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Role of phenolics as antioxidants, biomolecule protectors and as anti-diabetic factors – Evaluation on bark and empty pods of *Acacia auriculiformis*

Arumugam Sathya, Perumal Siddhuraju*

Bioresource Technology Lab, Department of Environmental Sciences, Bharathiar University, Coimbatore – 641046, TN, India

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ABSTRACT

Objective: To search for an efficient and inexpensive source of phytoconstituents with antioxidant potential and health promoting traits from bark and empty pods of *Acacia auriculiformis* (*A. auriculiformis*). **Methods:** Samples of bark and empty pod extracts were analyzed for bioactives (phenolics, flavonoids and proanthocyanidins) and subjected to free radical scavenging activity on DPPH[•], ABTS^{•+}, OH[•], O₂^{•-} and NO along with the determination of reducing power, iron chelating activity and peroxidation inhibition. Defensive action of extracts on biomolecules and cell membranes were evaluated by DNA nicking assay and haemolysis inhibition assay respectively. α -amylase and α -glucosidase inhibitory potentials were also determined. **Results:** All the bioactives analyzed were higher in bark (B) than empty pods (EP) [TPC: B (574.51±16.11); EP (96.80±3.45) mg GAE/g. TFC: B (94.71±7.65); EP (247.87±20.45) mg RE/g. Proanthocyanidins: B (2.81±0.31); EP (1.25±0.01) mg LE/100 g DM] except flavonoids. Both the extracts showed higher quenching capacity on DPPH and ABTS (DPPH: B (0.21±0.01); EP (1.51±0.17) g extract/g DPPH. ABTS: B (111 519.14±79 340.91); EP (80 232.55±32 894.12) mmol TE/g) with the FRAP of B (84 515.63±3 350.69) and EP (47 940.79±1 257.60) mmol Fe (II)/g. Iron chelation was not observed. In addition, they showed lower quenching activity on OH[•] (B (48.95±1.72); EP (34.94±1.62)%) and equivalent quenching on O₂^{•-} (B (53.47±3.92); EP (24.41±2.61)%), NO (B (49.04±5.04); EP (51.00±5.13)%), peroxidation inhibition (B (67.50±5.50); EP (55.1±2.3)%) and antihemolytic potential (B (87.60±6.84)%) towards authentic antioxidant standards. Interestingly, Empty pod extracts are devoid of antihemolytic activity. Both the extracts showed dose dependent DNA protection. Besides this, bark and empty pod extracts exhibited dual inhibiting potential against α -amylase and α -glucosidase enzymes. **Conclusions:** On summarization, it insinuated that both bark and empty pods can be used for the preparation of antioxidant/nutraceutical supplements and in anti-diabetic formulations.

1. Introduction

The molecules called free radicals have substantial roles in many physiological functions like defense, inflammation, signal transduction, cell-cell adhesion, cell proliferation, transcription and apoptosis. But exposure of the major biomolecules such as DNA, protein and lipid of the human body to excess of free radicals from both endogenous

(mitochondrial leak, respiratory burst, enzyme reactions and auto-oxidant reactions) and exogenous (environmental sources viz., cigarette smoke, pollutants, ultraviolet light, ionizing radiation and xenobiotics) sources leads to several pathophysiological conditions such as neurodegenerative disorders, cardiovascular diseases, diabetes and cancer^[1]. Besides this, the external sources also lead to the oxidation of foods especially with more lipids and in other oxidizable goods such as pharmaceuticals, cosmetics and plastics. Invariably all these systems require the supply of antioxidants, especially from natural sources rather than synthetic antioxidants due to its negative health impacts. Based on accumulative evidence, in recent decades, tremendous interest has considerably increased in finding out the natural substances especially from plants, one of the

*Corresponding author: Perumal Siddhuraju, Bioresource Technology Lab, Department of Environmental Sciences, Bharathiar University, Coimbatore – 641046, TN, India.

Tel: +91 422 2428394

Fax: +91 422 2422387

E-mail: siddhurajubrt@yahoo.com

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major kingdoms with potential bioactives and in precise with their inexpensive and residual parts^[2,3]. Hence this present study was aimed to evaluate the antioxidant potential of bark and empty pods of *Acacia auriculiformis* (*A. auriculiformis*).

