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Studies on antioxidant potential of methanol extract/fractions of *Acacia auriculiformis* A. Cunn

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Abstract

Antioxidant activities of the methanol extract/fractions of *Acacia auriculiformis* A. Cunn were evaluated by three *in vitro* experiments, namely, DPPH, relative reducing power and hydroxyl radical (site specific and non site specific) assays. The differential activities of methanol extract/fractions could be correlated with their respective total phenolic contents and compared with standards (BHT and L-ascorbic acid.). The bark powder of the plant was extracted with different solvents of increasing and decreasing polarity by a maceration extraction method and then the methanol extract was further partitioned with ethyl acetate and water. The scavenging activity of extract was found to be enhanced on fractionating the extract. Moreover, among the two fractions (ethyl acetate and water fraction) and the crude extract, the water fraction exhibited good scavenging responses of 72.0%, (57.2%), 1.76 (1.52), 88.0% (82.6%) and 93.6% (83.47) in DPPH, reducing power, site specific and non-site specific hydroxyl radical scavenging assay in increasing and (decreasing) order of solvent polarity at maximum concentration, respectively. Studies are in progress to evaluate the effect of extracts/fractions in other antioxidant assays and to identify the factors responsible for the activity.

Keywords: Free radicals; DPPH; Polyphenols; Hydroxyl radicals

1. Introduction

Reactive oxygen species (ROS) are involved in the organism's vital activities including phagocytosis, regulation of cell proliferation, intracellular signalling and synthesis of biologically active compounds and ATP (Halliwell & Gutteridge, 1989). With an insufficiency of the antioxidant protective system or under an intense influence of radical-initiating factors (ionizing radiation, hard ultraviolet radiation, xenobiotics, mineral dust), ROS are overproduced and oxidative stress develops. Oxidative stress is a specific feature in the pathogenesis of various diseases, including cancer, cardiovascular diseases, diabetes, tumors, rheumatoid arthritis and epilepsy (Ho, Chen, Shi, Zhang, & Rozen, 1992; Hof, Wiseman, Yang, & Tijburg, 1999).

In recent years, considerable attention has been paid to antioxidant properties of plants that may be used for human consumption. The comprehensive evaluation of antioxidant activity of natural products, using a battery of test methods, has been shown to be important in identifying both the antioxidant and pro-oxidant activities of these compounds. Phenolic compounds are attracting considerable interest in the field of chemistry, food and medicine due to their promising antioxidant potential (Okuda, Valentine, Shapiro, & Downing, 1994). It has also been reported that the extracts of the medicinal plants exhibit their protective effects through diverse mechanisms, e.g. preventing the formation of carcinogens by scavenging the free radicals and by acting as blocking agents or suppressing agents. Keeping in view the relevance of free radical theory of diseases as one of the objective parameters,

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the amenability of the extracts of *Acacia auriculiformis* was examined in the present study, following a previous antimutagenic/anticarcinogenic study (Kaur et al., 2002) done in our laboratory. The present investigation was undertaken to check the antioxidant effects/free radical scavenging activities of the methanol extract and its fractions, employing different assays, namely DPPH scavenging assay, deoxyribose degradation assay and reducing power toward heavy metals.

2. Materials and methods

2.1. Chemicals

1′,1′-Diphenylpicryl-hydrazyl (DPPH) and 2-thiobarbituric acid were obtained from Sigma Aldrich USA and deoxyribose was obtained from Lancaster. All other chemicals, namely potassium ferricyanide, trichloroacetic acid, ferric chloride, EDTA, hydrogen peroxide, L-ascorbic acid, sodium hydroxide, BHA, Folin–Ciocalteu reagent, sodium carbonate and other solvents were of analytical grade.

2.2. Preparation of extract

Acacia auriculiformis A. Cunn a vigorously growing deciduous or evergreen tree, possibly attaining 30 m in height, belongs to the family Mimosaceae and is commonly known as Black Wattle. The trees are widely distributed in India and are found growing on the roadside. It bears bright yellow-coloured flowers. Its bark is hard, fissured and dark brown in colour and is chewed as such to keep the teeth and gums healthy. It is found to be rich in methylglucuronic acid, glucuronic acid, galactose, arabinose and rhamnose (Anderson, 1978). It is reported to have central nervous system depressant, spermicidal and filaricidal activities due to the presence of tannins and triterpenoid saponins (Garai & Mahato, 1997; Ghosh, Sinha Babu, & Sukul, 1993; Parkashi, Ray, Pal, & Mahato, 1991).

