PRELIMINARY PHYTOCHEMICAL INVESTIGATION AND ANTIOXIDANT ACTIVITIES OF LEAVES EXTRACTS OF *ACHYRANTHES ASPERA* L

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Abstract

Plants synthesize compounds with biological activity, namely antioxidant, as secondary products, which are mainly phenolic compounds serving in plant defense mechanisms to counteract reactive oxygen species (ROS) in order to avoid oxidative damage. *Achyranthes aspera* Linn possess various therapeutic properties. In the present study investigated that the qualitative and quantitative phytochemicals are analyzed from different solvents extracts of *Achyranthes aspera* leaf. The phenol, flavonoids and alkaloids were found to be more in the ethanol extracts and followed by distilled water. The preliminary phytochemical screening, all the secondary metabolites were present in ethanol extracts only. The present study also conducted to evaluate the antioxidant activity of ethanol extracts of *Achyranthes aspera* leaf. This included the DPPH, reducing power assay and lipid peroxidation assay. The ethanol extracts of *A. aspera* leaf exhibited strong reducing power activity was 84.7 % and 89.1% at 500 µg/ml concentration of extracts and standards respectively. While the IC50 value was noted 90.7 µg/ml concentrations in ethanol extracts. The strong scavenging activity was noted in the all the assay with higher concentration of extracts.

Key words: DPPH, *Achyranthes aspera* Linn, Alkaloids, Phenol and Phytochemicals

INTRODUCTION

Medicinal plants are best owed with large number of pharmaceutically useful compounds which can be studied for investigation of new drugs for many serious diseases like cancer, tumours, AIDS and many human degenerative diseases. Medicinal plants are the local heritage with global importance. Plants and plant-based medicaments are the basis of many of the modern pharmaceuticals we use today for our various ailments. In plants, as a result of metabolic processes, many different kinds and types of organic compounds or metabolites are produced. These metabolites are grouped into primary and secondary metabolites. The primary metabolites like chlorophyll, amino acids, nucleotides, simple carbohydrates or membrane lipids, play recognised roles in photosynthesis, respiration, solute transport, translocation, nutrient assimilation and differentiation. The secondary metabolites also differ from primary metabolites in having a restricted distribution in the plant kingdom. That is, particular secondary metabolites are often found in only one plant species or a

taxonomically related group of species, whereas the basic primary metabolites are found throughout the plant kingdom (Taiz and Zeiger, 2006).

The human body has evolved with antioxidant systems to protect it against free radicals. These systems include some antioxidants produced in the body, obtained from the diet and repair antioxidant (proteases, lipase, transferases, and DNA repair enzymes). The ones produced in the body are enzymatic defences, such as Se-glutathione peroxidase, catalase, and superoxide dismutase, which metabolize superoxide, hydrogen peroxide and lipid peroxides, thus preventing most of the formation of the toxic hydroxyl radicals. Exogenous antioxidants consist of non-enzymatic defenses, such as glutathione, histidine-peptides, the ironbinding proteins transferring and ferritin, dihydrolipoic acid etc (Erkoç *et al.*,2003; Brahmachari and Gorai, 2006). Owing to the incomplete efficiency of our endogenous defence systems and the existence of some physiopathological situations in which ROS are produce in excess and at the wrong time and place, dietary antioxidants are needed for diminishing the cumulative effects of oxidative damage over the life span. The antioxidants derived from diet are vitamins C, E and A, and carotenoids. Other antioxidants of value to health derived from plants include phenols, phenolic acids, flavonoids, tannins and lignans (Pietta,2000). Antioxidants are those substances which possess free radical chain reaction breaking properties (Charles Lekhya Priya, 2010). Oxidative stress contributes to the development of a wide range of diseases including Alzheimer's disease, Parkinson's disease, the pathologies caused by Diabetes, a Rheumatoid Arthritis (Behl and Mosmann, 2002), Atherosclerosis, Ischemic heart disease, Ageing, Immune suppression, Neurodegenerative diseases, Cancer and others (Singh, 2003). The most effective path to eliminate and diminish the action of free radicals which cause the oxidative stress is "ant oxidative defense" mechanisms (Brand-Williams *et al.*, 2012).