Acacia, a cosmopolitan genus comprising more than 1 350 species and occurs in almost all habitat types^[4]. Although it is the second largest genus in the family Leguminosae, little research has been attempted in the field of antioxidant, phytochemicals and its therapeutics. Phytochemical constituents of *Acacia sensu-lato* alone have been demonstrated in detail^[5]. *A. auriculiformis* is one such valuable, vigorously growing tree up to the height of 30 m, commonly found in the road sides and parks of India. It gains importance mainly in agroforestry systems as its hybrids with *A. mangium* showed considerable hot rot resistance than the individual plants^[6]. Their saponins are unique in nature due to the presence of tridesmoside saponins (apart from the general phenomenon of monodesmosides or bidesmosides) proacaciaside-I, proacaciaside-II, acaciamine and triterpenoid trisaccharide also known as acacic acid lactone-3-O- β -D-glucopyranosyl (1-6)-[α -L-arabinopyranosyl (1-2)]- β -D-glucopyranoside, which is also a rare saponin that have arabinose at the terminal end^[7-9]. Apart from this, two acylated biglycoside saponins called Acaciaside A and B isolated from their funicles showed antimicrobial activity. A flavan glycoside called auriculoside from *A. auriculiformis* is found to have CNS depressant activity^[10,11]. Their antioxidant potential has also been validated^[12-14].

2. Materials and methods

2.1. Chemicals

All the chemicals used in this study were of analytical grade. ABTS, AAPH, BHA, DPPH, linoleic acid, ascorbic acid, catechin, rutin, tannic acid, trolox, quercetin, α -amylase, α -glucosidase and p-nitrophenyl- α -D-glucopyranoside were purchased from Sigma Chemicals Co (St. Louis, MO, USA). pBR322 is obtained from Genei (Bangalore, India). All the other chemicals were obtained from HiMedia Laboratories (Mumbai, India). The water was treated by arium 67316 reverse osmosis (Sartorius Stedim Biotech GmbH, Germany). All the spectrophotometric measurements were done using UV 100 (Cyberlab, USA).

2.2. Plant materials and solvent extraction

The bark and pods were collected during the month of June 2010, from a tree growing at Bharathiar University, Coimbatore, Tamilnadu. The pods were removed from the seeds and aril. Both were cleaned with tap water to wash out the sand particles and dried in an oven at 40 °C and ground into fine powder using laboratory blender (Remi Anupam Mixie Ltd, Mumbai, India) followed by ball mill MM400 (Retsch, Germany). The powdered samples were defatted with petroleum ether. The dried residues were extracted

with 70% acetone in the ratio of 1:5 (sample:solvent, w/v) by maceration. The bark and empty pod extracts were dried at 40 °C and the yield (%) was calculated. Hereafter, the empty pods are represented as pods.

2.3. Determination of phytochemicals

2.3.1. Total phenolic content (TPC)

The TPC of extracts were determined according to Folin-Ciocalteu method at 725 nm described by Siddhuraju^[15]. Aliquots of the extracts to final volume of 1 mL, was added with 0.5 mL of Folin-Ciocalteu reagent (1N) and 2.5 mL of sodium carbonate solution (20%). Soon after vortexing the reaction mixture, the tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm.

2.3.2. Total flavonoid content (TFC)

Total flavonoid content was measured according to the method of Zhishen *et al*^[16]. One mL of sample was added to 10 mL volumetric flask containing 4 mL water. 0.3 mL of 5% NaNO₂ was added to the flask. After 5 min, 0.3 mL of 10% AlCl₃ was added. After 6 min, 2 mL of 1 M NaOH was added to the mixture and mixed well. Immediately, the solution was diluted to the final volume of 10 mL with water, mixed thoroughly and the absorbance was measured at 510 nm. TFC was calculated by rutin calibration graph.

2.3.3. Proanthocyanidins

Proanthocyanidins (also called condensed tannins) were estimated by butanol-HCl method of Porter *et al*^[17]. 0.5 mL of extract, 3 mL of butanol-HCl (95:5, v/v) reagent and 0.1 mL of ferric ammonium sulphate reagent (2% in 2 N HCl) were added and kept at 90–100 °C for 60 min. Absorbance of the samples at 550 nm was subtracted with suitable sample blank (unheated mixture). It was calculated as leucocyanidin equivalents using the following formula: % Proanthocyanidins/DM = (OD value at 550 nm \times 78.26 \times Dilution factor)/(%dry matter).

2.4. Determination of antioxidant activity in vitro

2.4.1. Ferric reducing antioxidant power (FRAP) assay

FRAP was done by the method of Benzie and Strain^[18]. An aliquot of 30 μ L extracts were mixed with 90 μ L of water and 900 μ L of FRAP reagent [2.5 mL of 20 mmol/L TPTZ in 40 mmol HCl, 2.5 mL of 20 mmol/L ferric chloride, 25 mL of 0.3 mol/L acetate buffer (pH 3.6)] and incubated at 37 °C for 30 min. Absorbance was recorded at 593 nm and the reducing power was expressed as mmol Fe (II)/g extract.

