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Full Length Research Paper

Antioxidant activity of acetone and ethanolic leaves extracts of *Hippobromus pauciflorus (L.f.)* Radlk.

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This study focused on determining the antioxidant activities and phenolic contents of 70% acetone and ethanolic leave extracts of *Hippobromus pauciflorus (L.f.)* Radlk. The measured antioxidant properties included free radical scavenging activities against 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azinobis-3-ethylbenzothizoline 6-sulfonic acid (ABTS), ferric reducing power activities, hydrogen peroxide, nitric oxide scavenging properties and lipid peroxidation. The results show that the two extracts had good antioxidant potential. 70% acetone extract had higher ABTS, reducing power ability, hydrogen peroxide, nitric oxide and lipid peroxidation scavenging ability, but lower DPPH activities when compared with the ethanolic extract. The antioxidant capacity of the two extracts as determined by ABTS, DPPH, reducing power, hydrogen peroxide and nitric oxide scavenging ability were lower when compared with tannic acid and BHT standard. Phytochemical analyses revealed that the level of total flavonoid, flavonol, proanthocyanidin and tannins were relatively similar. On the other hand, 70% acetone extract had higher level of total phenolics than ethanol extract. The present results indicate that the extracts of *H. pauciflorus* possesses antioxidant properties and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants and could be used in the treatment and management of free radical-mediated diseases.

Keywords: Antioxidant activity, free radicals, *Hippobromus pauciflorus,* polyphenolic compounds.

INTRODUCTION

The etiology and pathogenesis of human diseases like diabetes mellitus, atherosclerosis, hypertension, ischemic diseases, Alzheimer's disease, Parkinsonism, cancer and inflammatory conditions are primarily due to the imbalance between pro-oxidants and antioxidants homeostasis (Halliwell and Whiteman, 2004). Pro-oxidant reactive oxygen species (ROS), for example, are normal products of aerobic metabolism (Lillian et al., 2007). However, excess free radicals can result from tissue damage and hypoxia, overexposure to environmental factors (smoking, ultraviolet radiation and pollutants), a lack of antioxidants or destruction of free radical scavengers (Heath, 2005).

The use of synthetic antioxidant such as butylated hydroxytoluene (BHT), tannic acid and propyl gallate has been reported to be harmful to human health (Pourmorad et al., 2006). Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants. Positive health effect of various plants extracts in the management of diseases associated with oxidative stress is attributed to bioactive substances often exhibiting a wide range of biological and pharmacological activities such as anti-inflammatory, anti-diabetes, antimicrobial, anti-atherosclerotic properties, anti-carcinogenic activities and anti-thrombotic (Girish et al., 2008).

These bioactive substances are naturally occurring phytochemicals (alkaloids, tannins, flavonoids and phenolic compounds), vitamins and minerals which give plants their color and flavor. Phytochemicals have been reported to protect the cell constituents against destructive oxidative damage, inhibition of hydrolytic and oxidative enzymes including lipid peroxidation, thus limiting the risk of various degenerative diseases associated with oxidative stress (Vinary et al., 2010).

Hippobromus pauciflorus is of particular interest because of it numerous uses in the management of diseases. *H.*

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pauciflorus has been reported to possess active agents against malaria (Clarkson et al., 2004) and eye infections (Hirotoshi et al., 2006; Fabiana et al., 2004). The root is also regarded as love charm by the Zulus in South Africa and is also used in the management of dysentery and diarrhea (Pendota et al., 2008). The leaves, stem bark and roots of *H. pauciflorus* have been reported to possess anti-inflammatory, analgesic, antipyretic and antimicrobial activities (Pendota et al., 2009). In spite of the numerous uses of this plant in the Eastern Cape Province of South Africa, no pharmacological work has been reported on this plant. In addition, the leaf of this plant has not been evaluated for its antioxidant potential along with polyphenolic contents. The aim of the present study was to assess and compare the total polyphenolic contents and antioxidant potentials in 70% acetone and ethanolic leaf extracts of *H. pauciflorus*.