Two methods of extraction were followed. In one method, the extraction was started with a highly polar solvent, i.e. water, which was followed by methanol, acetone, ethyl acetate, chloroform and hexane (Flow Chart 2). In the second method, the extraction was started with a less polar solvent (hexane), followed by chloroform, ethyl acetate, acetone, methanol and water. In both methods, the bark powder was soaked for 24 h in each solvent and, after recovering the supernatant, the respective solvents were added twice to the residue. The three supernatants obtained with each solvent were pooled and dried in a rotary vacuum evaporator. Extraction in each solvent was done thrice and the whole procedure was repeated twice (Flow Chart 1). The crude methanol extract, which was used in the present study, was obtained after extracting the bark powder in hexane, chloroform, ethyl acetate and acetone in the first method and, in second method, it was obtained after extracting the bark powder with water. The methanol extracts prepared by the two methods were separately fractionated with water and ethyl acetate to yield the water fraction and ethyl acetate fraction.

The methanol extract obtained by extraction of the bark powder in methanol was dried and weighed. The different concentrations of the dried methanol extract were made after dissolving it in double-distilled water in weight/volume. The concentrations were made as microgrammes/ml (μg/ml). While fractionating the crude methanol extract, it was redissolved in methanol and then partitioned by adding ethyl acetate and water. The ethyl acetate fraction and water fraction were dried and then the different concentrations were made by using water as solvent.

2.3. Determination of total phenolics

The amount of total phenolics in extracts was determined according to the Folin–Ciocalteu method (Singleton & Rossi, 1965). Samples (200 μl, three replicates) were introduced into test tubes containing 1.0 ml of Folin–Ciocalteu's reagent and 0.8 ml of sodium carbonate (7.5%). The contents in the tubes were mixed and allowed to stand for 30 min. Absorbance at 765 nm was measured (Systronic 2202 UV–VIS spectrophotometer). The total phenolic content was expressed as gallic acid equivalents (GAE) in grammes per kilogram dry plant material.

2.4. Antioxidant testing assays

2.4.1. DPPH scavenging assay

The extracts/fractions were measured in terms of hydrogen-donating or radical-scavenging ability using the stable radical DPPH$^{\cdot}$ (Blois, 1958). Scavenging of DPPH$^{\cdot}$ represents the free radical-reducing activity of extracts /fractions based on a one-electron reduction.

Briefly, the reaction mixture contained 300 μl of different extract/fraction concentrations (1–100 μg/ml) and 2 ml of DPPH (0.1 mM in methanolic solution). The reaction mixture was then placed in the cuvette holder of the spectrophotometer (Systronic 2202 UV–VIS spectrophotometer) against the blank, which did not contain the extract/fraction and read at 517 nm. The L-ascorbic acid was used as the positive control. The percent DPPH decolorisation of the sample was calculated by the equation:

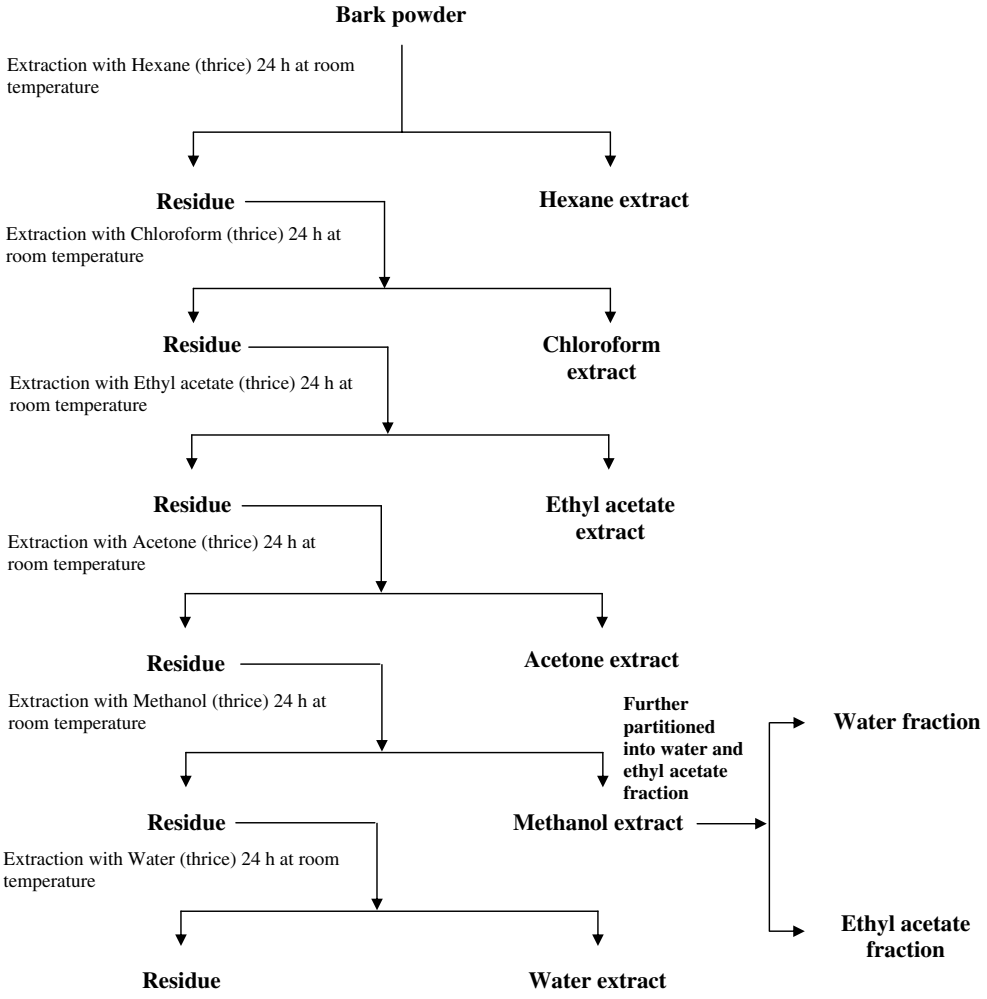
$$\text{Percent inhibition} = (\mathbf{B}_0 - \mathbf{B}_1/\mathbf{B}_0) \times 100$$

where $\mathbf{B}_0$ is the absorbance of negative control; $\mathbf{B}_1$ is the absorbance of reaction mixture.

The decoloration was plotted against the sample extract/fraction concentration in order to calculate the IC_{50} value (inhibitory concentration 50, μg/ml), which is the amount of sample necessary to decrease the absorbance of DPPH by 50%.

2.4.2. Reducing power assay

The reducing power of extract was determined by the method of Oyaizu (1986). Different concentrations of extract in 1 ml of distilled water were mixed with phosphate



Flow Chart 1. Maceration extraction of bark powder of *Acacia auriculiformis* by increasing order of solvent polarity.

buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. Aliquots (2.5 ml) of trichloroacetic acid (10%) were added to the mixture, which was then centrifuged for 10 min at 1036 g. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$(2.5 ml, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power.

2.4.3. Deoxyribose degradation assay

The non-site or site-specific deoxyribose assays were performed following the method of Halliwell, Gutteridge, and Aruoma (1987) Arouma, Grootveld, and Halliwell (1987) with slight modifications. In non-site-specific deoxyribose assay, briefly, the extracts (from 1 to 100 μg/ml) were mixed with a Haber–Weiss reaction buffer [10 mM $FeCl_3$, 1 mM EDTA (pH 7.4), 10 mM H_2O_2, 10 mM deoxyribose, and 1 mM L-ascorbic acid], and the final volume of all mixtures was made to 1.0 ml. The mixture was then incubated at 37 °C for 1 h and heated at 80^0C with 1 ml of 2-TBA (0.5% 2-TBA in 0.025 M NaOH, 0.02% BHA) and 1 ml of 10% trichloroacetic acid (TCA) in a water bath for 45 min. After cooling, absorbance of mixtures was measured at 532 nm. A site-specific assay was performed following slight modifications where the EDTA was replaced with the same volume of phosphate buffer. The percentage inhibition was calculated, employing the same formula as given for the DPPH·-scavenging assay.