2.4.2. Ferrous ion chelating activity

The ability of the extracts to chelate ferrous ions was determined by the method of Dinis *et al*^[19]. 0.1 mL of sample, 0.6 mL of distilled water and 0.1 mL of 0.2 mM FeCl₂ were mixed well and incubated for 30 s. 0.2 mL of 1 mM ferrozine was added to the above mixture, incubated for 10 min at room temperature and the absorbance was recorded at 562 nm. Results were expressed as mg EDTA equivalents/

g extract.

2.4.3. Free radical scavenging activity on DPPH[•]

The radical scavenging activity of extracts was measured using DPPH radical by the method of Sánchez–Moreno *et al*^[20] with slight modifications. Extract of 0.1 mL prepared in methanol was mixed with 3.9 mL of DPPH[•] (0.025 g/L) and incubated in dark for 30 min. Absorbance was read at 515 nm and the results were expressed as g extract/g DPPH[•].

2.4.4. Free radical scavenging activity on ABTS^{•+}

The ABTS cationic radical (ABTS^{•+}) decolorization assay was done by the method of Re *et al*^[21]. ABTS^{•+} was generated by adding 2.45 mM potassium persulphate (final concentration) to 7 mM ABTS and incubated in dark at room temperature for 12–16 h. This stock solution of ABTS^{•+} was diluted with ethanol to give an absorbance of 0.70±0.02 at 734 nm (working solution). 10 μL of extracts were mixed with 1.0 mL of working ABTS^{•+} solution and incubated at 30 °C for 30 min and the absorbance was measured at 734 nm. The results were expressed mmol trolox equivalents/g extract.

2.4.5. Hydroxyl radical scavenging activity

Hydroxyl radical (OH[•]) scavenging activity of extracts was measured using ascorbic acid–iron–EDTA model of Klein *et al*^[22]. Extract of 200 μg was mixed with 1 mL of iron–EDTA solution (0.13% ferrous ammonium sulphate in 0.26% EDTA), 0.5 mL of 0.018% EDTA and 1 mL of DMSO solution (0.85% in phosphate buffered saline 0.1 M, pH 7.4). The reaction was initiated by the addition of 0.5 mL of 0.22% ascorbic acid and incubated at 80–90 °C in water bath for 15 min. After the incubation, the reaction was terminated by the addition of 1 mL of ice cold TCA (17.5 w/v). 3 mL of Nash reagent (75 g of ammonium acetate, 3 mL of glacial acetic acid, 2 mL of acetyl acetone/L) was added to the above mixture and allowed to stand at room temperature for 15 min for color development. Absorbance values were recorded at 412 nm. The % hydroxyl radical scavenging activity (HRSA) was calculated using the following formula, HRSA% = 1–(Differences in absorbance of sample/Difference in absorbance of control) × 100.

2.4.6. Superoxide anion radical scavenging activity

The superoxide anion radical (O₂^{•-}) scavenging capacity of extracts were determined by the method of Martinez *et al*^[23] for the determination of superoxide dismutase with some modifications made by Dasgupta and De^[24] in the riboflavin–light–nitrobluetetrazolium system of Beauchamp and Fridovich^[25]. Each 3 mL of reaction mixture consists of 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, 75 μM NBT and 1 mL of extract (150 μg) is kept for 10 min of illumination under 20 W fluorescent lamps. The production of blue formazan was monitored and recorded at 560 nm. The degree of superoxide radical scavenging activity was calculated as follows,
% scavenging activity = (Ac–As)/Ac × 100

Where, Ac–Absorbance of control; As–Absorbance of sample.

2.4.7. Nitric oxide scavenging activity

Nitric oxide (NO) scavenging activity was done by the method of Marcocci *et al*^[26]. 250 μg of extracts were mixed with sodium nitroprusside (5 mM final concentration) in phosphate buffered saline, pH 7.4 to the final volume of 1 mL and incubated at 25 °C for 150 min. After incubation, the reaction mixture was mixed with Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% H₃PO₄). The absorbance was measured at 540 nm. The degree of NO scavenging activity (%) was calculated as mentioned for O₂^{•-} scavenging activity.

2.4.8. β–Carotene bleaching assay

Peroxidation inhibition (PI) capacity of the extracts was measured using β–carotene bleaching system of Taga *et al*^[27]. Stock solution of β–carotene–linoleic acid mixture was prepared as follows: 2 mg β–carotene was dissolved in 1 mL of chloroform along with 40 mg of linoleic acid and 400 mg of Tween 40. Tween 40 is added for its emulsifying property since β–carotene is not water soluble. Chloroform is allowed to evaporate. To this 100 mL of distilled water was added and the mixture was vigorously shaken until getting uniform distribution. 4.8 mL of this reaction mixture was mixed with 0.2 mL of extract (50 μg) and the absorbance was measured at 470 nm immediately against blank consists of reaction mixture without β–carotene. All the tubes were kept in a waterbath at 50 °C and the absorbance was measured at every 30 min interval for a total period of 120 min. PI was calculated as: PI% = [1–(A_{E0}–A_{C0})/(A_{E120}–A_{C120})], where A_{E0} and A_{C0} are the absorbance of values measured at zero time of the incubation for extract and control, respectively. A_{E120} and A_{C120} are the absorbance measured in the extract and control at 120 min respectively.

