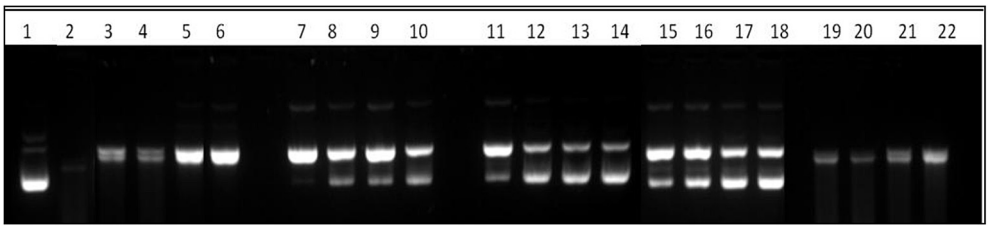

Fig. 2. DNA damage protective activity of *Terminalia arjuna* extracts on circular DNA. Lane 1 – Plasmid DNA; Lane 2 – H202 + UV treated DNA; Lane 3–6 – TAA; Lane 7–10 – TAD; Lane 11–14 – TAE; Lane 15–18 – TAB; Lane 19–22 – TAW. Four treatments were used for each extract i.e. 0.2, 0.4, 0.6 & 0.8 mg/ml respectively.

extract was reported as the percentage of inhibition of deoxyribose degradation and was calculated according to the following equation: Percent OH radical scavenged = [A cont−A sample]/A cont × 100, where A cont is the absorbance of the control and A sample is the absorbance of the arjuna fractions.

2.5.3. ABTS radical scavenging activity

The ABTS radical scavenging activity [2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical] was performed by Re et al.[20] The working reagent was prepared by mixing of 7 mM ABTS and 2.4 mM potassium per sulfate in equal quantities and then incubated for 12 h at room temperature in dark. For the above working reagent added different concentrations of arjuna extracts (different fractions separately) and then absorbance was measured at 734 nm % ABTS scavenging activity = [A cont−A sample]/A cont × 100, where A cont is the absorbance of the control and A sample is the absorbance of the arjuna fractions.

2.5.4. Metal chelating activity

Metal chelating activity was observed with the percentage inhibition of ferrozine-Fe^{2+} complex formation.[21] Briefly, the extract (25–100 μg/ml, different fractions separately) was added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM Ferrozine (0.2 ml) and then allowed to shake for 10 min at room temperature. Further absorbance was measured at 562 nm and inhibition of Ferrozine – Fe^{2+} complex formation was measured as: % inhibition = [A cont−A sample]/A cont × 100, where A cont is the absorbance of the control and A sample is the absorbance of the arjuna fractions.

2.5.5. Total antioxidant activity (FRAP assay)

Ferric reducing antioxidant property of arjuna fractions were determined by Benzie and Strain[22] with some modifications. Briefly, a mixture of 25 ml acetate buffer (300 mM; pH 3.6), 2.5 ml TPTZ (2, 4, 6-tripyridyl-*s*-triazine; 10 mM) in HCl (40 mM), 2.5 ml $FeCl_3 \cdot 6H_2O$ (20 mM) and arjuna extract (150 μl, different fraction separately). The mixture was then allowed to react with 2850 μl of the FRAP solution for 30 min in dark and then measured at 593 nm for the colored product of ferrous tripyridyltriazine complex.

2.5.6. Nitric oxide scavenging activity

Nitric oxide scavenging activity was measured by Griess reagent.[23] Briefly, sodium nitroprusside (10 mM in phosphate-buffered saline) was mixed with arjuna extracts (different fractions separately) and incubated for 150 min at room temperature. Further, Griess reagent was added to the sample mixture and absorbance was measured at 546 nm % inhibition = [A cont−A sample]/A cont × 100, where A cont is the absorbance of the control and A sample is the absorbance of the arjuna fractions.

2.5.7. Reducing power assay

Different concentrations of arjuna extracts were added with 2.5 ml of phosphate buffer (0.2 M, pH 6.6), 2.5 ml of potassium ferricyanide (1%). To the above mixture, 2.5 ml of trichloroacetic acid (10%) was added after 30 min incubation at 50 °C and centrifuged. Collected supernatant and mixed with equal amount of distilled water with 0.5 ml $FeCl_3$ (0.1%) and the absorbance was measured at 700 nm.[24] The increased absorbance of the reaction mixture was considered as reducing power.

2.6. Statistical analysis

Results were expressed as mean value ± Standard deviation ($n = 4$). Linear regression analysis was conducted to find out the correlation coefficient. Statistical significance was evaluated employing *t*-test and $P < 0.05$ was considered to be significant.

