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Free Radical Scavenging Potential of *Acrostichumaureum* L., Leaves

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ABSTRACT

Acrostichumaureum L. is a medicinal fern collected from Puthalam, Kanyakumari District, Tamil Nadu, India. This plants leaf is used by the local people for curing pharyngitis, chest pain and diabetics. Hence, in the present study, we evaluates the *in vitro* radical scavenging and antioxidant capacity of different concentrations of (20µg/ml, 100µg/ml, 200µg/ml, 400µg/ml, 800µg/ml) petroleum ether, benzene, ethyl acetate, ethanol and methanol extracts of the *A. aureum* leaves by using different *in vitro* analytical methodologies such as DPPH free radical scavenging, hydroxyl radical scavenging, superoxide anion radical scavenging, ABTS radical scavenging and total reducing ability methods. Ascorbic acid and trolox were used as the reference antioxidant radical scavenger compounds. In the present investigation, the antioxidant activity is increases with the increase in the concentration of the extracts. These analysis suggests that *A. aureum*(fern) leaves contains potentially health-protective phytochemical compounds with a potent source of natural antioxidant that can be used against free radical associated oxidative damage.

KEYWORDS: *Acrostichum aureum,* antioxidant activity, pteridophyte

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I.INTRODUCTION

A free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. Many radicals are unstable and highly reactive. They can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants[1]. Free radicals and other reactive oxygen species (ROS) are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals[2]. Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non-enzymatic reactions.Free radicals are known to cause various degenerative disorders, like mutagenesis, carcinogenesis, cardiovascular disturbances and ageing[3].

Antioxidants are the compounds, which combat the free radicals by intervening at any one of the three major steps of the free radical mediated oxidative process, viz., initiation, propagation and termination[4]. These antioxidants are also produced by biological system and occur naturally in many foods and the balance between oxidants and antioxidants decides the health and vigour[5]. Thus it is important to know the antioxidant content and their efficacy in foods, for preservation or protection against oxidative damage, to avoid deleterious changes and loss of commercial and nutritional value[6].

At present, the most commonly used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butylhydroquinone (TBHQ). Safety of these synthetic antioxidants has recently been questioned due to toxicity[7]. Besides, BHA and BHT have suspected of being responsible for liver damage and carcinogenesis[8]. Also, BHT had little effect on mutagenicity at low concentrations, but significantly increased their mutagenicity at high concentrations[9]. It was reported that BHT may cause internal and external haemorrhaging at high doses that is severe enough to cause death in some strains of mice and guinea-pigs[10]. Therefore, there is a growing interest on natural and safer antioxidants. Natural antioxidants are known to exhibit a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, anti-allergic, anti-thrombotic, vasodilatory, anti-atherosclerotic, antitumor, anti-mutagenic and anti-carcinogenic activities[11].

A.aureum is found in tropical and subtropical areas of the world. In Bangladesh, preparations from rhizomes and leaves of *A. aureum* are used to treat wounds, peptic ulcers and boils[12].In India, the frond (leaves) is applied over venomous snakebites as an antidote and the fertile fronds and roots are used traditionally for syphilitic ulcers[13]. Fijian people use it to treat asthma, constipation, elephantiasis, febrifuge and chest pain[14]. The native people of Costa Rica use leaves as emollients; whereas, the Panama and Colombian peoples use the young fiddleheads to extract fish bones from

the throat and as a medicine bath for infants[15]. The crude extract of a Japanese *A. aureum* specimen is reported to possess anti-oxidant, tyrosinase inhibiting activity[16]; while a Hainan specimen reported to have anti-tumour activity against cervical cancer cell line[17]. Methanol extracts from a Bangladeshi specimen of *A. aureum* is used to treat gastric, colon and breast cancer cells[18]. Baba *et al*[19] have included *A. aureum* as edible plants and as minor non-wood products. In Sri Lanka and Indonesia, young fronds are sold in the market as vegetable, sometimes consumed raw but more often, steamed or blanched[20].An interesting use of *A. aureum* was the harvesting of its stalks, which were sold to vegetable farmers as plant support in the Matang Mangroves, Malaysia[21]. In folklore medicine, the use of rhizomes and leaves of *A. aureum* for treatment of wounds and boils, and as worm remedy is most often reported[22].