MATERIALS AND METHODS

Collection and extraction of plant materials

The fresh leaves of *H. pauciflorus* were collected in February, 2010 from Sikusthwana village, near Alice, in Eastern Cape. The plant was previously authenticated by Pendota et al. (2009) and deposited at Giffens herbarium of the University Of Fort Hare. Air dried powder (100 g each) was first defatted and then extracted with 70% acetone and ethanol by shaking for 48 h in an orbital shaker. The extract was filtered using a Buchner funnel and Whatman No.1 filter paper. The filtrate was then concentrated to dryness under reduced pressure at 40°C using a rotary evaporator (Laborota 4000-efficient, Heidolph, Germany) to yield 20.20 g ethanol extract and 14.01 g 70% acetone extract.

Chemicals and reagents

1,1-Diphenyl-2-picryl-hydrazy (DPPH), absolute ethanol, ascorbic acid (AA), Folin-ciocalteu phenol reagent, sodium carbonate, aluminum chloride, vanillin, sodium acetate, phosphate buffer, $k_3Fe(CN)_6$, trichloroacetic acid (TCA), 2- thiobarbituric acid (TBA), potassium metabisulphite (PMS), nitro blue tetrazolium [2,2'-di-p-nitrophenyl-5,5'-diphenyl-(3,3'-dimethoxy-4,4'-diphenylene)-ditetrazolium chloride] (NBT), catechin, quercetin, 2,2'azino-bis (3-ethylbenthiazoline-6-sulphonic acid) (ABTS), potassium persulphate, sodium nitroprusside, hydrogen peroxide, sulfanilic acid, glacial acetic acid, butylated hydroxyl toluene (BHT) and tannic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Butanol-HCl reagents (butanol-HCl, 95:5 v/v) and ferric reagent were also purchased from Merck Chemical Supplies (Damstadt, Germany). All chemicals used, including the solvents were of analytical grade.

Determination of total phenolics

Total phenolic contents in the plant extracts were determined with the Folin-ciocalteu reagent according to the method of Wolfe et al. (2003), using tannic acid as a standard. Samples (200 µL, three replicates) were introduced into test cuvettes, then 1.0 ml of Folin-ciocalteu's reagent and 0.8 ml of Na_2CO_3 (7.5%) were added. The absorbance of acetone and ethanolic extracts was measured at 765 nm using the Shimadzu UV-Vis spectrophotometer after incubating at 30°C for 1.5 h. Total phenolic was expressed as mg/g of tannic acid using the following equation based on calibration curve: $y = 0.1216x$, $R^2 = 0.9365$; where x is the absorbance and y is the tannic acid equivalent (mg/g)

Determination of total tannin content

The total tannin content in the two extracts was determined by modified method of Polshelttiwar et al. (2007). The sample (0.1 ml) was mixed with 0.5 ml of Folin-Denis reagent followed by 1 ml of Na_2Co_3 (0.5% w/v) solution and distilled water (up to 5 ml). The absorbance was measured at 755 nm within 30 min of the reaction against the blank. The total tannin in the extract was expressed as the equivalent to tannic acid (g TE/g extract).

Determination of total proanthocyanidins

Determination of proanthocyanidin was based on the procedure reported by Sun et al. (1998). A volume of 0.5 ml of 0.1 mg/ml extract solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid. The mixture was allowed to stand for 15 min and then the absorbance was measured at 500 nm. Samples were evaluated at a final concentration of 0.1 mg/ml. Total proanthocyanidin contents were expressed as catechin equivalents (mg/g) using the following equation based on the calibration curve: $y = 0.5825$ x, $R^2 = 0.9277$, where x is the absorbance and y is the catechin equivalent (mg/g).

Determination of total flavonoids

Total flavonoids were estimated using the method of Ordonez et al. (2006). 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 ml of the extracts. After 1 h at room temperature, the absorbance was measured at 420 nm. The extract was evaluated at a final concentration of 0.1 mg/ml. Total flavonoid content was calculated as quercetin equivalent (mg/g) obtained from calibration curve using equation: $y = 0.0255$ x, $R^2 = 0.9812$, where x is the absorbance and y is the quercetin equivalent.

Determination of total flavonols

Total flavonols were estimated using the method of Kumaran and Karunakaran (2007). To 2.0 ml of sample (standard), 2.0 ml of 2% $AlCl_3$ ethanol and 3.0 ml (50 g/L) sodium acetate solutions were added. The absorption at 440 nm was read after 2.5 h at 20°C. Extract sample were evaluated at a final concentration of 0.1 mg/ml. Total flavonoid content was calculated as quercetin (mg/g) using the following equation based on the calibration curve: $y = 0.0255$ x, $R^2 = 0.9812$, where x is the absorbance and y is the quercetin equivalent (mg/g).

