Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2016; 8(9); 1497-1502

ISSN: 0975-4873

Research Article

In Vitro Antioxidant Activity and High Performance Liquid Chromatography (HPLC) Analysis of Methanolic Extract of *Platycerium angolense*

Obafemi T O*, Onasanya A, Olaoye O A, Afolabi O B, Awe O J, Anadozie S O, Ojo O A, Adedeji E

Biochemistry Unit, Department of Chemical Sciences, Afe Babalola University Ado-Ekiti, Ekiti State, Nigeria.

Available Online: 10th September, 2016

ABSTRACT

This study aims to elucidate the *in vitro* antioxidant activity and composition of methanolic extract of *Platycerium angolense* (MPA). Phytochemical screening was carried out on the extract while metal chelation and the radical scavenging activity of the extract against 2, 2-diphenyl -1-picrylhydrazyl (DPPH), $ABTS^{\cdot+}$and nitric oxide (NO) radicals was also evaluated. High Performance Liquid Chromatography (HPLC) was used to identify and quantify compounds present in the extract. Phytochemical screening showed that the extract contains saponins, flavonoids and cardiac glycosides. It was also observed that the extract has an excellent ability to chelate ferrous ion as well as a high DPPH radical scavenging activity. However a moderate scavenging activity was observed for other radicals tested in this study. Furthermore, HPLC analysis revealed the presence of flavonoids, phenolic acids and tannins. It could be safely concluded that the observed antioxidant activity observed in the extract might be as a result of its constituents.

Keywords: Chromatography, extract, phytochemical, scavenging.

INTRODUCTION

Reactive oxygen species including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl (OH^-), nitric oxide (NO) exert oxidative stress in the cells of human body rendering each cell to face about 10,000 oxidative hits per second [1, 2]. These reactive oxygen species production and oxidative stress have been shown to be linked to chronic diseases such as cancer, cardiovascular diseases and neurodegenerative diseases[3].Exogenous supply of antioxidants may protect against the damaging effect of free radicals and thereby prevent the incidence of many diseases[4]. Therefore interest in the search for new natural antioxidants has grown in recent times. These natural sources of antioxidants are believed to be much safer due to less toxicity and side effects[5]. Phytochemicals, especially phenolics are suggested to be the major bioactive compounds having health benefits. Their bioactivity may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals or prevent the adverse effects of reactive oxygen and nitrogen species (ROS/RNS) on normal physiological function in humans[6,7]. *Platycerium* are epiphytes, growing naturally on branches and trunks of trees in the tropical, subtropical jungles and rain forest of Southeast Asia, Phillippines, Indonesia, Australia, New Guinea, Africa and South America. They grow in overlapping layers with serrated or scalloped upper edges and stand erect forming an opened receptacle for water, dead leaves and organic debris that eventually decay and provide nutrients for the ferns[8,9]. Oyedapo *et al* discovered that the ethanolic extract of MPA contains saponins, anthraquinones, flavonoids and some other phytochemicals[10]. This study however sets out to investigate the *in-vitro* antioxidant activity as well as use HPLC to identify and quantify the compounds present in the extract.

MATERIALS AND METHODS

Chemicals

Acetonitrile, formic acid, gallic acid, chlorogenic acid, ellagic acid and caffeic acid were purchased from Merck (Darmstadt, Germany). Quercetin, quercitrin, isoquercitrin, rutin, catechin, epicatechin and kaempferol, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical, gallic acid, ascorbic acid, quercetin and Folin-Ciocalteau reagent were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

Plant material and extraction

Platycerium angolense were collected from the trunks of trees around Department of Chemistry, University of Ibadan, Oyo-state in South-Western Nigeria. The leaves were identified at Botany Department, University of Ibadan by Mr Donatus. The plant was air-dried for three weeks and pulverized. 700 g of the pulverized sample was extracted in 80% methanol by maceration for 72 hours. The methanolic extract was concentrated in a rotary evaporator, lyophilized and preserved for further use.