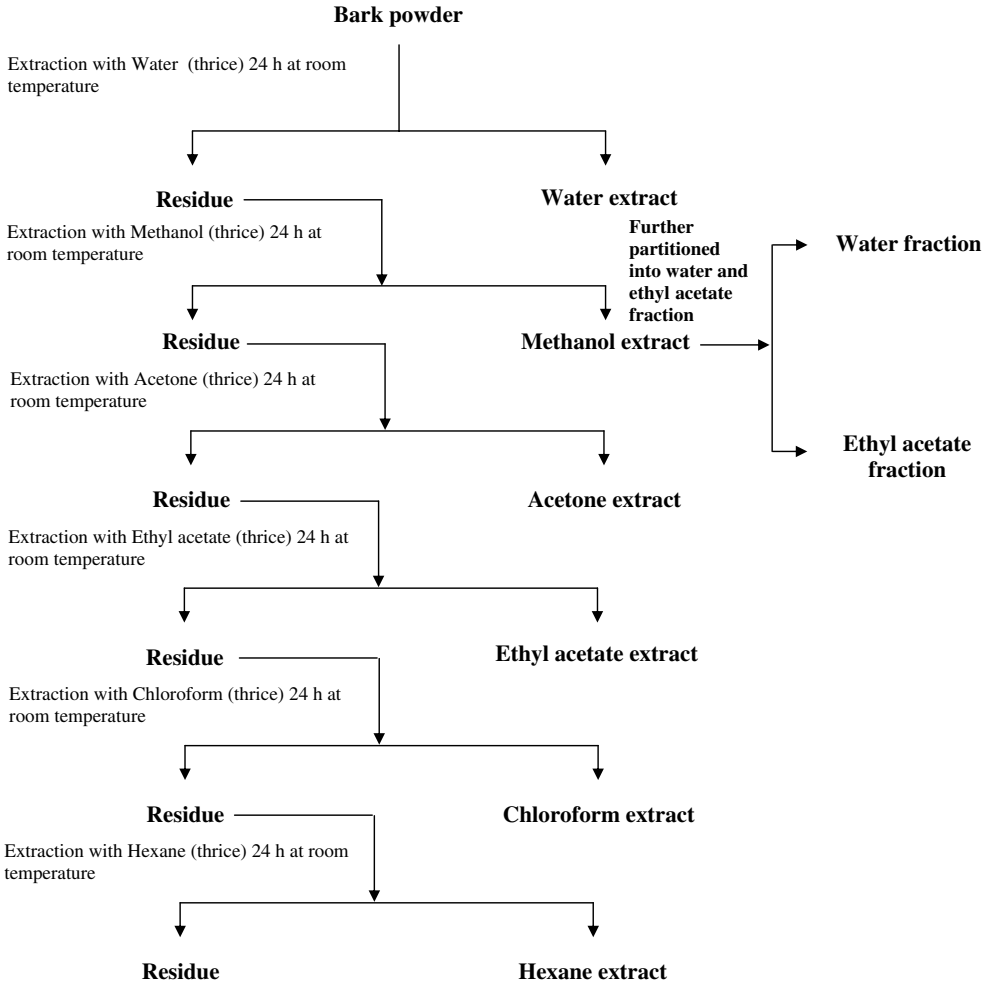
2.5. Statistical analysis

All experiments were repeated at least three times. Results are reported as means ± SE (not shown on the graphs). IC_{50} values were also calculated.

3. Results and discussion

3.1. Total phenolic content

The total phenolic contents of the methanol extract/fractions prepared by increasing and decreasing order of solvent polarity are shown in Table 1. It was noticed that the ethyl acetate and water fractions showed higher total phenolic contents than the crude methanol extract. The antioxidant capacity of the methanol extract /fractions of



Flow Chart 2. Maceration extraction of bark powder of *Acacia auriculiformis* by decreasing order of solvent polarity.

Table 1
Phenol content of methanol extract/fractions of *Acacia auriculiformis* in g/kg of plant material as equivalent to the standard gallic acid

Extract/fractions	Increasing order of polarity Phenol content (g/kg)	Decreasing order of polarity Phenol content (g/kg)
Crude extract	10.9	14.6
Et. Ace fraction	30.4	40.6
Water fraction	87.8	74.2

Acacia auriculiformis A. Cunn prepared by solvents, starting with high polarity and low polarity, was compared with the activities of known antioxidants, such as BHT, L-ascorbic acid, employing three *in vitro* complementary assays: DPPH$^{\bullet}$-reducing assay, deoxyribose degradation (site and non-site specific) assay and reducing power assay.