Ferric reducing power ability of the extracts

A modified method of Kuda et al. (2005) was adopted for the ferric reducing antioxidant power assay. 1.0 ml of the extract was mixed with 2.5 ml of phosphate buffer (50 mM, PH 7.0) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 20 min. After, 2.5 ml of trichloroacetic acid (10%) was added to the mixture and was then centrifuged at 3000 rpm for 10 min. Finally, 1.25 ml from the supernatant was missed with 1.25 ml of distilled water and 0.25 ml $Fecl_3$ solution (0.1%, w/v). The absorbance was measured at 700 nm. The assays were carried out in triplicate.

Results are expressed in µM Fe (II)/g dry mass and compared with that of BHT and tannic acid.

DPPH free radical scavenging activity

The effect of aqueous acetone and ethanol extract of *H. pauciflorus* on DPPH radical (1,1-diphenil-2-picrylhydrazyl) was investigated using the method described by Limei et al. (2008). Equal volume of diluted extract were mixed with an equal volume of DPPH 6×10^{-3} M in absolute ethanol and the obtained mixtures at 517nm was determined, in comparison with the control solution (maximum absorption). The ability to scavenge DPPH radical was calculated according to the following equation: DPPH radical scavenging activity (%) = $[(Abs_{control} - Abs_{test}) / (A_{control})] \times 100$, where $Abs_{control}$ is the absorbance of DPPH radical + ethanol; Abs_{sample} is the absorbance of DPPH radical + sample extract/ standard.

ABTS radical scavenging assay

For ABTS assay, the procedure of Nidhi et al. (2007) was used with some modifications. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solutions were then prepared by mixing the two stock solutions in equal quantities and left for 14 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using a spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and ascorbic acid and percentage inhibition calculated as ABTS radical scavenging activity: (%) = $[(Abs_{control} - Abs_{sample}) / (Abs_{control})] \times 100$, where $Abs_{control}$ is the absorbance of ABTS radical in methanol; Abs_{sample} is the absorbance of ABTS radical solution mixed with sample extract/standard. All determinations were performed in triplicate (n = 3).

Nitric oxide scavenging activity

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess reaction (Green et al., 1982). The reaction mixture (2 ml) containing sodium nitroprusside (10 mM) in phosphate buffer saline (PBS) and the extract from 0.05 to 0.25 mg/ml was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture was removed and 0.5 ml of Griess reagent (1% (w/v) sulfanilamide, 2% (v/v) H_3PO_4 and 0.1% (w/v) naphthylethylene diamine hydrochloride) was added. The absorbance of the chromophore formed was measured at 546 nm: inhibition of NO (%) = $[(A_0 - A_1) / A_0] \times 100$, where A_0 is the absorbance before reaction and A_1 is the absorbance after reaction has taken place.

Hydrogen peroxide scavenging activity

Scavenging activity of hydrogen peroxide by the plant extract was determined by the method of Ruch et al. (1998). Plant extract (2 ml) prepared in distilled water at various concentrations was mixed with 0.3 ml of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm against blank solution containing the plant extract without H_2O_2.

Lipid peroxidation assay

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed using egg yolk homogenates as lipid-rich media, as described by Ruberto et al. (2000). Lipid peroxidation was induced by $FeSO_4$. Malondialdehyde (MDA), produced by oxidation of polyunsaturated fatty acids, reacted with two molecules of thiobarbituric acid (TBA) yielding a pinkish red chromogen with an absorbance maximum at 532 nm which was measured. Percentage inhibition of lipid peroxidation by different concentration of the extract was calculated with the formula: inhibition of lipid peroxidation (%) = $[(A_0 - A_1) / A_0] \times 100$, where A_0 is the absorbance before reaction and A_1 is the absorbance after reaction has taken place.

Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. Where applicable, the data were subjected to one-way analysis of variance (ANOVA) and differences between samples were determined by Duncan's multiple range tests using the Statistical Analysis System (SAS) program. P values < 0.05 were regarded as significant and p values < 0.01 as very significant. Regression analysis was used to established correlation between phytochemical contents and antioxidant activity.