Quantification of compounds by HPLC-DAD

**Author for Correspondence*

Table 1: Phytochemical screening of methanolic extract of *Platycerium angolense.*

Phytochemical	Presence
Saponins	+ve
Tannins	-ve
Flavonoids	+ve
Alkaloids	-ve
Terpenoids	+ve
Anthraquinones	-ve
Cardiac glycosides	+ve
Steroids	-ve

Table 2: Composition of methanolic extract of*Platycerium angolense.*

Compounds	*Platycerium angolense* mg/g
Gallic acid	1.47 ± 0.01^{a}
Catechin	0.39 ± 0.03^{b}
Chlorogenic acid	4.61 ± 0.01^{c}
Caffeic acid	2.08 ± 0.02^{d}
Ellagic acid	0.47 ± 0.02^{b}
Rutin	0.43 ± 0.01^{b}
Quercitrin	2.16 ± 0.03^{d}
Quercetin	3.48 ± 0.04^{e}
Kaempferol	0.25 ± 0.01^{f}
Luteolin	3.51 ± 0.01^{e}

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.01$.

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm x 250 mm) packed with 5µm diameter particles. The mobile phase was water containing 1% formic acid (A) and acetonitrile (B), and the composition gradient was: 13% of B until 10 min and changed to obtain 20, 30, 50, 60, 70, 20 and 10% B at 20, 30, 40, 50, 60, 70 and 80 min, respectively[11] with slight modifications. *Platycerium angolense* methanolic extractwas analyzed dissolved in ethanol at a concentration of 20 mg/mL. The presence of eleven antioxidants compounds was investigated, namely, gallic acid, chlorogenic acid, caffeic acid, ellagic acid, catechin, epicatechin, quercetin, quercitrin, isoquercitrin, rutin and kaempferol. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.7 ml/min, injection volume 50 µl and the wavelength were 254 nm for gallic acid, 280 nm for catechin and epicatechin, 327 nm for caffeic, ellagic and chlorogenic acids, and 366 nm for quercetin, isoquercitrin, quercitrin, rutin and kaempferol. All the samples and mobile phase were filtered through 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.030 – 0.250 mg/ml for kaempferol, quercetin, isoquercitrin, quercitrin, catechin, epicatechin and rutin; and 0.030 – 0.250 mg/ml for gallic, caffeic, ellagic and chlorogenic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 400 nm). All chromatography operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve[12].

DPPH (1, 1, diphenyl 2-picryl hydrazyl) assay

The antioxidant activity by DPPH assay was assessed according to Molyneux method[13]. To 1 ml of various concentration of the extract 1 ml of DPPH 0.1mM was added in a test tube. Tannic acid was used as the standard for comparison. After incubation for 30 mins in dark at room temperature, absorbance was recorded at 517 nm. The percent DPPH radical scavenging was calculated with the equation:

% DPPH radical scavenging = [(Absorbance control – Absorbance sample)/ Absorbance control] × 100.

ABTS radical scavenging activity

A TEAC assay was conducted based on the method of Ramos *et al.,* (1999)[14]. The ABTS aqueous solution (7 mM) was oxidized with potassium peroxodisulfate (2.45 mM) for 16 hours in the dark at room temperature. The $ABTS^{\cdot+}$ solution was diluted with 95% ethanol to an absorbance of 0.75 ± 0.05 at 734 nm. An aliquot (20 µl) of each sample (125 µg/ml) was mixed with 180 µl ABTS·+ solution and the absorbance was read at 734 nm after 1 min. Trolox was used as a reference standard. The percent $ABTS^{\cdot+}$ radical scavenging was calculated with the equation:

% $ABTS^{\cdot+}$radical scavenging = [(Absorbance control – Absorbance sample)/ Absorbance control] × 100.

Nitric oxide scavenging activity

The nitric oxide scavenging activity was evaluated according to the modified method of Sreejayan and Rao (1997)[15]. Various concentrations of extract were prepared. Sodium nitroprusside (2.5 ml, 10 mM) in phosphate buffered saline (PBS) was added to 0.5 ml different concentrations of extracts. The reaction mixture was incubated at 25℃ for 150 min. After incubation, 0.5 ml aliquot 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 was measured at 546 nm. Ascorbic acid was used as reference standard and was treated the same way as that of fractions. Sodium nitroprusside in PBS (2 ml) was used as control. The nitric oxide radicals scavenging activity of the extracts and ascorbic acid was calculated according to the following equation:

Percentage of inhibition = $[(A_0 – A_1) / A_0] \times 100$, where $A_0$is the absorbance of sodium nitroprusside in PBS and A1 is the absorbance in the presence of the fractions and ascorbic acid.

