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# Evaluation of *in vitro* antioxidant activity of *Acrostichum aureum* Linn. Rachis

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**Abstract**
*Acrostichum aureum* Linn is a medicinal fern collected from Puthalam, Kanyakumari District, Tamil Nadu, India. This plant is used by the local people for curing pharyngitis, chest pain and diabetics. Hence, in the present study, petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of the rachis of *A. aureum* have been tested for antioxidant activity by using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical scavenging activity, antioxidant activity by radial cation ($ABTS^{\bullet+}$) and reducing power assays. The antioxidant potential is dose dependent in all the assays carried out. It is concluded that the *A. aureum* can be used as a medicine against free radical associated oxidative damage.

**Keywords:** medicinal plant, *Acrostichum aureum*, antioxidant, free radicals, DPPH, ABTS

## Introduction
Antioxidants may be defined as compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions [1]. Antioxidants can also protect the human body from free radicals and reactive oxygen species (ROS) effects. Antioxidants retard the progress of many chronic diseases as well as lipid peroxidation [2]. Also, antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods [3].
At the present time, the most commonly used antioxidants are butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT), propyl gallate (PG) and tert butyl hydroquinone (TBHQ). The safety of these antioxidants has recently been questioned due to toxicity [4]. Therefore, there is a growing interest on natural and safer antioxidants [5]. Development of safer natural antioxidants from extracts of oilseeds, spices and other plant materials that can replace synthetic antioxidants has been of interest [6].
Natural antioxidants are known to exhibit a wide range of biological effects including antibacterial, antiviral, antiinflammatory, ant allergic, antithrombotic and vasodilatory activities. Thus, there is a need of antioxidants of natural origin because they can protect the human body from the diseases caused by free radicals [7].
*Acrostichum aureum* Linn (Family- Pteridaceae), common name: Swamp Fern, Mangrove Fern, occurs Worldwide in mangrove swamps, salt marshes, canal margins, and low hammocks. It is widely distributed throughout South Florida [8], Brazil, South and West Mexico, Guyanas, Central America, Colombia, Venezuela, Ecuador, Paraguay, Barbados, Trinidad, South China, Taiwan, Japan, North Australia, India, Sri Lanka and Bangladesh [9]. It is an evergreen shrub, can be grown as annual which is locally used as choice of medicinal plant in the treatment of major and minor complaints. Several studies have reported the traditional use of *A. aureum's* rhizome for curing wounds, non-healing ulcers, boils, syphilitic ulcers, sore throat, chest pains, elephantiasis, purgative, febrifuge, cloudy urine in women, and rheumatism in Malaysia [10], Bangladesh [11], India [12], and Yap islands and Micronesia [13].
In the present study, the mode of antioxidant action in petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. aureum* was investigated. *In vitro* methods of assessment were used to determine the scavenging activity of the extract on 1, 1-diphenyl-2-picryl hydrazyl (DPPH), hydroxyl radical, superoxide scavenging, 2, 2-azinobis 3-ethylbenzothiazoline 6-sulfonate (ABTS) and reducing power.

## Materials and Methods
### Plant material
Rachis of *Acrostichum aureum* Linn were collected from Puthalam, Kanyakumari District, Tamil Nadu and was authenticated at Botanical Survey of India, Southern circle, Coimbatore, Tamil Nadu. The gathered rachis were cut into small pieces and shade dried at room

temperature. The shade dried rachis were ground into coarse powder using mechanic grinder and stored in air tight container for further use.

**Extract preparation**

The coarsely powdered rachis (20g) was extracted separately with petroleum ether, benzene, ethyl acetate, methanol and ethanol in a Soxhlet apparatus for 24h. The extracts obtained were filtered through Whatman No. 41 filter paper. Then the filtrates were concentrated under vacuum using rotary evaporator (Heidolph, Schwabach, Germany). The concentrated extracts were stored at 4ºC for further investigation of potential *in vitro* free radical scavenging activity.