3.2. DPPH scavenging assay

As shown in Fig. 3, the extracts obtained by the two extraction methods and their fractions and the reference chemicals were all able to reduce the stable radical DPPH$^{\bullet}$ to yellow-coloured diphenyl picrylhydrazine. The degree of discoloration indicates the scavenging potential of the extracts/fractions. It was observed that the water fraction of the methanol extract obtained by decreasing solvent polarity exhibited maximum free radical-scavenging activity (74.1%) at 100 μg/ml concentration. The potential of L-ascorbic acid to scavenge DPPH radical activity became almost stable after 50 μg/ml and there was no increase in scavenging activity from the concentrations 50 μg/ml (91.2) to 100 μg/ml (90.2). The IC_{50} value for the water fraction (increasing polarity) was observed to be 35.4 μg/ml. The activity of the extracts in the DPPH assay indicates their hydrogen-donating ability (Shimada, Fujikawa, Yahara, & Nakamura, 1992) as the free radicals are known to cause auto-oxidation of the unsaturated lipids in foods (Kaur & Perkins, 1991). On the other hand, antioxidants are also believed to intercept the free radical chain of oxidation and donate hydrogen from the phenolic hydroxyl groups, thereby forming a stable end-product, which does not initiate or propagate further oxidation of lipid (Sherwin, 1978). The data obtained show that methanol extract/fractions are free radical scavengers and may act as primary antioxidants, which can react with free radicals by donating hydrogen. In the present study, it is expected

that the DPPH radicals get stabilized by accepting the hydrogen donated by the hydroxyl groups present on the phenolic compounds.

3.3. Deoxyribose degradation (site and non site specific) assay

Figs. 1 and 2 show the results obtained in deoxyribose degradation assay in non-site-specific and site-specific assays, respectively. Deoxyribose assay was used to measure the site-specific (e.g. $Fe^{2+} + H_2O_2$) and non-site-specific ($Fe^{2+} + EDTA + H_2O_2$) ·OH-scavenging activity. Due to the affinity of deoxyribose for Fe^{2+}, the presence of the free form of Fe^{2+} in the site-specific assay enables Fe^{2+} to directly attack deoxyribose before the generation of ·OH from Fenton reactants. However, the presence of EDTA in the non-site-specific assay decreases the Fe^{2+} binding to deoxyribose and thus the formation of ·OH predominates in solution.

It was observed that extracts/fractions were very potent in scavenging the hydroxyl radicals and prevented the degradation of deoxyribose. Furthermore, the extracts/fractions were also expected to chelate with Fe^{2+} in the absence of EDTA, which indicates the chelating ability of extracts. The general chelating ability of the extract/fractions is probably related to the high nucleophilic chelation of the aromatic ring of the phenolic compounds rather than to specific chelating groups of the molecules (Aust & Koppenol, 1991; Becana & Klucas, 1992; Gelvan & Saltmen, 1991; Gutteridege, Rowley, & Halliwell, 1981). It was further observed that none of the extract/fractions exhibited pro-oxidant activity, if the ascorbate was omitted from the reaction mixture (data not shown). Table 2 shows the IC_{50} values of the extract/fractions, which were found to vary from 33.7 to 52.6 μg/ml in site-specific and from 21.6 to 60.6 μg/ml in the site-specific assay for the extract/fraction in increasing and decreasing order of solvent polarity, respectively.