Iron chelating ability assay

The in vitro Fe^{2+} chelating ability of plant extract was assayed according to the method of Minnoti and Aust,

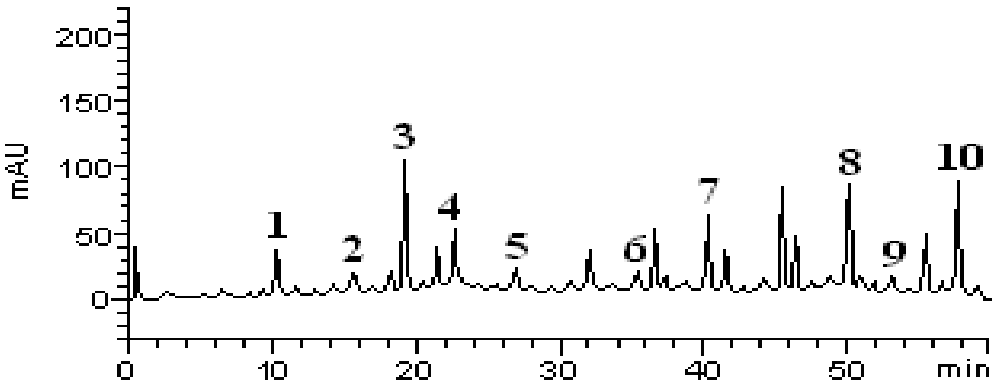

Figure 1: HPLC chromatogram of methanol leaf extract of *Platycerium angolense.*

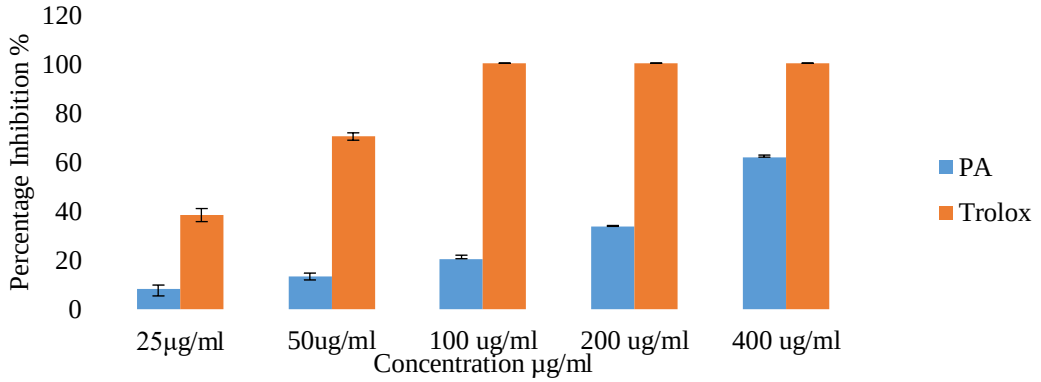

Figure 2: Percentage ABTS radical inhibition of methanolic leaf extract of MPA.

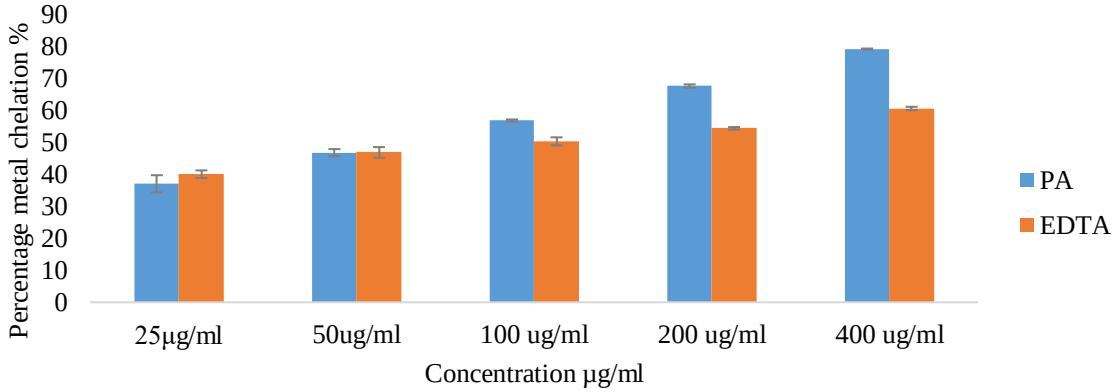

Figure 3: Percentage metal chelating ability of MPA.