Several wild edible plants are traditionally consumed along with staple foods, especially in rural areas and a few urban communities, in asian countries. These plants play a vital role in fulfilling the demand for nutritional, minerals and antioxidant compounds in the diet of indigenous communities (Afolayan and Jimoh,2009) besides these factors, they are also used in treating certain medical conditions, for example, diabetes, in these local tribes (Ocvirk *et al.*, 2013). Therefore, the present study was to assess the phytochemical investigation and antioxidant potential of the leaves *Achyranthes aspera* Linn. The genus *Achyranthes* belongs to the family *Amaranthaceae*. It is perennial stiff erect herb, growing up to 1000 m height. Stems are square, leaves elliptic, ovate or broadly rhombate. The plant is widespread in the world as a weed, in Baluchistan, Ceylon, Tropical Asia, Africa, Australia and America (Hariharan and Rangaswami, 1970). It is used in the treatment of fever, especially malaria fever, dysentery, asthma, hypertension and diabetes (Girach and Khan, 1992; Tang and Eisenbrand, 1992). The plant possesses various medicinal properties; the aim of the present study was to identify the phytocompounds and antioxidant activity of leaves of *A.aspera.*

MATERIALS AND METHODS

Collection of sample

Achyranthes aspera was collected from in and around region of Thanjavur, Tamil Nadu, India. The plant parts were identified taxonomically and authenticated according to various literatures, Flora of Madras Presidency and Wealth of India including other pertinent taxonomic literature. The

collected *A. aspera* leaves were cleaned well with distilled water to remove all the extraneous matter such as epiphytes, sand particles, pebbles and shells and brought to the laboratory in plastic bags.

Preparation of extract

Hot extraction method

The soxhlet apparatus was used for successive solvent extraction of the collected leaf materials of *A. aspera*. The collected leaves were shade dried to complete dryness and then the material was ground to fine powder in a mixer grinder. The powder was weighed in approximately 35g of plant material was extracted successively using sequential solvents ranging from non-polar to polar i.e. petroleum ether, chloroform, ethyl acetate, hexane, methanol and distilled water in a soxhlet. After complete extraction, the contents of each extraction were concentrated by distillation. The concentrated extracts were evaporated to dryness and stored at 4 ºC in air tight containers for further experimental studies.

Phytochemical screening

Qualitative Phytochemical analysis

The preliminary qualitative phytochemical investigation of *A. aspera* leaf .extract in different solvents such as, ethanol, petroleum ether and hexane were performed to detect the following phytoconstituents were performed by the standard procedure as described by Harborne (1973).

Detection of Tannins

a) Ferric chloride test: A small quantity of the extract was mixed with water and heated on water bath. The mixture was filtered and 0.1% ferric chloride was added to the filtrate. A dark green color formation indicates the presence of tannins.

Detection of Saponins

a) Froth test: about 0.2g of the various extracts was shaken with 5ml of distilled water. Formation of frothing (appearance of creamy stable persistent of small bubbles) shows the presence of saponins.
b) **Haemolysis test:** 1 ml of suspension of RBCs in normal saline was taken. In this equal volume of plant extract in normal saline was added. Solution was shaken gently. Clear red solution was obtained indicating haemolysis of RBCs (compared with blank).

Detection of Flavonoids

a) Lead acetate test: Each extracts were treated with few drops of lead acetate solution. Formation of yellow color precipitate indicates the presence of flavonoids.
b) H_2SO_4 test: Extracts were treated with few drops of H_2SO_4. Formation of orange colour indicates the presence of flavonoids.
c) **FeCl3 Test** To the alcoholic solution of the extract add few drops of neutral ferric chloride solution. Appearance of green colour indicates presence of flavanoids.