In view of the above, in the present study is focused on *A. aureum* leaves to determine their free radical scavenging properties by using DPPH scavenging assay, hydroxyl radical scavenging activity, superoxide scavenging assay, ABTS scavenging activity assay and reducing power assay.

II.MATERIALS AND METHODS

2.1Plant material

The plant material, *Acrostichumaureum* L., was collected from Puthalam, Kanyakumari District, Tamil Nadu and was authenticated at Botanical Survey of India, Southern circle, Coimbatore, Tamil Nadu. The leaves were removed from the plant, cut into small pieces and shade dried at room temperature. The shade dried leaves were ground to coarse powder using mechanic grinder and stored in air tight container for further use.

2.2 Extract preparation

The leaf powder (20g) was extracted sequentially by hot continuous percolation method using Soxhlet apparatus[23] using different polarities of solvents like petroleum ether, benzene, ethyl acetate, ethanol and methanol. The dried powder was packed in Soxhlet apparatus and successively extracted with petroleum ether, benzene, ethyl acetate, ethanol and methanol. The extracts were concentrated by using a rotary evaporator and subjected to freeze drying in a lyophilizer till dry powder was obtained. The dry extract powder was used for *in vitro* free radical scavenging activity assay.

2.3 Determination of DPPH radical scavenging activity

DPPH free radical scavenging activity of the extracts were measured *in vitro* using α, α-diphenyl-β-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, M = 394.33) assay described by Blois[24].50mg of the dry extract powder and ascorbic acid (standard) were dissolved in methanol separately and their final volume was made up to 50ml (stock - 1000µg/ml). Then different concentrations like 50, 100, 200,

400 and 800µg/ml were prepared separately by diluting with the methanol from the stock. 3ml of different concentrations (50, 100, 200, 400 and 800µg/ml) of test solutions and standard were taken in different test tubes. After that 1ml of DPPH working solution (0.1m*M* DPPH in methanol) was added to each test tubes and the mixtures were shaken vigorously and allowed to stand at room temperature for 30min. The absorbance was measured against methanol as blank at 517nm using a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation). The percentage inhibition was calculated by comparing the absorbance values of the test sample with those of the control (not treated with extract or ascorbic acid). The capability of scavenging the DPPH radical was calculated by using the following formula.

$$\text{DPPH scavenging effect (\% inhibition)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 refers to the absorbance of the DPPH control, and A_1 refers to the absorbance of DPPH in the presence of extract/ascorbic acid. The inhibitory concentration (IC_{50}) value was calculated. IC_{50} values denotes the concentration of extract/standard required to scavenge 50% of free radicals

2.4 Determination of hydroxyl radical scavenging activity

The effect of extracts on hydroxyl radical scavenging activity was assayed by using the deoxyribose method[25]with some modification. Stock solutions of EDTA (1m*M*), $FeCl_3$ (10m*M*), ascorbic acid (1m*M*), H_2O_2 (10m*M*) and deoxyribose (10m*M*) were prepared in deionized distilled water. 50mg of the dry extract powder and ascorbic acid (standard) were dissolved in deionized distilled water separately and their final volume was made up to 50ml (stock - 1000µg/ml). Then different concentrations like 50, 100, 200, 400 and 800µg/ml were prepared separately by diluting with the deionized distilled water from the stock. 1ml of different concentrations of test solutions and standard (ascorbic acid) were taken in different test tubes. To this 0.1ml EDTA, 0.01ml of $FeCl_3$, 0.1ml H_2O_2, 0.36ml of deoxyribose and 0.33ml of phosphate buffer (50m*M*, pH 7.4) were added and the mixture was incubated at 37ºC for 1h. 1ml of the incubated mixture was taken out and was mixed with 1ml of (10%) trichloroacetic acid and 1ml of (0.5%) thiobarbituric acid [in 0.025*M* NaOH containing 0.025% butylated hydroxyl anisole] to develop the pink chromogen. After that the absorbance of the test solutions and standard were measured at 532nm. Deionized distilled water was used as blank. The percentage inhibition was calculated by comparing the absorbance values of the test sample with those of the controls (not treated with extract or ascorbic acid). The hydroxyl radical scavenging activity of the extract was reported as percentage inhibition of deoxyribose. The degradation is calculated by using the following equation.