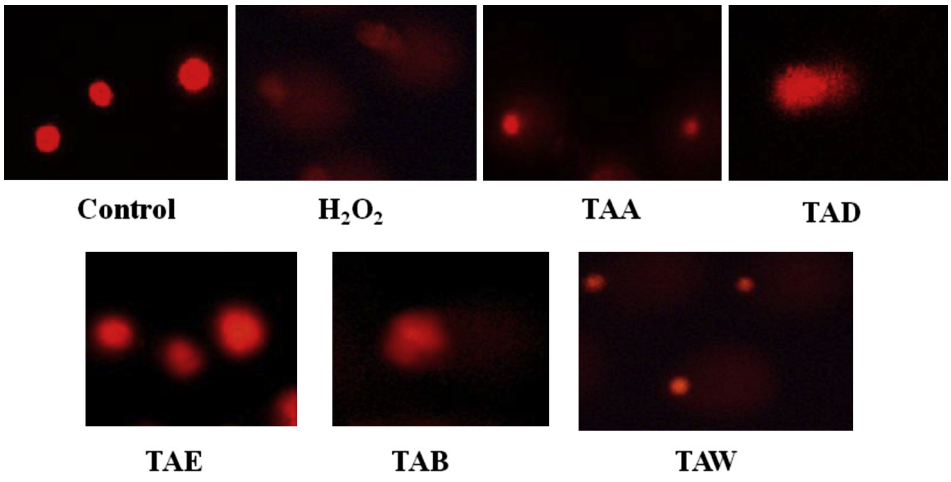

Fig. 3. DNA damage protection assay by single cell gel electrophoresis (SCGE).

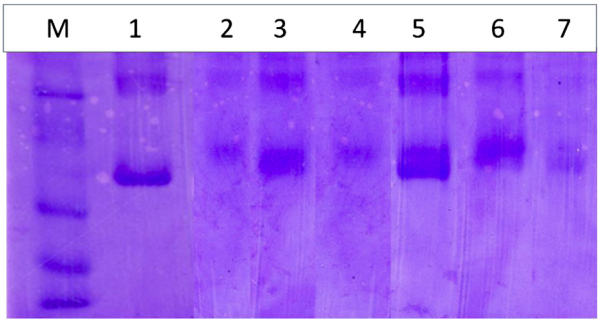

Fig. 4. The inhibitory activity of protein oxidations. Lane M – Marker (100 kD); Lane 1 – BSA (Positive control); Lane 2 – AAPH treatment (negative control); Lane 3 – TAA; Lane 4 – TAD; Lane 5 – TAE; Lane 6 – TAB; Lane 7 – TAW.

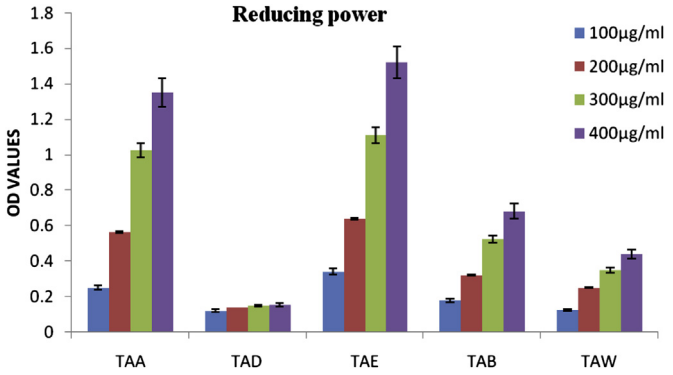

Fig. 5. Reducing power assay of *Terminalia arjuna* extracts.

3. Results

3.1. Effect of T. arjuna on super coiled DNA damage protection

The super coiled form of plasmid DNA pBR322 was the predominant band when the control DNA was run on an agarose gel (Fig. 2). *T. arjuna* extracts dose dependently inhibited hydrogen peroxide/UV induced DNA cleavage in the following manner: TAE > TAB > TAD > TAA > TAW (Fig. 2; lane 11–14, 15–18, 7–10, 2–6 and 19–22, respectively).

3.2. Effect of T. arjuna on PC-12 cell DNA damage protection

The supplementation of *T. arjuna* extracts with H_2O_2 to rat adrenal PC-12 cells revealed an observable enhancement in % Head DNA. The median tail moment of the control cell line treated with H_2O_2 alone (without extract) was assigned an arbitrary value of 1.0 in order to compare among the different fractions of arjuna extract treatments. The comet results showed the damage protective activity of arjuna extracts in the same way as plasmid DNA protection i.e. TAE > TAB > TAD > TAA > TAW (Fig. 3).

3.3. Inhibition of protein oxidation by T. arjuna

Results are presented in Fig. 4 showed that the BSA was completely degraded by 20 mM AAPH in the control as studied by SDS-PAGE electrophoresis. Pre-treatment of *T. arjuna* extracts showed protective effect significantly by restoring the band intensity upto 90% by TAE when compared with control. The inhibition activity protein oxidation by the different extracts as follows: TAE > TAB > TAA > TAD > TAW.

3.4. Quantification of polyphenols and flavonoids

Different extracts of arjuna bark were reported in a vast range of polyphenols from 50 ± 6 mg/g to 430 ± 18 mg/g GAE and the flavonoids were 4.1 ± 0.1–21 ± 0.2 mg/g RE. The results of phenolics and flavonoids of arjuna bark extracts/fractions were given in Table 1.