Detection of alkaloids

Extracts were dissolved individually in dilute hydrochloric acid and filtered. The filtrate was used to test the presence of alkaloids.

a) Mayer's test: Filtrates were treated with Mayer's reagent. Formation of a yellow cream precipitate indicates the presence of alkaloids.
Mayer's reagent: Mercuric chloride (1.358g) is dissolved in 60ml of water and potassium iodide (5g) is dissolved in 10ml of water. The two solutions are mixed and made up to 100ml with water.
b) Wagner's test: Filtrates were treated with wagner's reagent. Formation of brown/ reddish brown precipitate indicates the presence of alkaloids.
Wagner's reagent: Iodine (1.2g) and potassium iodide (2g) is dissolved in 5ml of water and made up to 100ml with distilled water.
c) **Dragendorff's test:** To a few ml of filtrate, 1 or 2 ml of Dragendorff's reagent was added by the side of the test tube. A prominent red precipitate indicates test as positive.

Detection of Steroids
a) Liebermann- Burchard test: 2ml of acetic anhydride was added to 0.5g of the extracts, each with 2ml of H_2SO_4. The color changed from violet to blue or green in some samples indicate the presence of steroids.

Detection of Terpenoids
a) Salkowski's test: 0.2g of the each extracts were mixed with 2ml of chloroform and concentrated H_2SO_4 (3ml) were carefully added to form a layer. A reddish brown coloration of the inner face was indicates the presence of terpenoids.

Detection of Anthroquinones
a) Borntrager's test: about 0.2g of the each extracts was boiled with 10% HCl for few minutes in a water bath. It was filtered and allowed to cool. Equal volume of $CHCl_3$ was added to the filtrate. Few drops of 10% NH_3 were added to the mixture and heated. Formation of pink color indicates the presence anthraquinones.

Detection of Phenols
a) Ferric chloride test: Extracts were treated with few drops of 5% ferric chloride solution. Formation of bluish black color indicates the presence of phenol.
b) Lead acetate test: The various extracts were treated with few drops of lead acetate solution. Formation of yellow color precipitate indicates the presence of phenol.

Test for Amino Acids
Ninhydrin test to the test solution added 1 ml of 0.2 % ninhydrin solution, violet color indicate the presence of amino acids in sample.
Millon's test: Added 5 drops of millon's reagent to 1 ml of test solution and heated on a water bath for 10 min, cooled and added 1% sodium nitrite solution. Appearance of red color confirmed the test.

Detection of Carbohydrates
a) Fehling's test: 0.2gm filtrate is boiled on water bath with 0.2ml each of Fehling solutions A and B. A red precipitate indicates the presence of sugar.
Fehling's solution A: Copper sulphate (34.66g) is dissolved in distilled water and made up to 500ml using distilled water.

Fehling's solution B: Pottassium sodium tartarate (173g) and sodium hydroxide (50g) is dissolved in water and made up to 500ml.

b) Benedict's test: : To a set of filtrates of various drugs' extracts, added equal volumes of Benedict's reagent and heated in boiling water bath for 5min. The appearance of green, yellow or red color indicated the presence of sugars.

Gum & Mucilage

Alcohol Precipitation All the test solutions were mixed with absolute alcohol and dried in air and the residues were tested for swelling properties and didn't get positive results.

Detection of Oils and Resins

a) **Spot test**: Test extracts were applied on filter paper. It develops a transparent appearance on the filter paper. It indicates the presence of oils and resins.

Quantitative Analysis of *A. aspera* leaf extract

Determination of Total phenols

Total phenolic content of the aqueous, 50% ethanolic and methanol extract of *A. aspera* stem was determined using the Folin-Ciocalteu reagent. The crude aqueous and methanol extracts were dilutedin methanol to obtain different concentrations. 50 µl of eachextract was mixed with 2.5 ml of Folin- Ciocalteu's reagent (1/10 dilution in purified water) and 2 ml of 7.5% Na2CO3 (w/v in purified water). The mixture was incubated at 45˚C for 15min. The absorbance was measured at 765 nm. Na2CO3 solution (2 ml of 7.5% Na2CO3 in2.55 ml of distilled water) was used as blank. The results were expressed as Gallic acid equivalence.