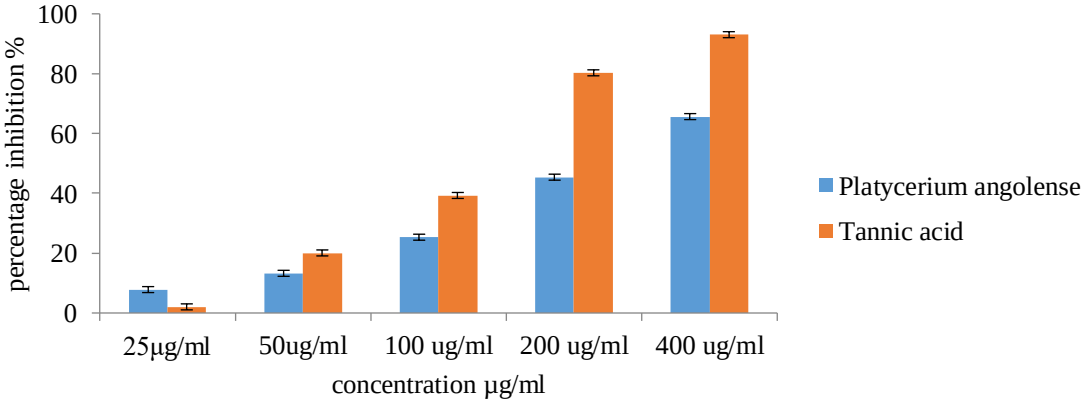

Figure 4: Percentage DPPH radical inhibitory activity of MPA.

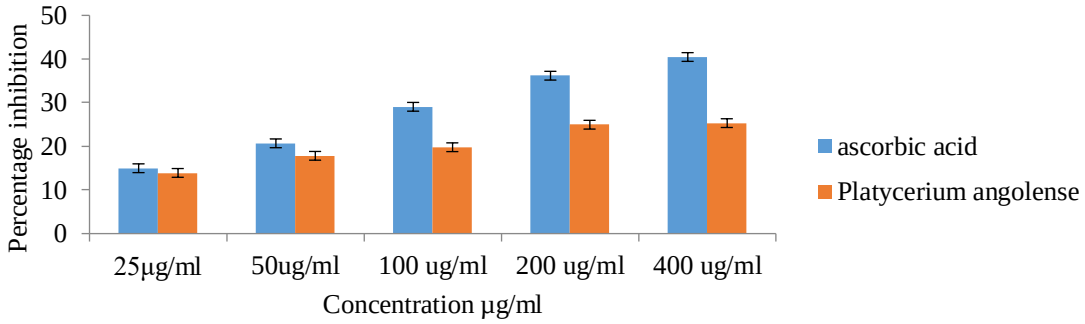

Figure 5: Percentage nitric oxide scavenging activity of MPA.

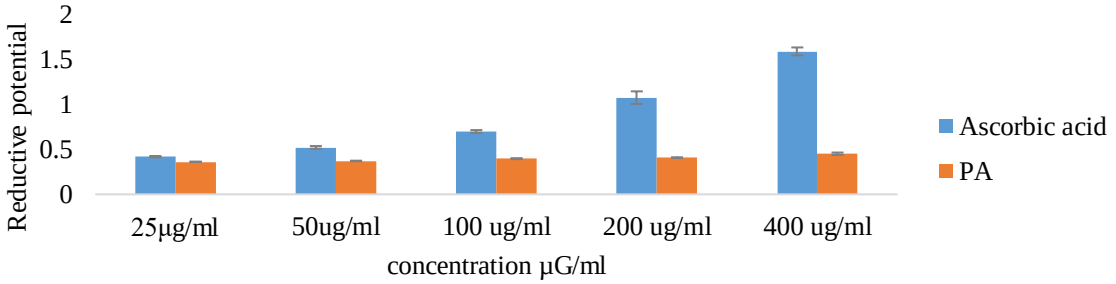

Figure 6: Reductive potential of MPA.