RESULTS AND DISCUSSION

Polyphenolic contents

The theurapeutic effects derived from several medicinal plants have been attributed to the presence of phenolic compounds such as flavanoids, phenolic acid, pro-anthocyanidins, diterpenes and tannis (Pourmorad et al., 2006). These compounds exhibit antioxidant activity by inactivating lipid free radicals or by preventing the decomposition of hydroperoxides into free radicals (Jimoh et al., 2008). Phenolic compounds are known to inhibit various types of oxidizing enzymes. These potential mechanisms make the diverse group of phenolic compounds an interesting target in the search for health beneficial phytochemicals (Akinpelu et al., 2010).

Results obtained from the present study revealed a significant level of phenolic compounds in both extracts of *H. pauciflorus* (Figure 1). The acetone extract had a higher concentration of total phenolic compounds than the ethanolic extract, while the amounts of flavonoid, flavonols, proanthocyanidins and tannins in both extracts were not significantly different (Figure 1). The difference in their phenolic compounds may be due to their respective polarities (Su et al., 2007)

Antioxidant activity

Ferric reducing power ability of the extracts

Several studies have revealed that ferric reducing power of bioactive compounds is associated with antioxidant activity (Shiddhuraju et al., 2002). It is a measure of the

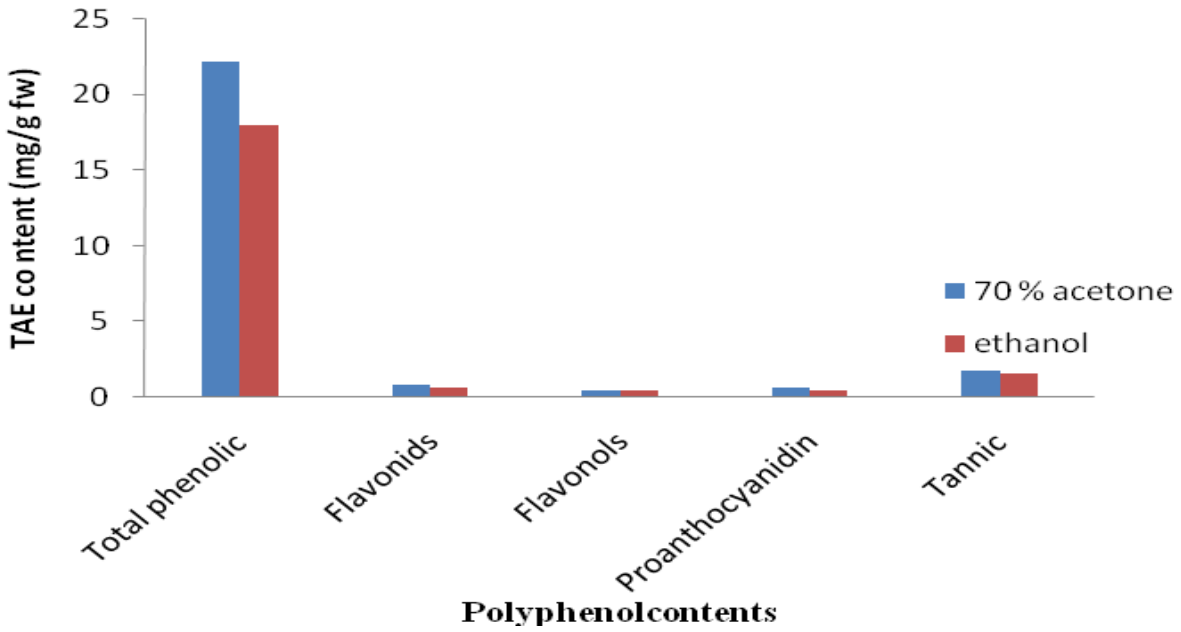

Figure 1. Total phenolic content expressed as tannic acid equivalents (TAE /mg/g. fw) of 70% acetone and ethanol extracts from the leaves of *Hippobromus pauciflorus*. Values are means ± SD from three determinations.

reductive ability of antioxidants and it is evaluated by the transformation of Fe^{3+} to Fe^{2+} in the presence of sample extracts (Huda-Faujan et al., 2009). Our results in this study showed that the ferric reducing ability of acetone and ethanolic extracts of *H. pauciflorus* increased with increase in their concentrations (Figure 4). Similar observation was reported by Huda-Faujan et al. (2009) who found that the reducing power of methanolic extracts of *Cosmos caudatus* and three others increased with concentration. Interestingly, the acetone extract of *H. paciflourus* still showed higher reducing power than the ethanolic extract eventhough both extracts exhibited higher reducing capacity than BHT at 0.1 mg/ml. It is plausible to assume that the phenolic compounds in the plant may be acting as electron donors thereby reducing free radical generation.