2.5. Determination of the extract efficiency in biomolecules and cell membrane protection

2.5.1. DNA nicking assay

The DNA nicking assay was performed using pBR322 plasmid DNA by the method of Hiramoto *et al*^[28] with some modifications. The reaction mixture (20 μL) consists of 2 μL pBR322 (200 ng), 2 μL of 50 mM AAPH (final concentration 5 mM) and 16 μL of extracts dissolved in buffer (final concentration of 25 and 50 μg) was incubated at room temperature for 10 min. Then the contents were analyzed on 1% agarose gel under 50 V for 1 h. The measurement of DNA damage was initially visualized by UV–transilluminator ECX (Vilber lourmat, France) and documented by Geldoc lab image IDL 320 (Medicare Scientific, India).

2.5.2. Oxidative hemolysis inhibition assay

Blood (15 mL) was collected from healthy human volunteers by venipuncture in a citrated tube. It was centrifuged immediately at 1 500 rpm for 10 min at 4 °C, the plasma and

buffy coat were then carefully discarded. Erythrocytes were washed three times with phosphate buffered saline (PBS, 0.02 M, pH 7.4), and then re-suspended to 2% using the same buffer. Free radical chain oxidation in erythrocytes was induced by the addition of AAPH (dissolved in PBS; final concentration 50 mM). To study the protective effects of the extracts against AAPH-induced hemolysis, erythrocyte suspension was pre-incubated with the bark and pod extracts (50 μ g) at 37 °C for 30 min, followed by incubation with and without AAPH. This reaction mixture was shaken gently while being incubated for 4 h at 37 °C. In all experiments, a negative control (erythrocytes in PBS), as well as extract controls (erythrocytes in PBS with extract) were used for comparison. The extent of hemolysis was determined spectrophotometrically by the procedure of Ko *et al.*[29]. Briefly, aliquots of the reaction mixture were taken at the end of incubation and centrifuged at 4 000 rpm for 10 min to separate the erythrocytes. The percentage of hemolysis was determined by measuring the absorbance of the supernatant (A) at 545 nm and compared with that of complete hemolysis (B) by treating an aliquot with the same volume of the reaction mixture with distilled water. The hemolysis percentage was calculated using the formula: $A/B \times 100$.

2.6. Analysis of anti-diabetic factors

2.6.1. α -amylase inhibition activity

The bark and pod extracts were mixed with 100 μ L of 0.02 M sodium phosphate buffer (pH 6.9) and 100 μ L of α -amylase solution (4.5 units/mL/min) and pre-incubated at 25 °C for 10 min. Then, 100 μ L of 1% starch solution was added and incubated at 25 °C for 30 min and the reaction was stopped by the addition of 1.0 mL of dinitrosalicylic acid reagent. The test tubes were then incubated in a boiling water bath for 5 min and then cooled to room temperature. The reaction mixture was then diluted 10-fold times with distilled water and the absorbance was measured at 540 nm. The readings were compared with the control (extract was replaced by buffer) and α -amylase inhibition activity (%) was calculated[30].

2.6.2. α -glucosidase inhibition activity

Both pod and bark extracts were mixed with 100 μ L of 0.1 M phosphate buffer (pH 6.9) and 100 μ L α -glucosidase solution (1 unit/mL/min) and incubated at 25 °C for 5 min. After the pre-incubation, 100 μ L of p-nitrophenyl- α -D-glucopyranoside (5 mM) solution was added and the reaction mixture was incubated at 25 °C for 10 min. After the incubation, the absorbance was recorded at 405 nm and α -glucosidase inhibition (%) was calculated[30].

2.7. Statistical analysis

The data were subjected to one-way analysis of variance

(ANOVA) and the significance of the difference between means was determined by Duncan's multiple-range test ($P < 0.05$) using SPSS (Statistical Package for the Social Sciences) version 13.0. (SPSS Inc., Chicago, Illinois, USA). Values are expressed as mean of triplicate determinations \pm standard deviation.