Hydroxyl radical scavenging activity = $\frac{A_0 - A_1}{A_0}$ x 100

Where, A_0 is the absorbance of the control and A_1 is the absorbance the test sample /standard.

2.5 Determination of superoxide radical scavenging activity

The superoxide anion scavenging activity was measured as described by Srinivasan *et al*[26]. 1ml of different concentrations (50, 100, 200, 400 and 800µg/ml were prepared with methanol) of the test solutions and standard (ascorbic acid) were taken in different test tubes. To this 0.5ml of 16mM Tris-HCl buffer pH 8, 0.5ml of 0.3m*M* nitroblue tetrazolium, 0.5ml of 0.936m*M* of Nicotinamide Adenine Dinucleotide Reduced and 0.5ml of 0.12mM of phenazine methosulphate were added and incubated at dark for 5min. The absorbance was measured at 560nm. Distilled water was used as blank. The percentage inhibition was calculated by comparing the absorbance values of the test sample with those of the controls (not treated with extract). The inhibition percentage was calculated as superoxide radical scavenging activity as follows.

Superoxide radical scavenging activity = $\frac{A_0 - A_1}{A_0}$ x 100

Where, A_0 is the absorbance of the control and A_1 is the absorbance of the test sample /standard.

2.6. Determination of ABTS scavenging activity

2, 2-Azinobis 3-Ethylbenzothiazoline 6-Sulfonate (ABTS) radical scavenging activity of *A. aureum* leafextracts was measured by Huang *et al*[27]method with some modifications. Unlike DPPH assay, the assay that involves scavenging of ABTS radicals required generation of the radicals. The ABTS radical cation ($ABTS^{\bullet+}$) was generated by mixing ABTS stock solution (7mM) with potassium persulfate (2.45mM). The reaction mixture left in the dark for 12h at room temperature and the resulting dark coloured solution was diluted using ethanol to an absorbance of 0.70 ± 0.02 at 734nm. 0.lml of different concentrations (50, 100, 200, 400 and 800µg/ml were prepared with methanol) of the test solutions and trolox (standard) were mixed with 3.9ml of radical solution in clean and labeled test tubes. The tubes were incubated in dark for 6min at room temperature followed by measuring the absorbance of the reaction mixture in spectrophotometer at 734nm. Methanol replacing the test sample / trolox served as control (i.e., 0.1ml methanol + 3.9ml ABTS radical solution). The ABTS radical scavenging activity of the sampleswere calculated by using the following formula and the results were expressed as trolox equivalent antioxidant capacity (TEAC) values.

ABTS radical scavenging activity = $\frac{A_0 - A_1}{A_0}$ x 100

Where, A_0 is the absorbance of the ABTS solution without sample/trolox and A_1 is the absorbance the ABTS solution in the presence of extract/trolox.