Estimation of total flavonoids

Total Flavonoid content was determined by AlCl3 colorimetric assay. Concentration of standard quercetin was prepared by serial dilution of stock solution. An aliquot of 1 ml quercetin of each concentration in MeOH was added to 10 ml v.f. containing 4 ml of double distilled water. At the zero time, 0.3 ml, 5% sodium nitrite was added to the flask. After 5 min, 0.3 ml of 10% AlCl3 was added to the flask. At 6 min, 2 ml of 1 M NaOH was added to the mixture. Immediately, the total volume of the mixture was made up to 10 ml by the addition of 2.4 ml double distilled water and mixed thoroughly. Absorbance of the pink colored mixture was determined at 510 nm versus a blank containing all reagents except quercetin. Absorbance values obtained at different concentrations of quercetin were used to plot the calibration curve

Estimation of Alkaloids

The plant extract (1 mg) was dissolved in dimethyl sulphoxide (DMSO), 1 ml of 2 N HCl was added and filtered. This solution was transferred to a separating funnel, 5 ml of bromocresol green solution and 5 ml of phosphate buffer were added. The mixture was shaken with 1, 2, 3 and 4 ml chloroform by vigorous shaking and collected in a 10-ml volumetric flask and diluted to the volume with chloroform. A set of reference standard solutions of atropine were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 470 nm with an UV/Vis spectrophotometer. The total alkaloid content was expressed as mg of AE/g of extract.

Anti-oxidant Activities

DPPH Radical Scavenging Activity

DPPH radical scavenging activity was carried out by the method of Ozturk *et al.,* (2011). To 1.0 ml of 100.0 µM DPPH solution in methanol, equal volume of the different concentration of ethanol extract of *A. aspera* leaf was added and incubated in dark for 30 minutes. The change in coloration was observed in terms of absorbance using a spectrophotometer at 514 nm. 1.0 ml of methanol instead of test sample was added to the control tube. The different concentration of ascorbic acid was used as reference compound. Percentage of inhibition was calculated from the equation [(Absorbance of control - Absorbance of test)/ Absorbance of control)] × 100. IC_{50} value was calculated using Graph pad prism 5.0.

Lipid peroxidation inhibitory activity

The degree of lipid peroxidation was evaluated by estimating the thiobarbituric acid reactive substances using the standard method. Briefly, different concentrations of the extract (100 – 500 microgram / ml in saline) were added to the liver homogenate (0.5 ml). Lipid peroxidation was initiated by adding 100 microliter of 15mM of $FeSO_4$ solution and then incubates at 37^0C for 30 minutes. After 30 minutes, 1.0 ml of 10% TCA was added and centrifuged. After 10 minutes to the supernatant, 1.0 ml of thiobarbituric acid was added. The tubes were then boiled for 20 minutes and the pink colour developed was read at 535 nm. Butylated hydroxyanisole were at different concentrations (1 – 5 nanomoles) was used as the standard. Control tube contain all chemicals except plant extract.

Reducing power assay

The sample together with Ascorbic acid solutions were spiked with 2.5ml of phosphate buffer (0.2 M, pH 6.6) and 2.5ml of 1% potassium ferricyanide. The mixture was kept in a 50°C water-bath for 20min. The resulting solution was cooled rapidly, spiked with 2.5ml of 10% trichloroacetic acid, and centrifuged at 3000rpm for 10 min. The supernatant (5ml) was mixed with 5ml of distilled water and 1ml of 0.1% ferric chloride and incubated for 10min. The absorbance was detected at 700nm on spectrophotometer. The extract concentration providing the absorbance was calculated from the graph of absorbance at 700 nm against extract concentration. Ascorbic acid was used as standard. Higher absorbance indicates higher reducing power.

RESULTS AND DISCUSSION

Qualitative phytochemical analysis

In the present study, the phytochemical screening was performed the sequential extraction of some solvents such as hexane, petroleum ether, ethyl acetate, chloroform, ethanol and distilled water extracts of ***A. aspera*** . phytochemical analysis of 12 different chemical compounds (alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, phenols, anthroquanine, oil resin, amino acids, gums and mucilage and carbohydrates) were tested in six different extracts. The results of the preliminary phytochemical investigation are summarized in Table 1. Among the extracts, ethanol extracts showed maximum compounds and followed by distilled water. The results reveal the presence of medicinally active phytoconstituents studied in the different kind of extracts.