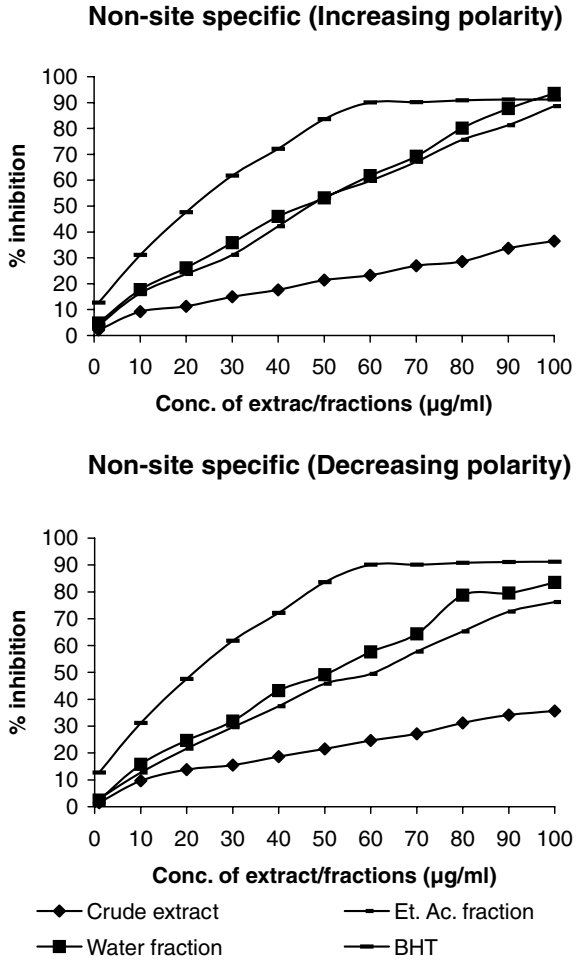


Fig. 1. Scavenging of hydroxyl radicals (non site specific) by methanol extract/fraction of *Acacia auriculiformis* in deoxyribose degradation assay.

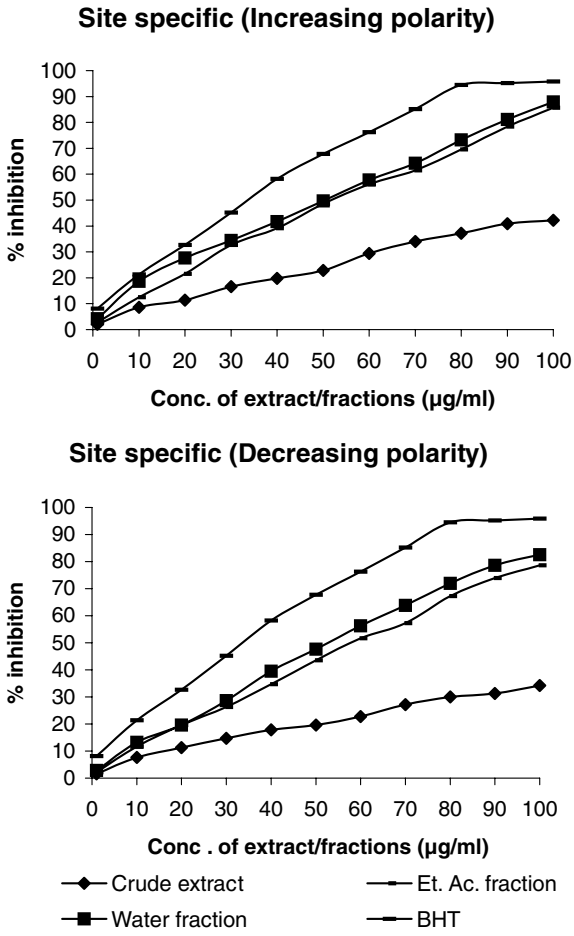


Fig. 2. Scavenging of hydroxyl radicals (site specific) by methanol extract/fraction of *Acacia auriculiformis* in deoxyribose degradation assay.

3.4. Reducing power assay

Fig. 4 shows heavy metal reducing capacities of the extracts/fractions and BHT. It was observed that the water fraction of the methanol extract obtained by increasing the polarity showed maximum heavy metal reducing capacity

Table 2
IC_{50} values of methanol extract/fractions of *Acacia auriculiformis* in different antioxidant systems

S. No.	Assay	Extract/fraction/standard	IC_{50} value (μg/ml)	
			Increasing polarity	Decreasing polarity
1.	DPPH radical-scavenging assay	L-Ascorbic acid	15.7	15.7
		Crude extract	NA	NA
		Water fraction	35.4	88.2
		Et. Ace. fraction	51.3	74.3
2.	Deoxyribose degradation assay (site-specific)	BHT	33.7	33.7
		Crude extract	NA	NA
		Water fraction	50.3	52.6
		Et. Ace. fraction	51.7	51.7
3.	Deoxyribose degradation assay (non-site-specific)	BHT	21.6	21.6
		Crude extract	NA	NA
		Water fraction	45.4	50.8
		Et. Ace. fraction	46.9	60.6

(1.76) (compared to other extracts/fractions). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Yıldırım et al., 2000). Heavy metal reducing power of the extract/fractions is employed because the antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Ramarathnam, Ochi, & Takeuchi, 1997; Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995). However, the activity of antioxidants has been assigned to various mechanisms, such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical-scavenging