3. Results

3.1. Antioxidative phytochemicals

The yield of bark and empty pod extracts was found to be 33.44% and 34.80% respectively and no variation was found between their extractability with the solvent acetone. The TPC, TFC and proanthocyanidin content of bark and pod extracts were calculated from the standard calibration curves and expressed as mg gallic acid equivalents/g extract (GAE) (standard curve equation: $Y = 0.078X + 0.004$, $r^2 = 0.99$), mg rutin equivalents/g extract (RE) (standard curve equation: $Y = 0.0061X - 0.0068$, $r^2 = 0.99$) and g leucocyanidin equivalents/100 g DM respectively. The TPC of bark and pod extract was found to be (574.51 \pm 16.11) and (96.80 \pm 3.45) mg GAE/g extract with the flavonoid content of (94.71 \pm 7.65) and (247.87 \pm 20.45) mg rutin equivalents/g extract and proanthocyanidins of (2.81 \pm 0.31) and (1.25 \pm 0.01) g leucocyanidin equivalents/100 g DM respectively. All the phytochemicals estimated were found to be higher in bark than pod except for the flavonoids.

3.2. In vitro antioxidant potential

3.2.1. Reducing power and iron chelating activity

Reducing power (RP) of bark and pod extracts along with positive controls have been summarized in Table 1. It is found in the decreasing order of tannic acid > BHA > rutin > bark > pod. There is no statistical significant ($P < 0.05$) difference between bark [(84 515.63 \pm 3 350.69) mmol Fe(II)/g extract] and pod [(47 940.79 \pm 1 257.60) mmol Fe(II)/g extract] but with authentic standards. Iron chelating activity was not observed in either bark or pod extracts.

3.2.2. Free radical scavenging activity on DPPH \cdot and ABTS \cdot^+

As it can be seen from the Table 1, both the extracts were capable of scavenging DPPH \cdot radicals with the activity of (0.21 \pm 0.01) and (1.51 \pm 0.17) g extract/g DPPH \cdot for bark and pod extract respectively. There is no statistical significant ($P < 0.05$) difference found between bark extract and authentic standards like BHA, rutin and tannic acid. Pods showed lower activity than others and hence the significant difference also. Similar to DPPH system, ABTS scavenging potential is also found in the decreasing order of tannic acid > BHA > rutin > bark > pod (Table 1). Even though the order of scavenging potential is the same, statistical significant

Table 1Reducing power and free radical scavenging activity of bark and empty pod extracts of *A. auriculiformis* (n=3).

Samples	FRAP (mmol Fe (II)/g extract)	DPPH [•] (g extract/g DPPH) [†]	ABTS ^{•+} (mmol TE/g extract)
BHA	350 760.45±72 476.70 ^b	0.16±0.01 ^a	655 137.01±61 415.86 ^a
Rutin	174 032.83±26 869.47 ^c	0.19±0.01 ^a	433 569.06±23 178.34 ^b
Tannic acid	562 955.03±42 130.92 ^a	0.11±0.01 ^a	751 735.57±62 890.85 ^a
Bark	84 515.63±3 350.69 ^c	0.21±0.01 ^a	111 519.14±9 340.91 ^c
Pod	47 940.79±1 257.60 ^c	1.51±0.17 ^b	80 232.55±8 294.12 ^c

Values are representatives of mean±S.D. Values followed by different superscripts in a column are significantly different ($P<0.05$). [†] – Amount of sample required to decrease by 50% of initial DPPH radical concentration.

($P<0.05$) difference was not observed between bark and pod extracts. However, bark ((111 519.14±9 340.91) mmol TE/g extract) showed the higher activity than pods ((80 232.55±8 294.12) mmol TE/g extract).

3.2.3. Quenching potential against biologically important radicals

The bark and pod extracts of *A. auriculiformis* showed quenching potential against OH[•], O₂^{•-} and NO, the radicals which are generated during the physiological reactions of human system and have biological significance. The decreasing order of OH[•] quenching potential was found to be (82.46±2.29)% (catechin)>(48.95±1.72)% (bark)>(34.94±1.62)% (pod). Statistically significant ($P<0.05$) difference was observed between the samples and also with the standard. On the other hand, scavenging potential of bark and pod extracts on superoxide anion radical was registered in the increasing order of pod (24.41±2.61)%<trolox (49.87±1.31)%<bark (53.47±3.92)%<BHA (69.29±2.71)%<rutin (76.72±2.82)%. Interestingly bark extracts showed comparable activity with the standard trolox without statistically significant ($P<0.05$) difference. Again pod extract showed lower activity than the bark and the positive controls. Figure 1 depicts comparable NO quenching capacity of both bark and pod extracts towards the positive control of quercetin and ascorbic acid. It is worth noting that pod extract (51.00±5.13)% has equivalent radical scavenging capacity with bark extract (49.04±5.04)%.