Quantitative analysis of phytocompounds

The results of the phenol, flavonoid and alkaloids were presented in table. 2. The phenolic content of the *A. aspera* leaf was found to be maximum in ethanol (275.21 mg GAE/g) extracts and followed by chloroform (263.41 mg GAE/g) and distilled water (249.34 mg GAE/g). The moderate amount was found to be ethyl acetate (225.58 mg GAE/g), petroleum ether (216. 24 mg GAE/g) and hexane (203.45 mg GAE/g). The total flavonoid content was found to be high in ethanol (140.4 mg QE /g) extracts and followed by chloroform (117.3 mg QE /g) extracts of *A. aspera* leaf. The least flavonoid content was present in petroleum ether (58.5 mg QE /g extracts and moderate amount found to be ethyl acetate (98.5 mg QE /g) and distilled water (90.5 mg QE /g) extracts. The Alkaloid content was found to be more in ethanol (48.6 mg AE/ g) extracts of *A. aspera* leaf and followed by distilled water (36.8 mg AE/ g) and petroleum ether (34.2 mg AE/ g). The less alkaloid content was presented in chloroform (14.5 mg AE/ g) extracts of *A. aspera* leaf. The secondary metabolites are compounds, which are responsible for therapeutic efficacy of the drugs. Therefore, the current preliminary phytochemicals screening might be proved valuable in the detection and further quantitative analysis of these therapeutically important compounds. The phenolic and flavonoid compounds are important antioxidants, which also include antimicrobial, antiallergic, antiinflammatory and anticancer agents. These secondary metabolites play a vital role in reproduction and growth. These compounds also provide protection against harmful pathogenic microbes and predators (Rice-Evans *et al.,* 1996). According to the literatures, flavonoids, phenolic and saponins are known to exhibit antifertility activity (Jain *et al*., 2005). Therefore, quantitative analysis of such vital compounds is extremely significant to determine the quality of drugs. In previous study Soni *et al.,* (2018) revealed that the *B. arundinacea* leaves, bark of *B. racemosa* and *F. racemosa* extracts contain significant amount of phenols, flavonoids, tannins and saponins. The outcome of these findings might be useful as a diagnostic tool for the evaluation of these antifertility herbs.

Antioxidant activity

DPPH Scavenging activity

Total free radical scavenging method is based on the reduction of DPPH, a stable free radical and any molecule that can donate an electron or hydrogen to DPPH can react with it and thereby bleach the DPPH absorption. Because of its odd electron, DPPH gives a strong absorption maximum at 514nm by visible spectroscopy. As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, that is, a free radical scavenging antioxidant, the absorption strength is decreased and the resulting decolorization is stoichiometric with respect to the number of electrons captured (Blios, 1958). The ethanol extracts of *A. aspera* leaf exhibited strong scavenging activity was 90.4 % at 500 µg/ml concentration of extracts. While the standard ascorbic acid was exhibited the radical scavenging activity was 93.5% at 500 µg/ml concentrations (Fig.1). The DPPH radical scavenging activity was found to be increasing as dose increases. The results of the DPPH radical scavenging activities of the extracts revealed that the plant extracts contain free radical scavenging activity which could exert a beneficial action against pathological alterations caused by generation of free radicals.

Lipid peroxidise assay

lipid peroxidation was measured by FTC and TBA methods. The FTC method measures the amount of peroxide produced during the initial stages of lipid oxidation. Subsequently, at a later stage of lipid oxidation, peroxide decomposes to form carbonyl compounds that are measured by the TBA method. The present study, higher activity of LPO was found to be 78.5% in ethanol leaf extracts of *A. aspera* and 80.4% in standard at 500 µg/ml concentrations (Fig. 2). This might suggest that the amount of peroxide in the initial stage of lipid per oxidation is less than the amount of peroxide in the secondary stage. Furthermore, the secondary product is much more stable for a period of time.