Lipid peroxidation inhibition (evaluated in egg-yolk homogenates)

The two extratcs of *H. pauciflorus* leaves inhibited lipid peroxidation induced by ferrous sulfate in egg-yolk homogenates in concentration-dependent manner (Table 1). Lipid peroxidation contains a series of free radical mediated chain reaction processes and is also associated with several types of biological damages (Perry et al., 2000). The percentage lipid inhibition of acetone extract of the plant was significantly higher than the ethanolic extract, but significantly lower than gallic acid at the highest concentration of 75 µg/ml. The EC_{50} values were in the range of 64.7 to 84.6 µg/ml. This activity was higher than that of ethanolic and hexane extract of *Ziziphus mauratiana* and *Z. spina-christi* reported by Abalake et al. (2011) using egg yolk as media of peroxidation. Our results suggest that *H. pauciflorus* leaf extracts could play a role in protecting the physicochemical properties of membrane bilayers of cells from severe free radical-induced cellular dysfunction.

Radical- scavanging activity

Reacting oxygen species, which include free radicals such as superoxide anion radicals ($O^{\cdot}{}_2^-$), hydroxyl radicals ($OH^{\cdot}$), non free-radical species like H_2O_2 and singlet oxygen ($^1O_2$), are various forms of activated oxygen. These molecules are exacerbating factors in cellular injury and aging process (Gülçin et al., 2003). The results of DPPH and ABTS scavenging radical activities assay showed that the two extracts in this study were fast and effective radical scavengers (Figures 2 and 3). The activity of 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) of the acetone extract was significantly higher than that of ethanolic extract and BHT at all concentrations (Figure 3). DPPH radical scavenging assay of the acetone and ethanolic leaf extracts of the plant show a dose response pattern, which was comparably lower than that of the BHT and tannic acid respectively (Figure 2).

However, it was observed that the percentage DPPH scavenging activity of the ethanolic extract was significantly higher than that of acetone extract. The discrepancy in the results of ABTS and DPPH scavenging

Table 1. Inhibition of lipid peroxidation induced by $FeSO_4$ using egg-yolk homogenates as lipid-rich media by two extracts of from leaf of *H. pauciflorus*.

Concentration (µg/ml)	Percentage inhibition (mean ± SD)		
	70% ACE	EtOH E	Gallic
15	38.5 ± 0.05^{F}	29.0 ± 0.02^{A}	28.4.0 ± 0.03^{A}
30	40.8 ± 0.02^{D}	33.6 ± 0.01^{B}	35.2 ± 0.05^{B}
45	43.5 ± 0.07^{D}	38.3 ± 0.03^{C}	45.8 ± 0.02^{D}
60	46.4 ± 0.03^{M}	40.2 ± 0.04^{R}	55.7 ± 0.02^{F}
75	51.3 ± 0.01^{R}	44.4 ± 0.01^{E}	63.5 ± 0.02^{P}
R^2	0.976	0.978	0.995
EC_{50}	64.7^{A}	84.6^{B}	49.1^{C}

70% ACE and EtOH E represents acetone and ethanol, respectively. Means with different letters within a column are significantly different ($p < 0.05$), while means with different letters within a row are significantly different ($p < 0.05$).