2.7 Determination of reducing power

The reducing power of the extract was determined by the method of Kumar and Hemalatha[28]. 1ml of different concentrations (50, 100, 200, 400 and 800µg/ml was prepared with methanol) of test samples and standard (ascorbic acid) were mixed with 5ml of sodium phosphate buffer (0.2*M*, pH 6.6) and 5ml of 1% potassium ferric cyanide and the mixture was incubated at 50ºC for 20min. After incubation, 5ml of 10% trichloroacetic acid was added to the mixture and centrifuged at 980*g* for 10min in a refrigerator centrifuge. About 5ml of supernatant of solution was taken and diluted with 5ml of distilled water and shaken with 1ml of freshly prepared 0.1% ferric chloride and the absorbance was measured at 700nm in UV-VIS spectrophotometer. A blank was prepared without adding extract. This result indicates that increase in absorbance of reaction mixture indicates increase in reducing power.

2.8. IC_{50} determination

The inhibitory concentration (IC_{50}) values denotes the concentration of extract/standard required to scavenge 50% of free radicals. GrapPad PRISM software (version 4.03) was used for calculating IC_{50} values for DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical scavenging activity and antioxidant activity by radical cation ($ABTS^{\bullet+}$).

2.9 Statistical analysis

Antioxidant activities like DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical activity, ABTS radical cation scavenging activity and reducing powers were estimated in triplicate determinations. Data were analyzed using the statistical analysis system SPSS (SPSS software for windows release 17.5; SPSS Inc., Chicago IL, USA). The results were expressed as mean.

III.RESULTS AND DISCUSSION

3.1 DPPH radical scavenging assay

DPPH free radical scavenging method offers the first approach for evaluating the antioxidant potential of a compound, an extract or other biological sources. This is the simplest method, wherein the prospective compound or extract is mixed with DPPH solution and absorbance is recorded after a defined period[29].This method is based on thereduction of DPPH in methanol solution in the presenceof a hydrogen–donating antioxidant due to the formationof the non-radical form DPPH-H[24].

This transformation results in a colour change from purple toyellow, which is measured spectrophotometrically. The disappearance of the purple colour is monitored at 517nm.

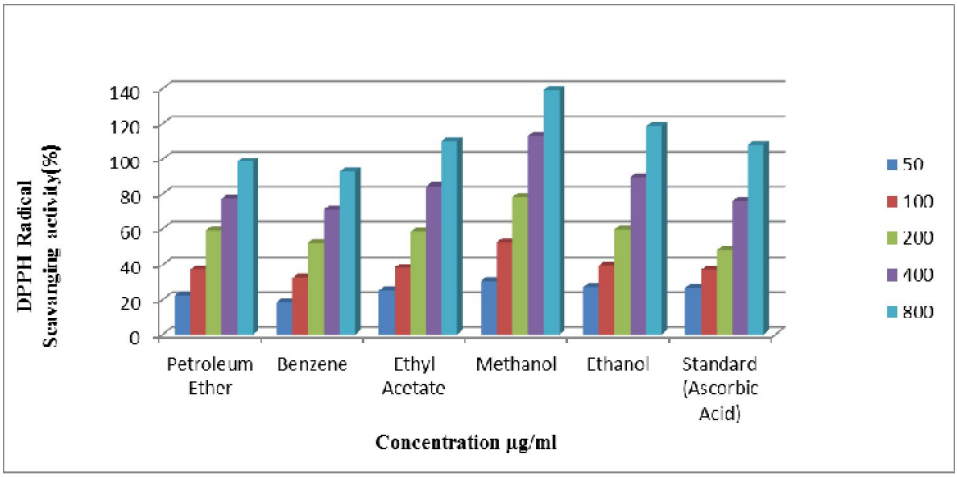

Fig 1: DPPH radical scavenging activity of different leaf extracts of *A.aureum*

The DPPH radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. aureum* leaves were shown in figure 1. The results of this study indicated that all the solvent tested had noticeable effect on DPPH radical. The scavenging effect was increased with the concentration of extracts and standard (ascorbic acid).