3.5. Antioxidant scavenging activity of T. arjuna

The antioxidant activities of all extracts were increased with an increasing concentration of the extract. The IC_{50} values of DPPH, ABTS, hydroxyl and nitric oxide radical scavenging activities, metal chelating activity were showed higher activity with TAE (Table 1). The FRAP assay, which is expressed in units of µmol Fe (II)/g were given in Table 1. The reducing power of different extracts of *T. arjuna* bark are summarized in (Fig. 5).

4. Discussion

Defence mechanism against oxidants, toxins, pathogens and other causative agents of stress are balanced by the body with the help of different antioxidant mechanisms like, protein expression, gene expression, DNA repair, stress enzymes like SOD, CAT, GPx etc.[25] DNA damage is one of the biological marker to see the antioxidant capacity of any medicinal plant.[26] The present study confirmed that TAE and TAA were able to protect PC-12 cells in the comet assay and pBR322 plasmid DNA against H_2O_2 induced DNA damage. Previous reports of Sivalokanathan[27] support the present results and further extending the application of *T. arjuna* bark extract against DNA damage of human hepatoma cell line HepG2 along with PC-12 cells. In the present study *T. arjuna* extracts (TAA, TAE and TAB) showed protection against AAPH induced protein

Table 1
Antioxidant activity and quantification of polyphenol, and flavonoid contents.

Parameter	TAA	TAD	TAE	TAB	TAW
DPPH radical scavenging activity[a]	283 ± 15	>500	270 ± 12	363 ± 18	>500
Hydroxyl radical scavenging activity[a]	185 ± 9	>500	175 ± 11	441 ± 21	>500
ABTS radical scavenging activity[a]	31 ± 2	212 ± 10	25 ± 1.2	125 ± 8	251 ± 14
Metal chelating activity[a]	421 ± 11	>500	405 ± 15	475 ± 29	>500
FRAP assay[b]	425 ± 25	>500	310 ± 15	365 ± 11	465 ± 21
Nitric oxide scavenging activity[a]	110 ± 4	99 ± 7	82 ± 4	85 ± 5	121 ± 4
Poly phenols[c]	430 ± 18	50 ± 6	380 ± 13	101 ± 5	190 ± 8
Flavonoids[d]	11 ± 0.6	4.1 ± 0.1	21 ± 0.2	5.6 ± 0.4	1.5 ± 0.05

[a] Expressed in IC_{50} values in µg/ml.
[b] Expressed in units of µmol Fe (II)/g.
[c] Expressed in units of mg/g of gallic acid equivalents.
[d] Expressed in units of mg/g of rutin equivalents.

oxidation. These results are encouraging and also may have positive role in inhibiting several stress/toxic induced protein oxidation.

Phenolic compounds are the major class among the antioxidant agents, which are also considered as a major group of medicinal plant free radical scavengers. In the present study, we found that the TAA contains higher concentration of polyphenols and flavonoids (43% and 1.1%, respectively). However, Mety and Mathad[28] have observed quite low concentrations of phenolic contents in methanolic and ethanolic extracts of *T. arjuna* bark when compared with the present results. Reactive oxygen species (ROS) such as hydroxyl radical (•OH), superoxide ($O_2-\bullet$), hydrogen peroxide (H_2O_2) and reactive nitrogen species (RNS) like peroxynitrite (ONOO−), nitric oxide (NO•) and other radicals are the major sources of oxidative stress in cells, which can damage lipids, proteins and DNA.[29] Hence, prevention of oxidative stress is a significant property of any medicinal plant or phytochemical for the management of stress related disorders. In the present study, all the arjuna fractions showed free radical scavenging activities against DPPH, ABTS, •OH, NO•, FRAP, metal chelation and reducing power. Moreover, TAE inhibited maximum free radicals and then followed by TAA and TAB. We speculate that arjuna extracts protect against oxidative stress-induced damage because of its high total phenolic content, free radical scavenging activity, DNA protection activity as well as its ability to prevent AAPH induced protein damage. Previous studies on experimental animal models[30] also suggest that the *T. arjuna* alcoholic extract may help in altering the levels of cytochrome P-450 and other antioxidant enzymes like SOD, CAT, GST and GSH in different organs. The exogenous antioxidants from *T. arjuna* extracts may act directly or indirectly with the internal antioxidant system for synergistic effects to protect several diseases linked to free radicals such as, coronary heart diseases, neurodisorders and other stress related disorders.

5. Conclusion

In the present study, alcoholic extract of *T. arjuna* bark and its extracts were evaluated for DNA protection, protein oxidation and free radical scavenging activity. The results of the present research work leads to the conclusion that *T. arjuna* extracts (TAE, TAD, TAE, TAB and TAW) have significant antioxidant activity and potential to prevent protein oxidation, DNA damage protection (by pBR322 DNA and SCGE assay). The potent antioxidative activity and DNA protection ability of *T. arjuna* bark extracts may be endorsed with phenolic/flavonoid compounds. Moreover, there was a significant correlation was observed between free radical scavenging activity, *in vitro* DNA damage activity and the total phenolic/flavonoid content. Further isolation and characterizations of these extracts is in progress so as to identify the specific antioxidative/genoprotective compounds.

Conflicts of interest

All authors have none to declare.

Acknowledgment

Authors are thankful to Director, DFRL, Mysore for his kind support.

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