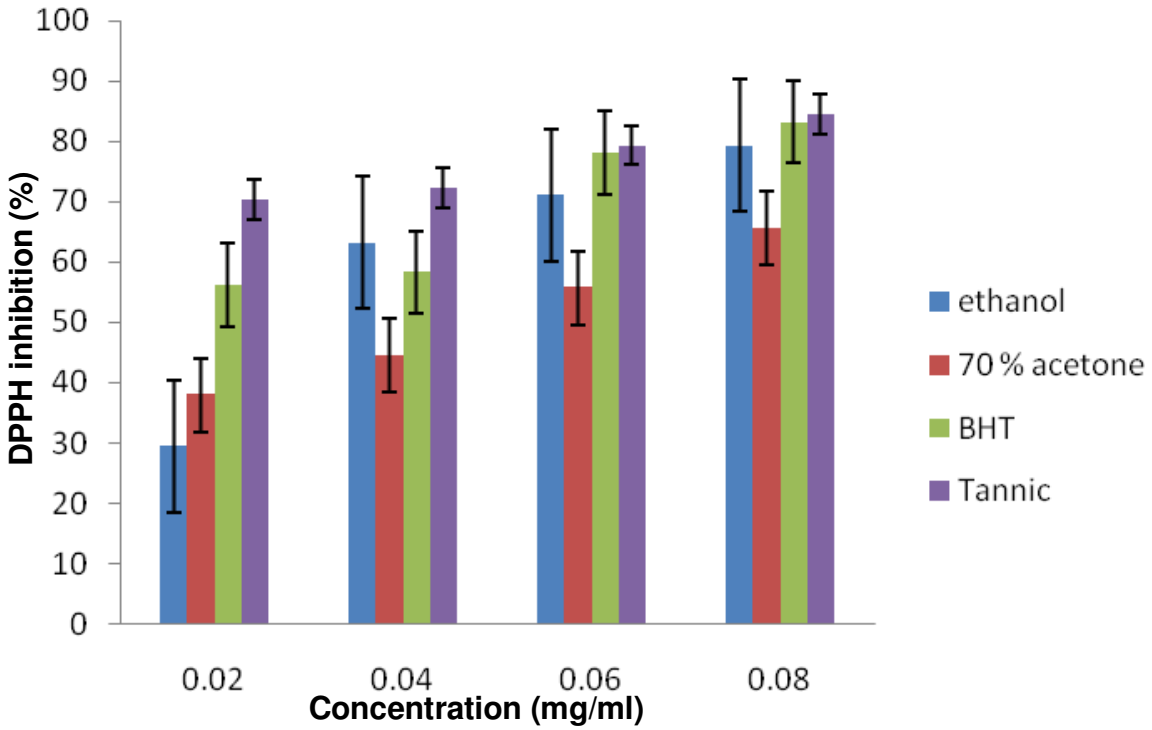

Figure 2. DPPH scavenging activity of extracts from leaves of *H. pauciflorus* using tannic acid and BHT as reference compounds.

ability of the acetone and ethanolic extracts might be due to their different mechanisms of reaction. ABTS is based on hydrogen transfer reaction, while DPPH is based on electron transfer (Prior et al., 2005). Hydrogen peroxide is an important reactive oxygen species formed *in vivo* by many oxidizing enzymes such as superoxide dismutase. It has a strong oxidizing property with the ability to penetrate biological membranes. Hydrogen peroxide itself is not very reactive, but can sometimes be toxic to cells when it gives rise to hydroxyl radicals (Gülçin et al., 2010). As shown in Table 2, the ethanolic and acetone leaf extracts of *H. pauciflorus* were capable of scavenging hydrogen peroxide in concentration-dependant manner. This property may be attributed to their phenolic contents that donate electrons to H_2O_2, thus reducing it to water.

More also, the EC_{50} values of the two extracts and BHT were similar in hydrogen peroxide scavenging abilities, but were significantly lower than the value obtained for tannic acid (Table 2). The effectiveness in antioxidant properties of the two extracts is less than tannic acid. Table 3 shows that the two extracts can effectively inhibit nitric oxide (NO) production. Nitric oxide is an essential molecule required for several physiological processes like