Table -1 shows the DPPH radical scavenging activity of different solvent extracts which was expressed in terms of IC_{50} value with respect to ascorbic acid as standard. Lower IC_{50} value shows more antioxidant potential. The IC_{50} value for benzene extract was 27.18µg/ml which was comparatively lower than the IC_{50} (32.84µg/ml) of ascorbic acid, showed that benzene extract of *A. aureum*was more effective as antioxidant than that of standard, ascorbic acid.According to Phongpaichit *et al*[30], extracts which possess IC_{50} values ranging from 50 to 100µg/ml is considered to exhibit intermediate antioxidant activity. Meanwhile, extracts with IC_{50} value ranging between 10 to 50µg/ml is considered to possess strong antioxidant activity.

Table 1: IC_{50} values of different solvent extracts of leaves of *A. aureum*

Solvent	DPPH assay	Hydroxyl radicals assay	ABTS assay	Superoxide Dismutase assay
Petroleum ether	29.22	20.67	21.86	25.16
Benzene	27.18	30.11	24.56	22.06
Ethyl acetate	33.16	27.84	29.16	31.11
Methanol	41.36	31.06	28.56	34.12
Ethanol	34.56	28.16	32.96	32.18
Ascorbic Acid	32.84	29.93	-	30.15
Trolox	-	-	33.06	

3.2 Hydroxyl radical scavenging assay

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell[31].

The hydroxyl radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A.aureum* leaves were shown in figure 2.The results showed that there was a dose-dependent radical scavenging activity.The IC_{50} of a compound is inversely related to its antioxidant capacity, as it expresses the amount of antioxidant required to decrease the hydroxyl radical concentration by 50%, which is obtained by interpolation from a linear regression analysis[32]. A lower IC_{50} indicates a higher antioxidant activity of a compound. Table 1 shows the IC_{50} values in the hydroxyl radical scavenging activity assay of the extracts. It was found that the petroleum ether extract possesses the strongest hydroxyl radical activity (20.67µg/ml) compared to standard ascorbic acid (29.93µg/ml).

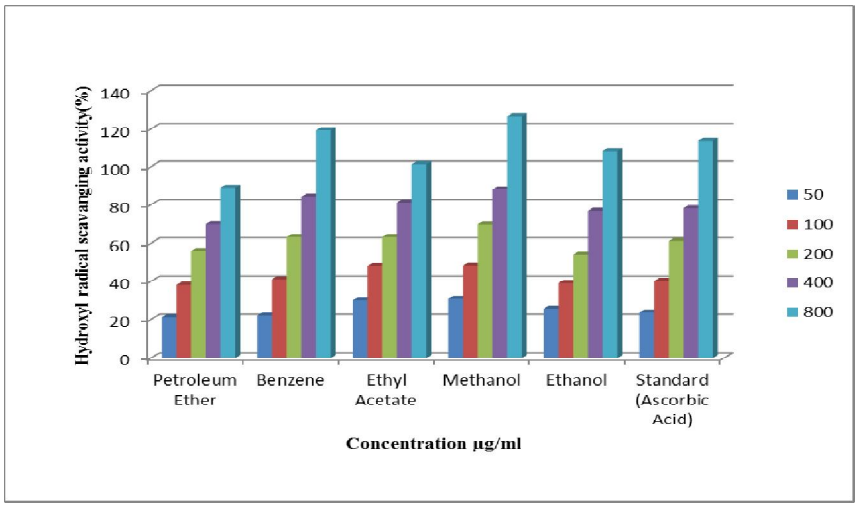

Fig 2: Hydroxyl radical scavenging activity of different leaf extracts of *A. aureum*

3.3 ABTS radical cation scavenging assay

The assay is based on interaction between antioxidant and ABTS radical cation ($ABTS^{\bullet+}$). ABTS assay measures the relative ability of antioxidant to scavenge the $ABTS^{\bullet+}$ generated in aqueous phase, as compared with a trolox(water soluble vitamin E analogue) standard. The $ABTS^{\bullet+}$ is generatedby reacting a strong oxidizing agent (e.g.potassium permanganateor potassium persulfate) with the ABTS salt. ABTS assay is frequently used by the food industry and agricultural researchers to measure the antioxidant capacities of foods[33].