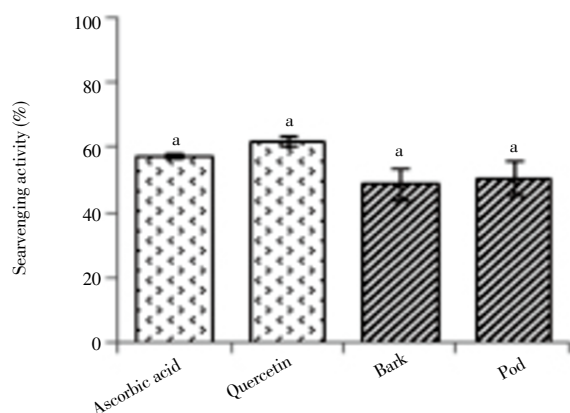


Figure 1. Nitric oxide scavenging activity of bark and empty pod extracts of *A. auriculiformis*.

Values are representatives of mean±S.D (n=3). Bars having different letters are significantly different ($P<0.05$).

3.2.4. Lipid peroxidation inhibition

The capacity of bark and pod extract against lipid peroxidation is depicted in Figure 2 along with the standards BHA, rutin and ascorbic acid. Attractively, both the extracts showed higher activity than rutin and in addition bark showed analogous activity with BHA. In addition none of the extract showed pro-oxidant activity as like ascorbic acid.

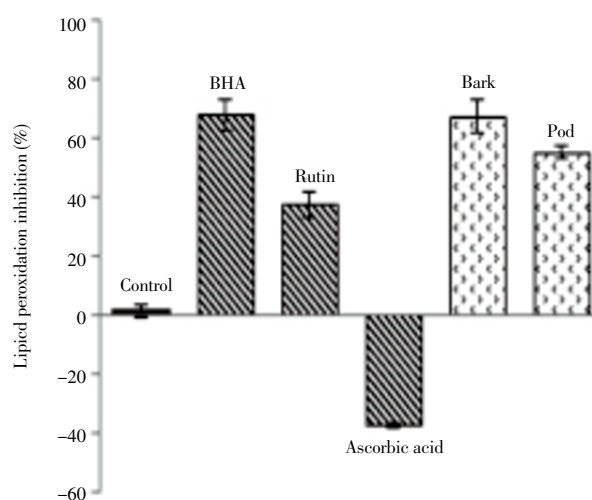


Figure 2. Peroxidation inhibition capacity of bark and empty pod extracts of *A. auriculiformis* measured by β -carotene bleaching system.

Values are representatives of mean±S.D (n=3).

3.3. Protective action on biomolecules and cell membranes against oxidation

Figure 3 depicts the electrophoretic pattern pBR322 plasmid DNA, its cleaved products and protection conferred by bark and pod extra-cts against the devastating effects of peroxy radicals. In the agarose gel electrophorogram, lane 1 shows the intact fast moving supercoiled (native form) form of plasmid DNA. Exposure of AAPH and its further action on plasmid DNA converted the native form into slow moving open circular form followed by linear forms as shown in lane 2. This indicates AAPH induced DNA strand scission. Lane 3–6 showed dose dependent protective effects of both the extracts by reducing the fragmentation rate (not the fragmentation process) and showed dense supercoiled form rather than open-circular and linear forms.

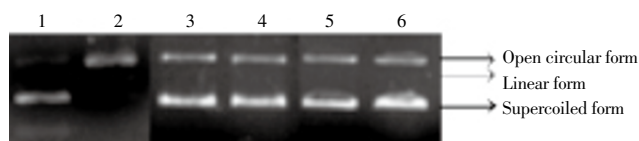


Figure 3. Agarose gel electrophoresis of various forms of pBR322. Lane 1 – untreated plasmid DNA (DNA+water); Lane 2 – treated plasmid DNA (DNA+AAPH); Lane 3 & 4 – treated plasmid DNA with 25 and 50 μ g of bark extract respectively; Lane 5 & 6 – treated plasmid DNA with 25 and 50 μ g of pod extract respectively.

Among the two extracts, bark extract registered the protective effect of (87.60 \pm 6.84)% against hemolysis of human RBC's induced by AAPH. It is comparable with the standards BHA (91.06 \pm 2.41)% and tannic acid (89.91 \pm 0.87)% without statistical significant ($P < 0.05$) difference. It is worth noting that, pod extract did not show protection against hemolysis, instead it leads to hemolysis in a contrary context.

3.4. Inhibition of α -amylase and α -glucosidase enzymes

Both bark and pod extracts showed dual inhibiting potential against α -amylase and α -glucosidase enzymes. Bark extract scored higher inhibition of (64.55 \pm 5.12)% and (95.12 \pm 4.75)% on α -amylase and α -glucosidase at a concentration of 50 μ g and 2.5 μ g respectively. Pod extract showed comparable activity towards bark with the inhibition of (50.57 \pm 5.12)% and (79.1 \pm 6.5)% at a concentration of 50 μ g and 5 μ g on α -amylase and α -glucosidase respectively. It is noticed that inhibition of α -amylase requires high quantity of extracts than α -glucosidase.