**Determination of DPPH radical scavenging activity**

DPPH free radical scavenging activity of the extracts were measured *in vitro* using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay described by Blois [14]. 50mg of the extract and ascorbic acid (standard) were taken and dissolve in methanol and final volume make up to 50ml which was used as a stock solution with the concentration 1000µg/ml. Then different concentrations like 50, 100, 200, 400 and 800µg/ml were prepared by diluting with the methanol from stock solution. 3ml of different concentration (50, 100, 200, 400 and 800µg/ml) of test solution and standard was taken in different test tubes. To this add 1ml DPPH working solution (0.1mM DPPH in methanol) 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 controls (not treated with extract). 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 (IC50) value was calculated. IC50 values denotes the concentration of extract/standard required to scavenge 50% of free radicals

**Determination of hydroxyl radical scavenging activity**

The effect of extracts on hydroxyl radical scavenging activity was assayed by using the deoxyribose method [15] with some modification. Stock solutions of EDTA (1mM), $FeCl_3$ (10mM), ascorbic acid (1mM), $H_2O_2$ (10mM) and deoxyribose (10mM), were prepared in deionized distilled water. 1ml of different concentration (50, 100, 200, 400 and 800µg/ml prepared with deionized distilled water) of test solution and control (ascorbic acid) was 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 (50mM, pH 7.4) were added and the mixture was then 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 (TBA) [in 0.025M NaOH containing 0.025% butylated hydroxyl anisole (BHA)] to develop the pink chromogen. The absorbance of the test solution 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). The hydroxyl radical scavenging activity of the extract was reported as percentage inhibition of deoxyribose. The degradation is calculated by using the following equation.

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

Where, $A_0$ is the absorbance of the control, and $A_1$ is the absorbance test samples and reference.

**Determination of superoxide radical scavenging activity**

The superoxide anion scavenging activity was measured as described by Srinivasan *et al* [16]. 1ml of different concentration (50, 100, 200, 400 and 800µg/ml prepared with methanol) of test solution and control (ascorbic acid) was taken in different test tubes. To this 0.5ml of 16mM Tris-HCl buffer pH 8, 0.5ml of 0.3mM nitroblue tetrazolium (NBT), 0.5ml of 0.936mM of Nicotinamide Adinie Dinucleotide Reduced (NADH) and 0.5ml of 0.12mM of phenazine methosulphate (PMS) were added and incubated at dark for 5 min. The absorbance was measured at 560nm. Distilled water 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.

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

Where, $A_0$ is the absorbance of the control, and $A_1$ is the absorbance test samples and reference.

**Determination of ABTS scavenging activity**

2, 2-Azinobis 3-Ethylbenzothiazoline 6-Sulfonate (ABTS) radical scavenging activity of *A. aureum* extracts was measured by Huang *et al* [17] 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 prepared with methanol) of extracts and trolox (reference standard) was 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 extract / trolox served as control (i.e., 0.1ml methanol + 3.9ml ABTS radical solution). The ABTS radical scavenging activity of the extracts was calculated using the following formula and the results were expressed as trolox equivalent antioxidant capacity (TEAC) values.

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

Where, $A_0$ is the absorbance of the ABTS solution without extract/trolox and $A_1$ is the absorbance the ABTS solution in the presence of extract/trolox. The $IC_{50}$ value was calculated.

$IC_{50}$ denotes the concentration of extract required to scavenge 50% of the radicals.

**Determination of reducing power**

The reducing power of the extract was determined by the method of Kumar and Hemalatha [18]. 1ml of each extract of different concentration (50, 100, 200, 400 and 800µg/ml prepared with methanol) and standard (ascorbic acid) were mixed with 5ml of sodium phosphate buffer (0.2M, pH 6.6) and 5ml of 1% potassium ferricyanide and the mixture was incubated at 50ºC for 20min. After incubation, 5ml of 10% trichloroacetic acid (TCA) 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.

**Statistical analysis**

Grap Pad 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 ($ABTS^{\bullet+}$). The results were expressed as mean and all the experiments were done in triplicate.