Reducing power assay

Reducing power was measured by direct electron donation in the reduction of Fe3+(CN-)6-Fe2+(CN-)6. The product was visualized by forming the intense Prussian blue color complex and then measured at 700nm. The ethanol extracts of *A. aspera* leaf exhibited strong reducing power activity was 84.7 % and 89.1% at 500 µg/ml concentration of extracts and standards respectively. While the IC50 value was noted 90.7 µg/ml concentrations in ethanol extracts (Fig.3). The extract showed concentration-dependent reducing power. Antioxidant compounds are able to donate electrons to reactive radicals, reducing them into more stable and un reactive species. Polyphenols of plant kingdom are one of the most effective antioxidative constituents. It is important to estimate phenolic contents of plant extracts so as to justify their controls with to antioxidant activity (Tilak *et al* . 2004). In earlier study, Rajakumar and Saranya Raju, (2017) reported the total antioxidant activity, Reducing power assay, DPPH radical scavenging activity and Lipoxygenase activity that increased with increasing amount of the extract concentrations. With these antioxidant properties, can be effectively used in the development of new pharmaceutical medicine for oxidative stress mediated problems.

CONCLUSION

Various solvents are used for the analysis of preliminary phytochemical screening of *A. aspera* leaves. Of these the ethanol extracts was exhibited the most phytocompounds when compared with other. The present results of phytochemical, polyphenol and antioxidant were reveals that the plant extract was vital and protective mechanism in vivo. The maximum polyphenol and phytochemical is present in ethanol extracts of *A. aspera* . Furthermore, some pharmacological activities have to be performed to establish the importance of certain phytoconstituents having protective action.

Table. 1 Qualitative Phytochemical analysis of various extracts of *A. aspera leaf*

S.No	Phytoconstituents	Solvents					
		CF	EA	HX	PE	ET	DW
1	Tannins						
	a) Ferric chloride test	-	+	-	+	+	+
2	Saponins						
	a) Froth test	+	+	+	+	+	+
	b) Haemolysis test	+	++		+	+	+++
3	Flavonoids						
	a) Lead acetate test	+	+	-	+	+	+
	b) H_2SO_4 test	+	-	-	+	++	++++

	c) FeCl3 Test	+	-	-	-	+	+
4	Alkaloids						
	a) Mayer's test	-	+	+	++	+	+
	b) Wagner's test	+	+	+	+	-	+
	c) Dragendorff's test	+	+	+	+	+	+++
5	Steroids						
	a) Liebermann-Burchard test	-	+	+	-	+	+
6	Terpenoids						
	a) Salkowski's test	+	-	+	++	++	+
7	Anthroquinones	-	-	+	-	+	+
	a) Borntrager's test						
8	Phenols						
	a) Ferric chloride test	+	+	+	++	++	+++
	b) Lead acetate test	+	+	+	+	+	+
9	Amino Acids						
	a) Ninhydrin test	+	++	+	+	++	+++
	b) Millon's test	+	+	-	+	+	+
10	Carbohydrates						
	a) Fehling's test	-	+	+	-	+	++
	b) Benedict's test	+	+	+	++	+	+
11	Gum & Mucilage						
	a) Alcohol Precipitation	-	-	-	-	-	-
12	Oils and Resins						
	a) Spot test	-	-	-	+	-	-

CF-Chloroform, EA-Ethyl acetate, HX-Hexane, PE-Petroleum ether, ET-Ethanol, DW-Distilled water

Table. 2 **Quantitative phytochemical analysis for the extracts of *A. aspera leaf***

S.No	**Phytoconstituents**	**Solvents**					
		CF	**EA**	**HX**	**PE**	**ET**	**DW**
1	Flavonoids (mg QE /g)	117.3	98.5	64.2	58.5	140.4	90.5
2	Alkaloids (mg AE/ g)	14.5	23.5	27.6	34.2	48.6	36.8
3	Phenols (mg GAE/g)	263.41	225.58	203.45	216. 24	275.21	249.34

CF-Chloroform, EA-Ethyl acetate, HX-Hexane, PE-Petroleum ether, ET-Ethanol, DW-Distilled water

Fig.1 DPPH radical scavenging activity for ethanol extracts of *A. aspera* leaf and standard