1987 with slight modification[16]. Briefly, 900 µl of aqueous $FeSO_4$ (500 µM) and 150µl of extract were incubated for 5min at room temperature, 78 µl of 1, 10- phenanthroline (0.25 % ,w/v , aqueous) was added. The absorbance of the orange colour solution was read at 510nm with a spectrophotometer. The principle of the assay is based on distruption of o-phenanthroline - Fe^{2+} complex in the presence of chelating agent. The in vitro of Fe2+ chelating ability of the sample was calculated by using the following formula:

Chelating ability (%) = $(A_{control} - A_{sample})/ A_{control} \times 100$. Where;

$A_{control}$ = The absorbance of the control (reaction mixture in the absence of sample) ($FeSO_4$ alone).

A_{sample} = The absorbance of the reaction mixture (sample, $FeSO_4$ and 1, 10-phenanthroline)

Reductive potential

Reducing power of the extracts were measured by the direct reduction of $Fe^{3+}(CN^-)^6$ to $Fe^{2+}(CN^-)_6$ and was determined by absorbance measurement of the formation of the Perl's Prussian Blue complex following the addition of excess Fe^{3+} (Oyaizu, 1986), as described previously[17]. Different concentrations of extracts in 0.5mL of distilled water were mixed with 1.25 ml of 0.2 M, pH 6.6 sodium phosphate buffer and 1.25 ml of potassium ferricyanide $[K_3Fe(CN)_6]$ (1%) the mixture was incubated at 50 °C for 20 min. After 20 min incubation, the reaction mixture was acidified with 1.25 ml of trichloroacetic acid (10%). Finally, 0.5 ml of $FeCl_3$ (0.1%) was added to this solution and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates greater reduction capability.

Statistical analysis

All statistical analyses were performed using Microsoft excel package 2013 version. Results are expressed as mean ± standard deviation. Independent sample T-test was used for data analysis. Linear regression analysis was used to calculate the IC_{50} values.

RESULTS

Platycerium angolense and *Synsepalum dulcificum* extracts; detection UV was at 325 nm.Gallic acid (peak 1), Catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak5), rutin (peak 6), quercitrin (peak 7), quercetin (peak 8), Kaempferol (peak 9) and luteolin (peak10).

DISCUSSION

Phytochemicals present in plants are known to be biologically active and contribute to the antioxidant capacity of the plants in which they are found[18]. In our study as shown in table 1 it was discovered that methanolic extract of *Platycerium angolense* contains flavonoids, saponins, cardiac glycosides and terpenoids. These phytochemicals could therefore contribute to the overall antioxidant potential observed in the extract. HPLC is a chromatographic technique that can separate a mixture of compounds and is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components of the mixture[19,20]. It has been suggested that HPLC fingerprinting is the best way for characterizingchemicals[21]. Our result presented in table 2 shows the quantity of various phenolic compounds identified in the extract. Among the compounds, chlorogenic acid, luteolin and quercetin are the most abundant. Many sources indicate good correlations between phenolics' content and antioxidant capacity