**Result and Discussion**

**DPPH radical scavenging activity**

Free radicals are molecules, usually of oxygen, which have lost an electron and are continuously generated during human body metabolism. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule which is widely used to investigate to investigate radical scavenging activity. In DPPH radical scavenging assay, antioxidants react with DPPH (deep violet colour) and convert it to yellow coloured α, α-diphenyl-β-picryl hydrazine. The degree of discoloration indicate the radical-scavenging potential of the antioxidant [14].

The effect of different solvent extracts of rachis of *A. aureum* and standard ascorbic acid on DPPH radical scavenging activity were shown in figure 1.

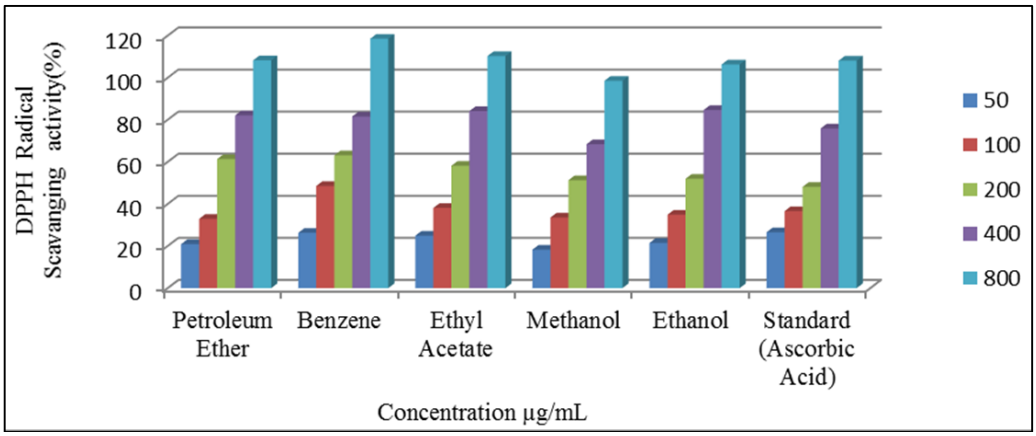


**Fig 1:** DPPH radical scavenging activity of different extracts of *Acrostic hum aureum* rachis

The DPPH radical scavenging activity of all the samples was highly depend on concentration, namely, antioxidant activity increased with increase in extract concentration. Among the tested solvents, benzene extract of rachis of *A. aureum* exhibited high DPPH radical scavenging activity. Results shows that 800µg/ml concentration of benzene extract from the rachis of *A. aureum* exhibited the highest DPPH (118.56%) scavenging activity compared to other extracts. The $IC_{50}$ value of ascorbic acid (Table 1) was 32.84µg/ml; whereas, methanol extract was found to contain 36.54µg/ml $IC_{50}$ value.

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

| Solvent | DPPH | Hydroxyl radicals | ABTS | Superoxide |
|---|---|---|---|---|
| Petroleum ether | 31.56 | 25.16 | 26.12 | 26.18 |
| Benzene | 34.13 | 30.18 | 22.46 | 24.16 |
| Ethyl acetate | 30.36 | 31.48 | 27.16 | 28.16 |
| Methanol | 36.54 | 32.16 | 30.11 | 30.96 |
| Ethanol | 32.16 | 30.84 | 28.36 | 34.84 |
| Ascorbic acid | 32.84 | 29.93 | 33.06 | 30.15 |

**Hydroxyl radical scavenging activity**

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extracts for hydroxyl radicals generated from $Fe^{2+}/EDTA/H_2O_2$ system (Fenton reaction). The hydroxyl radicals attack deoxyribose, which eventually results in the formation of thiobarbituric acid reacting substance (TBARS) [19].

Among the reactive oxygen species, the hydroxyl radicals are the most reactive and predominant radicals generated endogenous during aerobic metabolism. A single hydroxyl radical results in the formation of many molecules of lipid hydroperoxides in the cell membrane which may severely, disrupts its function and leads to cell death [20].

The petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of rachis of *A. aureum* were found to possess concentration dependent scavenging activity on hydroxyl radicals and the results were given in figure 2.

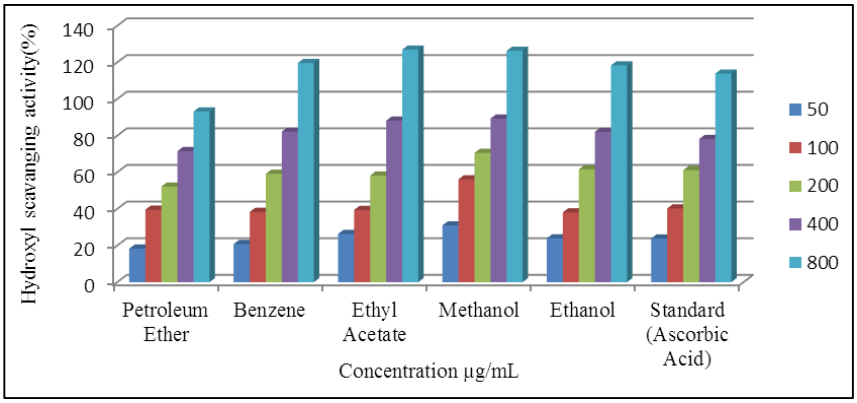


**Fig 2:** Hydroxyl radical scavenging activity of different extracts of *Acrostichum aureum* rachis

Among all the tested extracts, ethyl acetate and methanol extracts (800µg/ml) showed high levels of hydroxyl radical scavenging activity (126.92% and 126.32% respectively).The $IC_{50}$ value of ascorbic acid was 31.48%; whereas, $IC_{50}$ value of methanol extract was found to be 32.16%.

**ABTS radical cation scavenging activity**

The effect of *A. aureum* rachis extracts and standard ascorbic acid on ABTS radical cation were compared and shown in figure 3. At 800µg/ml concentration of methanol, ethanol, ethyl acetate extracts of *A. aureum* rachis possessed 119.22%, 108.16%, 106.32% scavenging activity on ABTS. All the concentration of *A. aureum* rachis extract showed lower activity than the standard ascorbic acid 121.36%. The $IC_{50}$ value of ascorbic acid was 33.06% whereas methanol extract was found to be 30.11%. This scavenging activity of ABTS radical by the plant extracts were found to be appreciable; this implies that the plant extract useful for treating radical related pathological damage especially at higher concentration [21].

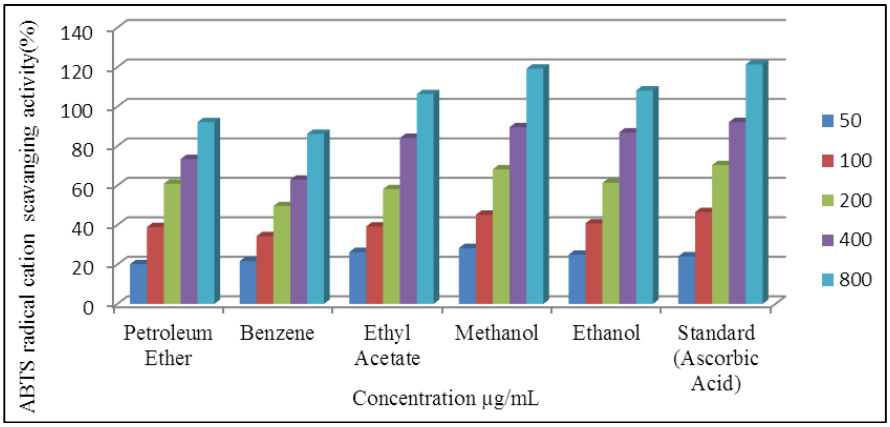


**Fig 3:** ABTS radical cation scavenging activity of different extracts of *Acrostichum aureum* rachis

**Superoxide radical scavenging activity**

Superoxide anion plays an important role in the formation of more reactive species such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA [22]. Therefore, estimating the scavenging activity of plant extracts on superoxide radical is one of the most important ways of clarifying the mechanism of antioxidant activity.