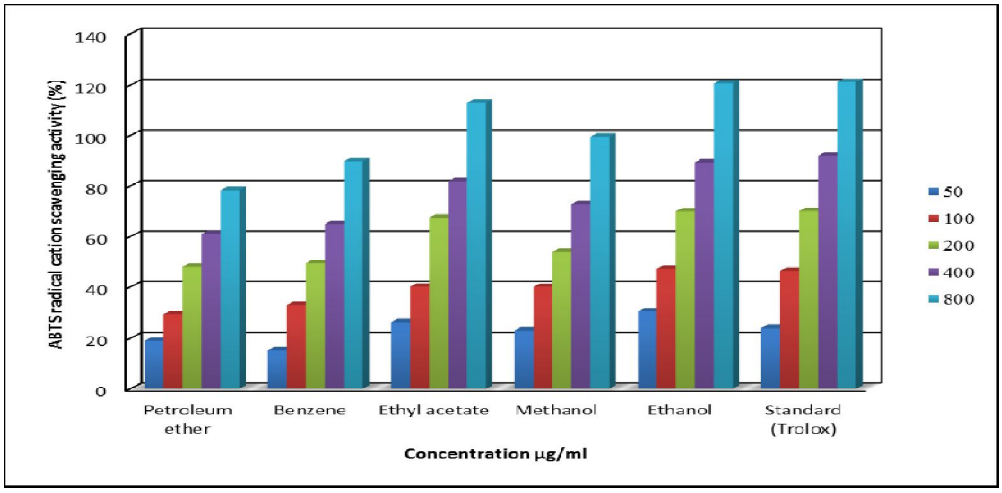

Fig: 3 ABTS radical cation scavenging activity of different leaf extracts of *A. aureum*

The ABTS radical cation scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. aureum* leaves were shown in figure 3. The scavenging effect was increased with the concentration of extracts and standard (trolox).

Table -1 shows the ABTS radical scavenging activity of different solvent extracts which is expressed in terms of IC_{50} value with respect to trolox as standard. Lower IC_{50} value shows more antioxidant potential. The IC_{50} value for petroleum ether extract was 21.86µg/ml which was comparatively lower than the IC_{50} (33.06µg/ml) of trolox, showed that petroleum ether extract of *A. aureum* is more effective as antioxidant compared to ascorbic acid.

3.4 Superoxide radical scavenging assay

Superoxide radical anion ($O2^{\bullet-}$) is produced as a result of the donation of one electron to oxygen. This radical arises either from several metabolic processes or following oxygen activation by irradiation. $O2^{\bullet-}$ is generated using a non-enzymatic reaction of phenazine methosulphate in the presence of nicotinamide adenine dinucleotide (NADH). In this generation system, $O2^{\bullet-}$ may reduce nitroblue tetrazolium into formazan, which is spectrophotometrically monitored at 560 nm[34].