evaluated by different techniques[22,23]. Figure 1 is a chromatogram showing the peaks for the compounds identified in the extract. ABTS radical scavenging activity of plant extracts is a method commonly used for investigating *in vitro* antioxidant capacity. The ABTS assay is a decolourization assay where the extent of is a measure radical scavenging ability of the antioxidant Our result as presented in figure 2 shows that MPA has a moderate ABTS radical scavenging activity when compared with trolox. Iron is known to be the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron is also involved in the perpetuation of the chain reaction of lipid peroxidation[24]. Haber and Weiss[25] asserted that free iron can catalyze the production of reactive oxygen species such as superoxide anion radical, hydrogen peroxide and hydroxyl radical. In our result presented in figure 3, it was evident that MPA has an excellent metal chelating ability in a dose dependent manner that is even higher than that of ethylenediamine tetraacetic acid (EDTA). This is an indication that the extract could protect against oxidative damage by sequestering Fe^{2+} ions.It was suggested that no single assay accurately describes the mechanism of action of all antioxidants or radical sources in a complex system[26]. More than one method should therefore be employed in elucidating the total antioxidant activity of extracts[27]. According to Philips *et al*, the ability of plant extracts and other natural compounds to scavenge DPPH radical is a widely used method for testing their *in vitro* antioxidant activity[28]. In the present study, the ability of MPA to scavenge DPPH was compared with tannic acid. Our result shown in figure 4 showed that MPA compared favourably with the standard antioxidant in a dose dependent manner. Nitric oxide (NO) reacts with superoxide anion to form peroxynitrite which is a potentially cytotoxic molecule capable of oxidatively damaging proteins[29]. In our study as reported in figure 5, we observed a moderate NO scavenging activity for MPA. This is an indication that the extract might be able to prevent oxidative damage to proteins, which is implicated in several chronic diseases such as Alzheimer's disease, respiratory distress syndrome, muscular dystrophy, cataractogenesis, rheumatoid arthritis, progeria, and Werner's syndrome[30]. The reducing capacity of medicinal plants is associated with the presence of reductones which exerts their antioxidant action by breaking free radical chains through the donation of hydrogen atoms[31]. Figure 6 shows the reductive capacity of MPA. It was observed that the extract showed a significantly lower reductive potential when compared with ascorbic acid, the standard antioxidant. Amoo *et al* opined that the low reductive potential of extracts may be ascribed to low level of antioxidant compounds with electron donating ability, in an impure form[32]. This fact could account for the low reductive potential observed in the extract.

CONCLUSION

The result of this study suggested that the observed *in vitro* antioxidant activity of methanolic extract of *Platycerium angolense* might be as a result of its phytochemical composition as well as the presence of some phenolic compounds found in it. However, further studies are required to characterize the active principles in the extract. This will facilitate in vivo studies that could delineate the mechanism of action of such principles in models of diseases in which oxidants are the major culprit.

REFERENCES

1. Halliwell B. Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. British journal of experimental pathology 1989; 70: 737-757.
2. Lata H, Ahuja GK. Role of free radicals in health and disease. Indian Journal of physiology and Allied Sciences 2003; 57: 124.
3. Fatma A, Sokindra K, Shah AK. Estimation of total phenolic content, in-vitro antioxidant and anti-inflammatory activity of flowers of *Moringa oleifera*. Asian Pacific Journal of Tropical Biomedicine 2013; 3: 623-627.
4. Ghazanfar SA, Al Sabahi AMA. Medicinal plants of Northern and central Oman. Economic Botany 1993; 47: 89-98.
5. Moyo B, Masika PJ, Hugo A, Muchenje V. Nutritional characterization of Moringa (*Moringa Oleifera* Lam) leaves. African Journal of Biotechnology 2011; 10(60): 12925-12933.
6. Halliwell B, Gutteridge JM. The definition and measurement of antioxidants in biomounts may either delay or inhibit the initiation. Free Radical Biology and Medicine 1995; 18: 125-126.
7. Huang R, Mendis E, Kim SK. Factors affecting the free radical scavenging behavior of chitosan sulfate. International Journal of Biological Macromolecules 2005; 36: 120-127.
8. Huntington A. *Platycerium elephantosis*- Elephant Staghhorn Fern. Albert's Greenhouse. Madagascar Tropical: A *Platycerium* Fern Nursery, Altadena, California- Los Angeles Country, USA, 2007 626: 791-3600.
9. Benzing DH. Vascular epiphytes: general biology and related biota. Cambridge University Press. Cambridge, 1990.
10. **S**ikiru OB, Oluboade OO. Biological activities and phytoconstituents of the lower plant *Platycerium angolense*, Welwex Hook. Journal of Medicinal Plants Research 2011; 5(8): 1321-1329.
11. Boligon AA, Brum TF, Frolhich JK, Froeder ALF, Athayde ML. HPLC/DAD profile and determination of total phenolics, flavonoids, tannins and alkaloids contents of *Scutia buxifolia* Reissek stem bark. Research Journal of Phytochemistry 2012 6 (3): 84-91.
12. Boligon AA, Kubiça TF, Mario DN, Brum TF, Piana M, Weiblen R, Lovato L, Alves SH, Santos RC, Alves CFS, Athayde ML. Antimicrobial and antiviral activity-guided fractionation from *Scutia buxifolia* Reissek extracts. Acta Physiologiae Plantarum 2013: 35: 2229-2239.
13. Molyneux P. The use of stable free radical diphenylpicryl-hydrazyl (DPPH) for estimating