In the present investigation, different solvent extracts of rachis of *A. aureum* were found to possess concentration dependent scavenging activity on superoxide and the results were given in figure 4. Among all the solvent extracts, ethanol extracts (800µg/ml) of the plant material exhibited the highest superoxide radical scavenging activity; whereas, the standard ascorbic acid had 109.54% of superoxide radical scavenging activity. The mean IC50 value of ascorbic acid was 30.15%; whereas, ethanol extract was found to be 34.84%.

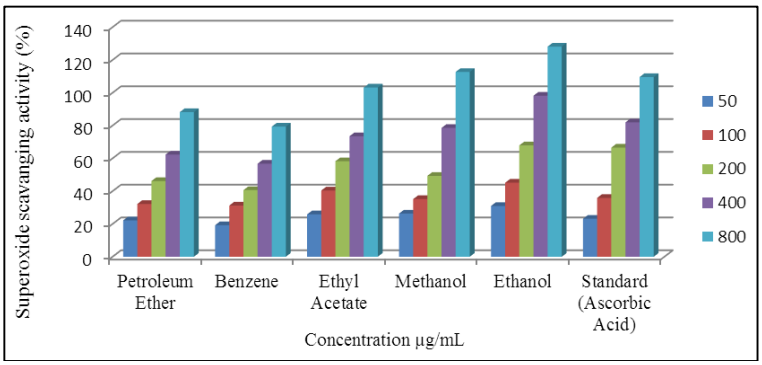


**Fig 4:** Superoxide radical scavenging activity of different extracts of *Acrostichum aureum* rachis.

**Reducing power**

Reducing power reflects the electron donating capacity of its bioactive compounds, which serves as a significant indicator of its antioxidant activity. Reduced $Fe^{3+}$ / ferricyanide complex to the ferrous form, which indicated existence of reductants in the sample solution. The reductants have been exhibiting antioxidative potential by breaking the free radical chain and donating a hydrogen atom [23]. The reducing power of the extracts of *A. aureum* rachis was shown in figure 5. The reducing ability of the extract increased with increasing concentration, our results were in accordance to studies done by Vijaya e*t al* [24]. In the present study, 800µg/ml concentration of methanol extract showed the highest reducing power than the ascorbic acid.

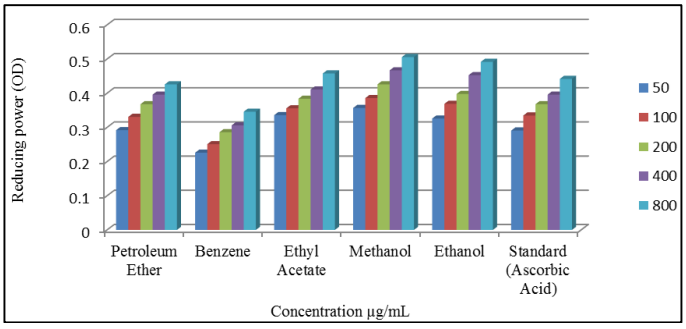


**Fig 5:** Reducing power ability of different extracts of *Acrostichum aureum* rachis

**Conclusion**

In the present study, it can be concluded that different solvent extracts of rachis of *A. aureum* have wide range of antioxidant and/or free radicals scavenging activity. The antioxidant activity of plant may vary depending upon the geographical area, seasonal variation and also the method and solvent used for extraction. Literature survey reveals that flavonoids [25] and phenolic compounds [26] are responsible for antioxidant activity. However, we do not know what components in the plant extracts show these activity. More detailed studies on chemical composition of the plant extracts, as well as *in vivo* assays are essential to characterize them as biological antioxidant. It should be kept in mind that antioxidant activity measured by *in vitro* methods may not reflect *in vivo* effects of antioxidant [27]. Many other factors such as absorption/metabolism are also important.

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