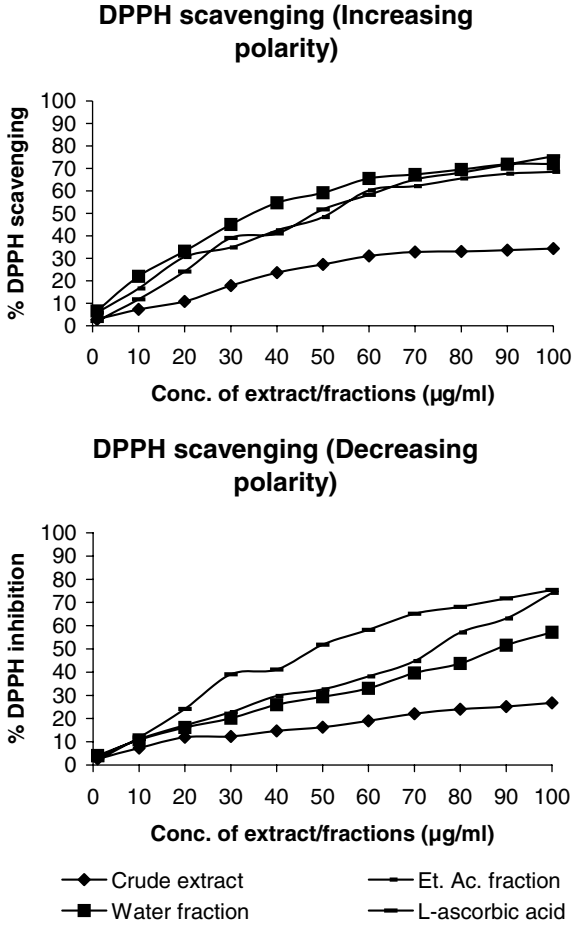


Fig. 3. Scavenging of the DPPH radical of different extract/fraction of *Acacia auriculiformis* by DPPH and reducing power assay.

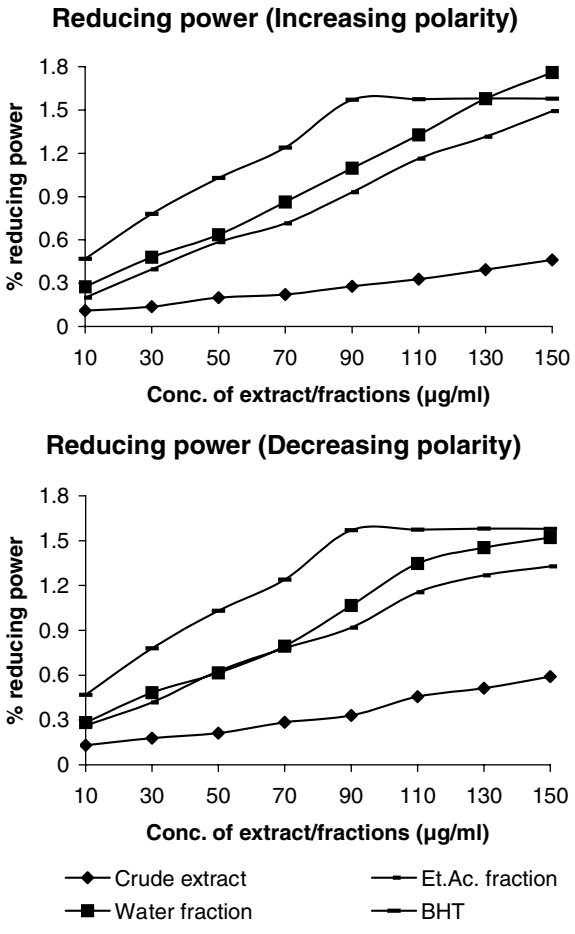


Fig. 4. Scavenging of the reducing power potential of different extract/fraction of *Acacia auriculiformis* by DPPH and reducing power assay.

(Diplock, 1997; Meir, Kanner, Akiri, & Hadas, 1995). Among the extract/fractions tested, the water fraction, which showed maximum total phenolic content, exhibited greatest reducing power, indicating that hydrophilic polyphenolic compounds may be the cause of greater reducing power.

Further studies are in progress to isolate and elucidate the chemical nature of the active antioxidant principles, employing HPLC, LC-Mass and NMR techniques.

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