antioxidant activity. Songklanakarin Journal of Science and Technology, 2004; 26:212-219.
14. Ramos, F, Takaishi Y, Kawazoe K, Osorio C, Duque C, Acuña R, *et al*. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 1999; 26: 1231-1237.
15. Sreejayan, Rao MN. Nitric oxide scavenging by curcuminoids. Journal of Pharmacy and Pharmacology 1997; 49: 105-107.
16. Minnoti G, Aust SD. An investigation into the mechanism of citrate –Fe^{2+}dependent lipid peroxidation. Free Radical Biology and Medicine 1987; 3: 379–387.
17. Gulcin I. Antioxidant and antiradical activities of L-carnitine. Life Sciences 2006b; 78: 803- 811.
18. Gulcin I, Topal F, Ozturk Sarikaya SB, Bursal E, Birsel G, Goren AC. Polyphenolcontents and antioxidant properties of Medlar (*Mespilus germanica* L.). Records of Natural Products Journal 2011; 5:158–75.
19. Cannell RJP. Natural Products Isolation. Human Press Inc. New Jersey. 1998; 165-208.
20. Piana M, Zadra M, de Brum TF, Boligon AA, Gonçalves AF, da Cruz RC, et al. Analysis of rutin in the extract and gel of *Viola tricolor*. Journal of chromatographic science 2013; 51: 406-411.
21. Hemadri SR, Sheikha RA, Wafaa IA. Study on Phytochemical Screening, HPLC Analysis of Phenols and In vivo Assay on Mice by Using Traditional Herbal Medicinal Plant in Oman. International Journal of Scientific and Research Publications 2015; 4: 1-7
22. Benzie FF, Strain JJ. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods in Enzymology 1999; 299:15-27.
23. Brand-Williams W, Cuvelier ME, Berset C. Use of a freeradical method to evaluate antioxidant activity. LWT - Food Science and Technology 1995; 28:25-30.
24. Halliwell B. Reactive oxygen species in living systems. Source biochemistry and role in human disease. American Journal of Medicine 1991; 91: 14-21.
25. Haber F, Weiss J. The catalytic decomposition of hydrogen peroxide by iron salts. Proceedings of Royal Society of London. Series A 1934; 147: 332-351.
26. Prior RL, Wu X, Schaich K. Standardized methods for thedetermination of antioxidant capacity and phenolics in foodsand dietary supplements. Journal of Agricultural and Food Chemistry 2005; 53: 4290-4302.
27. Ilhami GI, Haci AA, Mehmet C. Determination of in VitroAntioxidant and Radical Scavenging Activities of Propofol. Chemical and Pharmaceutical Bulletin 2005; 53: 281-285.
28. Philips A, Philips S, Arul V, Padmakeerthiga B, Renju V, Santha S *et al*. Free radical scavenging activity of leaf extracts of *Indigofera aspalathoides*- An *in vitro* analysis. Pharmaceutical Science Research 2010; 2: 322-328.
29. Szabó C, Ischiropoulos H, Radi R. Peroxynitrite: Biochemistry, pathophysiology and development of therapeutics. Drug Discovery 2007; 6: 662.
30. Schuessler H, Schilling K. Oxygen effect in the radiolysis of proteins. Part 2. Bovine serum albumin. International journal of radiation biology and related studies in physics, chemistry, and medicine. 1984,45(3): 267–281
31. Gordon MH, In: BJF. Hudson (Ed.), Food antioxidants (Elsevier Applied Science, London, 1990; 1-18.
32. Amoo SO, Ndhlala AR, Finnie JF, Van Staden J. Antifungal, acetylcholinesterase inhibition, antioxidant and phytochemical properties of three Barleria species. South African Journal of Botany 2011; 77:435–45.