4. Discussion

The extract yields reported here for bark and pod extracts were higher than bark extracts of other legume trees such as *A. nilotica* and *Cassia fistula*[31,32]. The presence of concentrated active principles in the extracts is due to its prior extraction with non-polar solvents which aids the removal of interfering substances[33]. Phenolics, flavonoids and proanthocyanidins are the principal compounds accounting for antioxidant potential and multiple biological effects. The TPC of bark extracts observed in the present study is higher than the contents of already available reports on *A. auriculiformis*, *A. confusa*, *A. nilotica*[12–14,32,34,35] and analogous to *C. fistula*[31]. Similarly the observed TPC of pods was higher than the empty pods of *A. pennatula* and comparable towards *A. nilotica* pods[32,36]. Contrast to phenolics, flavonoids was found to be higher in pod extracts rather than bark in the present report. Sultana et al[32] and Feregrino-Pérez et al[36] registered the TFC in the range of 2.14–4.93 g catechin equivalents/100 g DM and 37.87–76.46

mg CE/g extract for *A. nilotica* bark and *A. pennatula* pods respectively. Some of the reported phenolics and flavonoids of *Acacia* spp are gallic acid, caffeic acid, ferulic acid, catechin, epicatechin, rutin, quercetin, myricetin, kaempferol and also gallotannins[37–39].

Proanthocyanidins of bark and pod extracts are comparable to those observed in *Ziziphus mucronata* bark extracts[40] and lower than *A. confusa* bark. Propelargonidin, procyanidin and prodelfinidin are some of the identified proanthocyanidins[41]. This lower concentration of condensed tannins recorded in the present study may impart their use in livestock as feed substitute after the clarification with fibre and protein bound proanthocyanidins which were not included in the estimation[42]. It is noticed that, among the phytoconstituents estimated in the present report, phenolics was found to be the highest followed by flavonoids and proanthocyanidins are the least in bark extracts and this trend is similar to those observed by Olajuyigbe and Afolayan[40], in *Z. mucronata* bark extracts. Concentration of simple phenolic compounds such as caffeic acid ferulic acid etc. are generally higher in younger tissues, later different phenolic acids condense to form complex phenolic compounds such as flavonoids, tannins and lignin etc. Hence, bark accumulates phenolic compounds with the maturity of the plant, possess relatively higher amounts than other plant organs which mimic our present report[31,43].

Reducing power (RP) of a compound serves as a significant indicator of its antioxidant potential. The reductones terminate the free radical chain reaction by donating hydrogen atoms to the radical molecules. Similar to the present report, Singh et al[12–14,38] also showed lower reducing power for *A. auriculiformis* bark and *A. nilotica* pods than the standard BHA and quercetin. On the other hand, the RP of pods in the present study is higher than *A. pennatula* empty pods[36]. RP of other *Acacia* spp. have also been documented[33,38,39]. However, adequate comparison is not possible due to the expression units since most of the literatures have been documented with the absorbance values. However, all the extracts showed potential RP due to the presence of high content of oxidizable components[44].

In consonance with our report on DPPH, Singh et al[12–14] and Feregrino-Pérez et al[36] showed lower quenching potential of *A. auriculiformis* bark and *A. pennatula* pods than the standards gallic acid, ascorbic acid and trolox. The scavenging potential of other tree barks and pods (*A. nilotica* and *C. fistula*) have also been reported[31,32,38]. ABTS is another nitrogen centered cationic radical with the absorption maxima of 760 nm, a wavelength usually not encountered with plant compounds like anthocyanins (λ max= 470–580) and carotenoids (λ max= 400–500) and hence the interferences can be nullified. But DPPH radical chromogen (λ max= 515) has the limitation in this point.

Even though they are less stable than DPPH, they are more reactive than DPPH and the reaction takes place in milliseconds. So free radical scavenging activity using ABTS can give a fast and reliable measurement^[45]. Similar findings related to our report on ABTS was given by Feregrino-Pérez *et al*^[36] in which the pod extracts of *A. pennatula* also showed lower scavenging activity than gallic acid and trolox. ABTS scavenging activity of *A. catechu* was reported as 17.3 ascorbic acid equivalents (%) and only minimal literatures are available on this radical quenching^[44].

Hydroxyl radical, an extremely reactive radical of biological systems, has been effectively quenched by both bark and pod extracts. OH[•] scavenging capacity of leaves and green pods of *A. nilotica* and isolated compounds like kaempferol and umbelliferone from *A. nilotica* bark have been documented by site-specific and non-site-specific deoxyribose degradation assays. By this connection, Singh *et al*^[37,38,46] revealed that, the quenching potential was aided by both the mechanisms of direct OH[•] scavenging (non-site-specific) and iron chelating potential (site-specific). In contrast, Kalaivani and Lazar Mathew^[33] disclosed the presence of OH[•] scavengers rather than iron chelators. Our results are in good agreement with this report, since both bark and pod extracts showed the existence of OH[•] quenchers and absence of iron chelators. This reveals the difference in the constitution of phytochemicals in various plant parts of the same tree species.