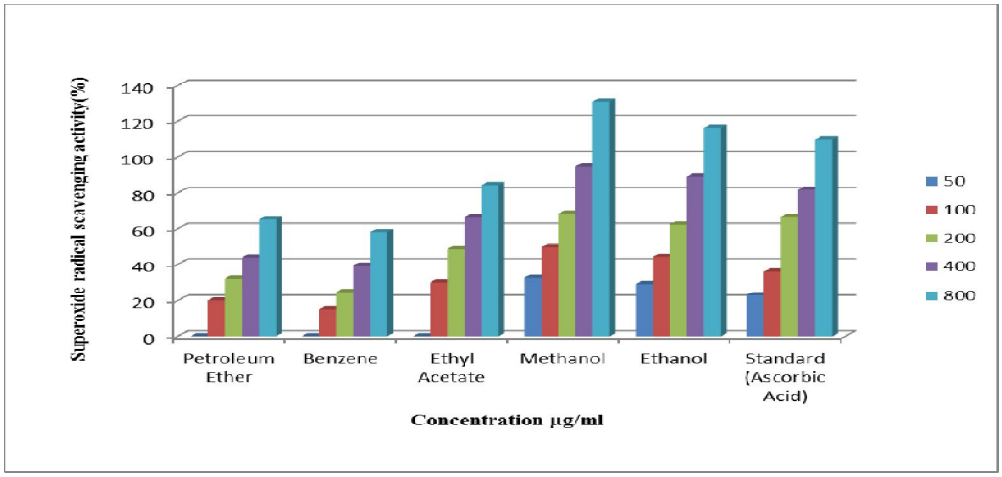

Fig 4: Superoxide radical scavenging activity of different leaf extracts of *A. aureum*.

The superoxide radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. aureum* leaves was shown in figure 4. Methanol extract of *A. aureum* leafwas found to have the highest superoxide radical scavenging activity. 800µg/ml concentration of methanol extract of *A. aureum* leaves recorded 131.16% of scavenging activity; nonetheless the standard, ascorbic acid had 109.54% of scavenging activity.

3.5 Reducing power assay

The reducing power of the extract, which may serve as a reflection of its antioxidant activity, was determined using a modified Fe^{3+} to Fe^{2+} reduction assay, whereby the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of the sample. The presence of antioxidants in the sample causes the reduction of Fe^{2+}/ferric cyanide complex to the Fe^{2+} form, which is monitored by measuring the formation of Perl's Prussian blue at 700 nm[35].

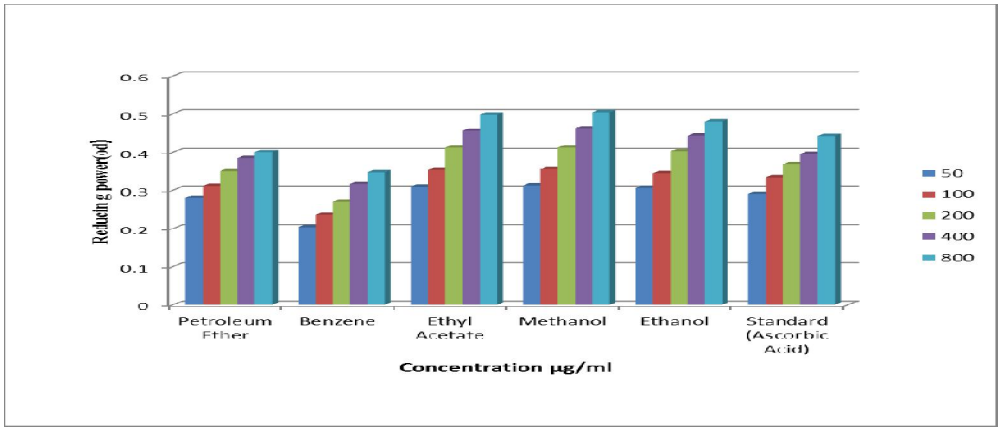

Fig 5: Reducing power ability of different leaf extracts of *A. aureum*.

In figure 5, all the extracts shows some degree of electron-donating capacity in a concentration-dependent manner. Increasing the absorbance at 700nm indicated an increase in reductive ability. The 800µg/ml concentration of methanol extract gave the highest reducing power and is higher than that of the other extracts at all concentrations studied. The extracts showed good reducing power that was comparable with that of ascorbic acid.

IV.CONCLUSION

Decisively, results of this study signify that the extracts of *A. aureum* leaf is an important source of natural antioxidants which can play vital role in reducing the oxidative stress and preventing certain degenerative diseases. Purification of the extract may lead to increased activity of the compounds. The present findings appears to be useful in leading to further study in theidentification and characterization of specific compounds responsible for the relatively high antioxidant activities in these fern. These studies are now in progress.

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