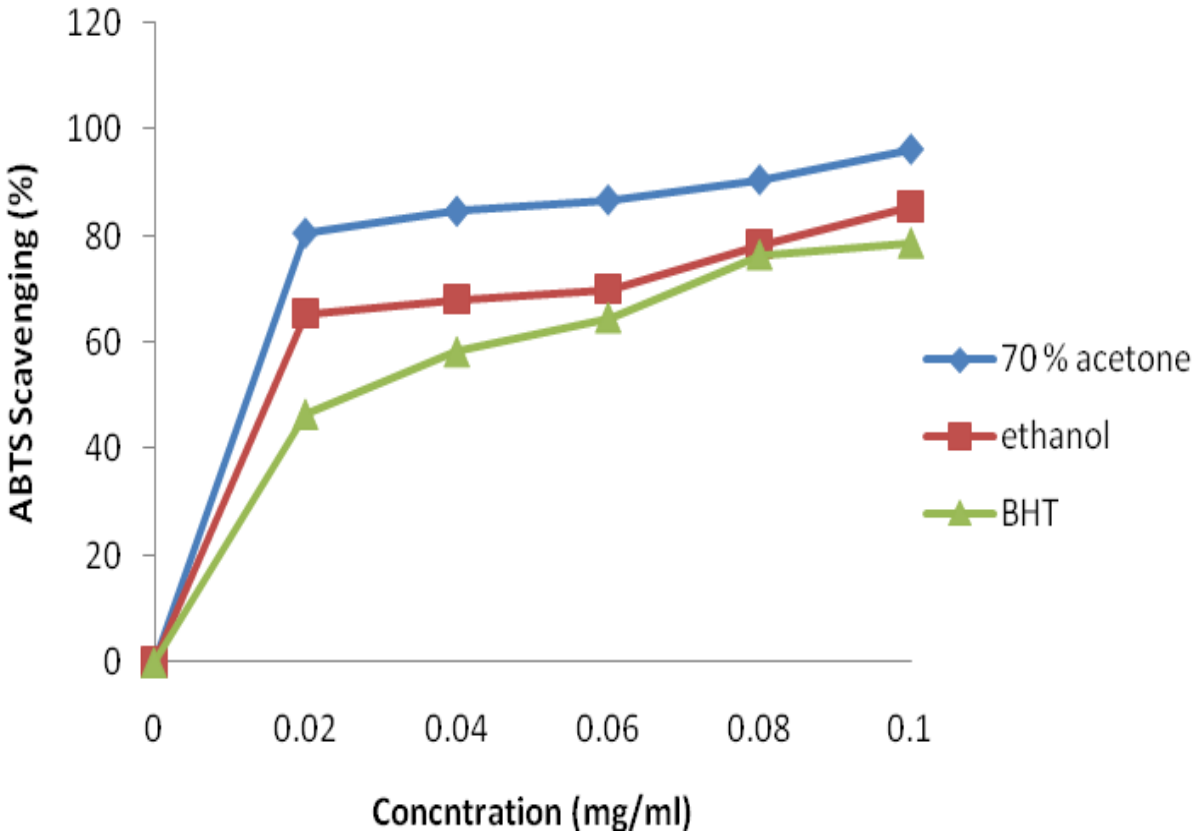

Figure 3. ABTS scavenging activity of extracts from leaves of *H. pauciflorus* using BHT as ABTS assay references.