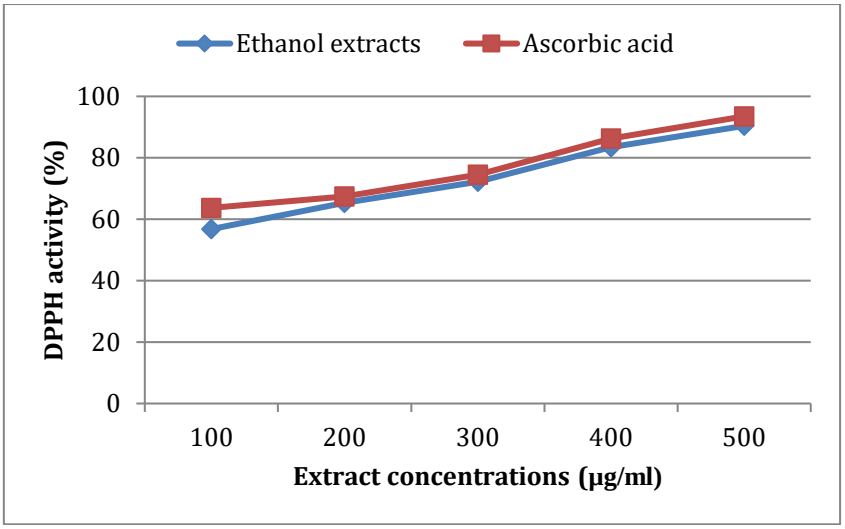


Fig. 2 Lipid peroxidise assay for ethanol extracts of *A. aspera* leaf and standard

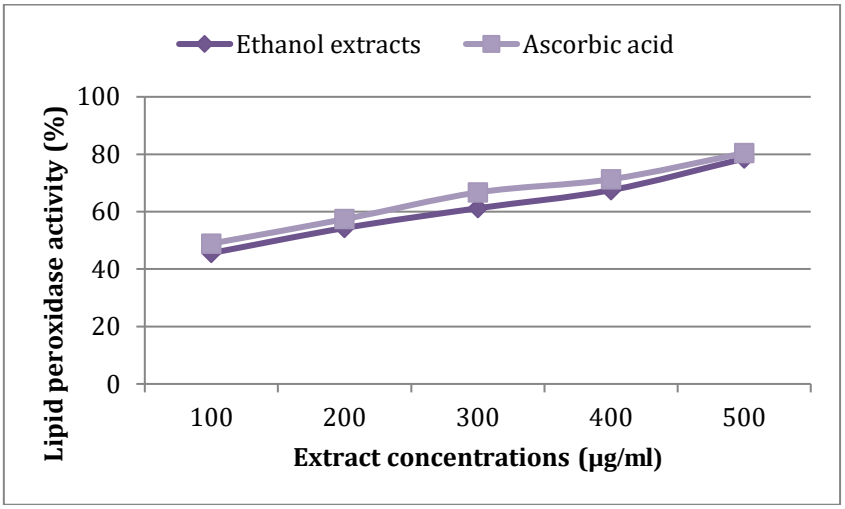
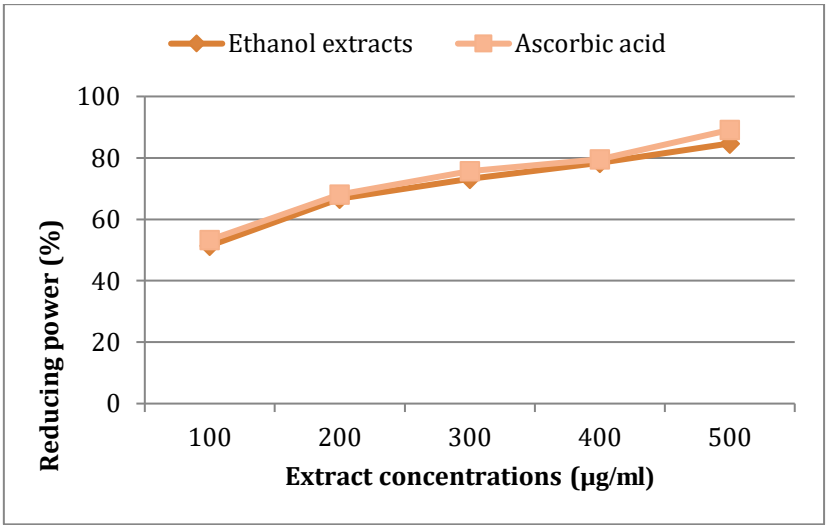


Fig. 3 Reducing power assay for ethanol extracts of *A. aspera* leaf and standard



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