Superoxide anion radical (O₂^{•-}), the one which have indirect attack on biomolecules and metabolically important enzymes has been quenched by both the extracts. In parallel with our findings, *Pinus radiata* and *P. maritima* bark extracts also showed higher scavenging power than the standards calcium ascorbate, vitamin C and trolox^[47]. O₂^{•-} scavenging potential was also recorded in leaves and green pods of *A. nilotica*^[38,39]. Dose dependent quenching potential was also noticed in stem barks of *C. fistula*^[31].

Nitric oxide, an important chemical mediator generated by endothelial cells with several physiological roles in nervous system, vascular system, lung vasodilation and gastrointestinal function has its negative play on mitochondrial enzymes and proteins upon their excess production. Comparable activity of the both bark and pod extracts towards authentic standards against this deleterious radical is of enormous importance. There is no report on this radical for *Acacia* species.

Peroxidation of lipids and their further development into foam cells by a series of chain reactions are the major contributing factors for cardiovascular diseases. Both bark and pod extracts conferred the potential against peroxidation during the initiation step^[48–51]. In addition, both the extracts showed its potential against peroxidation inhibition (PI) in ammonium thiocyanate model and on liposomes (data not shown). Contrary to our present findings (*ie.*, equivalent

PI activity to BHA and higher activity than rutin), Singh *et al*^[12,14] reported lower PI of *A. auriculiformis* bark extract than BHT. The effectiveness of various plant parts (bark, green pods and leaves) and isolated compounds (kaempferol and umbelliferone) of *A. nilotica* against lipid peroxidation have been documented in liver homogenates^[32,33,37,38,46].

Erythrocytes are considered as major targets for free radical attack owing to the presence of high membrane concentration of polyunsaturated fatty acid (PUFA) and to their specific role as oxygen carriers^[52]. Even though, both the extracts showed PI against linoleic acid and in liposomes, validation of this effect in human cell-based model is extremely important. Therefore, the *in vitro* oxidative damage of human erythrocytes in a metabolically simplified model system was used to evaluate the protective effect against AAPH, generator of peroxy radicals. The results obtained with this system is partially fit to those observed in linoleic acid model except for pod extracts, which showed hemolysis and this is suspected to be aided by the action of saponins (data not shown) rather than the absence of peroxy radical scavenging activity. But bark extracts showed mimicking activity in both the systems by exhibiting analogous activity to BHA. Antihaemolytic activity of pressure cooked seeds of *A. auriculiformis* is the only report available for *Acacia* spp^[53].

DNA protection of bark and pod extracts was verified by determining their ability to protect supercoiled pBR322 plasmid DNA from oxidative insult by AAPH. The damage of plasmid DNA results in a cleavage of one of the phosphodiester chains of the supercoiled DNA and produces a relaxed open-circular form. Further cleavage near the first fracture results in linear double-stranded DNA molecules. Both the extracts showed good protection against DNA oxidation. As far as our literature survey, no report is available on DNA damage protection for *Acacia* spp. using this kind of plasmid DNA system. Therefore, the results reported here could be assumed as the first report. However DNA protection in human lymphoblast cell K652 for *A. salicina* leaves is available^[54].

α -amylase and α -glucosidase are the important enzymes in the carbohydrate metabolism and as targets for the therapeutics of diabetes, a major metabolic disorder of developing and developed countries due to changes in people's lifestyle and dietary habits. Inhibition of α -glucosidase and α -amylase results in delayed carbohydrate digestion and glucose absorption with attenuation of postprandial hyperglycaemic excursions. Acarbose (the first dual inhibitor), miglitol, metformin and voglibose are commercially available enzyme inhibitors for type II diabetes. However, these drugs are reported to cause various side effects such as abdominal distention, flatulence and possibly diarrhea^[55]. Search for safe and effective inhibitors from natural sources are of emerging interest.

Surprisingly, both bark and pod extracts showed dual inhibitory potential against the target enzymes which might be due to the presence of specific phenolics^{56–59}.

From the present findings, it is concluded that the plant parts bark and empty pods of *A. auriculiformis* have potential antioxidant activity by the influence of phenolics, flavonoids and proanthocyanidins. Apart from this they have the capacity to protect biomolecules and cell membranes with the additional role as remedy for type II diabetes. Hence the phytochemicals of both bark and empty pods can be promoted for enlightening the human health.

Conflict of interest statement

We declare that we have no conflict of interest.

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