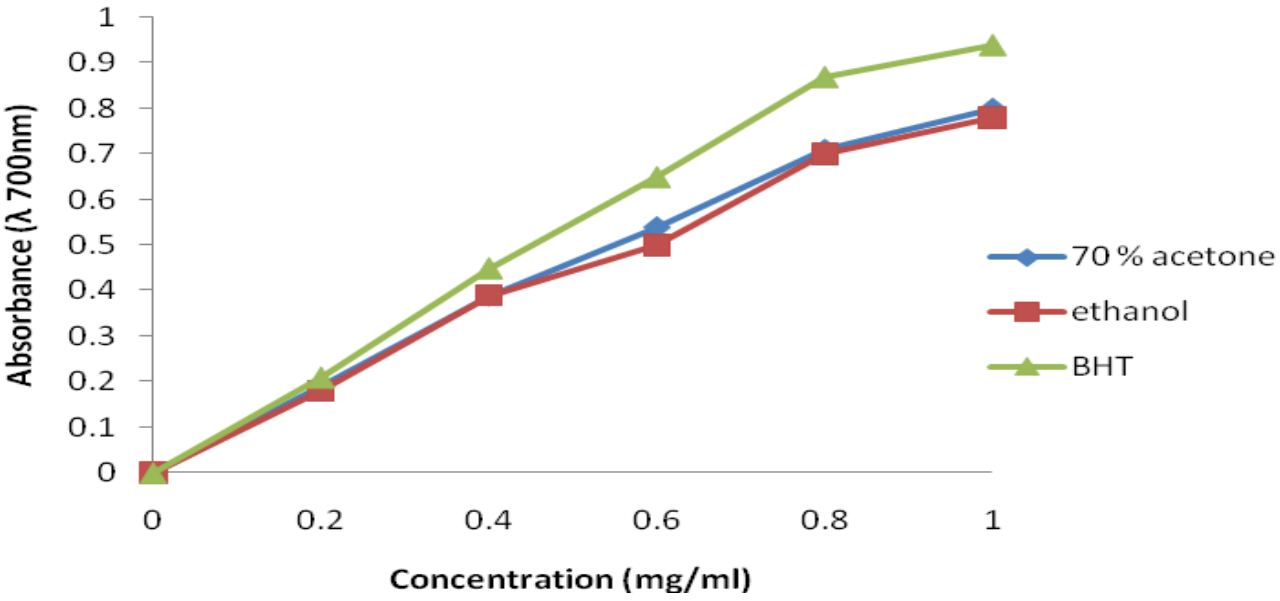

Figure 4. Ferric reducing power of extracts from leaves of *H. pauciflorus*. Values are means ± SD of three replicates.

neural signal transmission, vasodilation immune response and blood pressure (Wink et al., 1991). However, high concentration of NO may result in several pathological conditions including cancer and inflammation

Table 2. Hydrogen peroxide scavenging activity of extracts from leaf of *H. pauciflorus*

Concentration (µg/ml)	Percentage inhibition (n = 3, mean ± SD)			
	70% ACE	EtOH E	Tannin	BHT
15	49.5 ± 0.05^{D}	48.0 ± 0.02^{D}	63.0 ± 0.01^{A}	a48.8 ± 0.01^{D}
30	52.1 ± 0.03^{E}	53.0 ± 0.01^{E}	65.9 ± 0.02^{A}	51.2 ± 0.02^{E}
45	55.2 ± 0.20^{F}	55.6 ± 0.03^{F}	68.0 ± 0.02 ^{A}	52.4 ± 0.05^{F}
60	61.7 ± 0.01^{G}	60.2 ± 0.04^{G}	70.1 ± 0.01^{A}	54.9 ± 0.03^{B}
75	65.5 ± 0.04^{B}	62.1 ± 0.05^{B}	73.8 ± 0.02^{A}	56.5 ± 0.01^{C}
R^2	0.985	0.987	0.988	0.986
EC_{50}	15.1^{A}	15.6^{A}	11.9^{B}	15.3^{A}

70% ACE and EtOH E represents acetone and ethanol, respectively. Means with different capital letters within a column are significantly different ($p < 0.05$), while means with different small letters within a row are significantly different ($p < 0.05$).

Table 3. Nitric oxide radical-scavenging activity of extracts from leaf of *H. pauciflorus*.

Concentration (µg/ml)	Percentage inhibition (mean ± SD)			
	70% ACE	EtOH E	Rutin	BHT
15	35.1 ± 0.02^{F}	28.1 ± 0.01^{E}	73.0 ± 0.01^{G}	80.1 ± 0.01^{A}
30	40.5 ± 0.01^{E}	31.1 ± 0.04^{D}	78.4 ± 0.02^{F}	83.2 ± 0.02^{B}
45	43.0 ± 0.02^{D}	40.1 ± 0.03^{C}	80.0 ± 0.01^{A}	87.5 ± 0.05^{E}
60	48.1 ± 0.03^{C}	45.9 ± 0.05^{F}	83.0 ± 0.01^{B}	90.1 ± 0.03^{G}
75	52.5 ± 0.04^{B}	48.1 ± 0.05^{A}	90.1 ± 0.02^{D}	92.5 ± 0.01^{C}
R^2	0.977	0.961	0.949	0.996
EC_{50}	62.4^{A}	78.0^{B}	10.2^{C}	9.36^{D}

70% ACE and EtOH E represents acetone and ethanol, respectively. Means with different letters within a column are significantly different ($p < 0.05$), while those with different letters within a row are significantly different ($p < 0.05$).

(Patil et al., 2009). The leaf extracts in this study showed significant inhibition of NO with EC_{50} values ranging from 62.4 to 78.0 µg/ml. The observe activity might be due to the phytochemical consituents in the plant.

In conclusion, the data obtained from the present study showed that the leaf extracts of *H. pauciflorus* is a potential source of natural antioxidant which might help in preventing the progress of various oxidative stresses.

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