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Free-radical scavenging activity of stem and leaf of *Atalantia monophylla* (L.) Corr. Serr

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Abstract
The ethnobotanical and folk medicine used of plant *A. monophylla* (L.) Corr. Serr. Itis used for Respiratory, Rheumatism etc. To evaluate the plant's leaves and stem of *Atalantia monophylla* extract of pet ether, chloroform, acetone and methanol for, total antioxidant and free radical scavenging ability. The ability of the plant extract to act as hydrogen/electrons donor or scavenger of radicals were determined by *in-vitro* antioxidant assays using 2,2-diphenyl-2-picryl-hydrazyl free radical (DPPH·) scavenging, then reducing power assay, superoxide radical (O_2^{*-}) scavenging activity, phosphomolybdenum assay, ferric Reducing antioxidant power assay, and metal chelating activity, were performed to know the antioxidant potency of the plant extract of leaves and stem of *Atalantia monophylla*. The study also revealed significant free radical scavenging ability of the plant leaves as assessed by DPPH, reducing power, phosphomolybdenum assay, FRAP, and metal chelating activity. Scavenging ability of the plant leaves as assessed by DPPH, reducing power, phosphomolybdenum assay, FRAP, and metal chelating activity.

Keywords: *Atalantia monophylla* (L.) Corr. Serr. DPPH assay, reducing power assay, superoxide radical (O_2^{*-}) scavenging activity, phosphomolybdenum assay, and ferric reducing antioxidant Power assay

1. Introduction
Medicinal plants correspond to a wealthy source of antimicrobial agents. The plant materials used in traditional medicine are readily available in rural areas at relatively cheaper than modern medicine [1, 2]. Plants naturally produce a wide variety of secondary metabolites which have a good antibacterial, antifungal and hence are of pharmaceutical importance. These compounds are also responsible for antioxidant potential which offered a defensive mechanism against oxidant produced in living systems such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). The ROS and RNS are produced as by-products in aerobic organisms during oxidation which was considered to be vital phenomenon to many forms of life for their normal physiological function. But now it is believed that most of ROS and RNS, produced as by product of cellular metabolism are very harmful to body regarding cell and tissue damage. The ROS such as superoxide anions (O-2), hydroxyl radical (OH) and nitric oxide (NO) initiate degenerative processes in body by inactivating enzymes and destroying important cellular components and have been implicated in the pathology of a vast variety of diseases including cancer, atherosclerosis, diabetic mellitus, hypertension, AIDS and aging. Therefore, antioxidant potential of medicinal plant is important in view of the free radical theory of aging and associated diseases [3-5].
Reactive oxygenic species in the form of superoxide anion (O2−.), hydrogen peroxide (H2O2) and hydroxyl radical (HO.) are natural by-products of our body's metabolism. They are dangerous, however, when present in excess, and can attack biological molecules such as lipids, proteins, enzymes, DNA and RNA, leading to cell or tissue injury associated with degenerative diseases [6-8]. Although the mammalian body has certain defence mechanisms to combat and reduce oxidative damage, epidemiological evidence indicates that the consumption of foodstuffs containing antioxidant phytonutrients—notably flavonoids and other polyphenolics—is advantageous for our health [9, 10].
This study therefore sought to investigate the free-radical scavenging properties like DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical, DPPH, reducing power, phosphomolybdenum assay, FRAP, superoxide radical (O_2^{*-}) scavenging activity and metal chelating activity in pet-ether, chloroform, Acetone, and methanol extract of leaves of *Atalantia monophylla* and its used by Maruthamalai tribes of Coimbatore District of Tamilnadu. The results obtained from the study could be crucial for understanding the role played by the green leafy vegetables in the prevention and treatment of several diseases.

2. Material and methods

Collection and Preparation of Sample Leaf and stem of *Atalantia monophylla* were collected fresh from Maruthamalai forest a tail of Western Ghats, Coimbatore district. These were shade dried, ground well using mechanical blender in to fine powder and transferred to airtight containers for future studies.

2.1 Extraction from plant parts

The powdered plant material was extracted in Soxhlet extractor successively with petroleum ether, chloroform, acetone and methanol. Each time before extracting with the next solvent, the thimble was dried in hot air oven below 40 ºC. The different solvent extracts were concentrated by rotary vacuum evaporator and then air dried. The dried extract obtained with each solvent was weighed. The percentage yield was expressed in terms of air dried weight of plant material. The crude concentrated extract was again weighed and used for further biochemical studies. The extract was used for analysis of free-radical scavenging activities. The different free-radical scavenging properties like DPPH, Reducing power, Phosphomolybdenum assay, Superoxide radical (O_2^{*-}) scavenging activity, FRAP, Metal chelating activity and ferric reducing power activity were studied. The extracts were freeze dried and stored in desiccators until further analysis.

2.2 Chemicals

Riboflavin, EDTA (Ethylene Diamide Tetra Acetic acid), NBT (Nitro-blue tetrazolium), $FeCl_3$ (Ferric Chloride), ascorbic acid, Butylated hydroxyanisole (BHA),
Butylatedhydroxy toluene (BHT), Sodium nitro prusside, Gallic Acid, Potassium ferricyanide, DPPH(1, 1-diphenyl-2-picryl hydrazyl), DMSO (Dimethyl Sulphoxide).

2.3 *In vitro* antioxidant studies

2.3.1 DPPH radical scavenging activity (Shimade *et al.*, 1992)

The antioxidant activity of the extracts was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$),according to the method of Blois, (1958). Plants extracts at various concentrations (20 - 100 µL) was added to 5 mL of 0.1 mM methanolic solution of $DPPH^{\cdot}$ and allowed to stand for 20 min at 27 ºC. The absorbance of the sample was measured at 517 nm. Radical scavenging activity of the sample was expressed as IC_{50}. Concentration of DPPH by 50 %(IC_{50}) under the experimental condition was determined. Methanol was served as blank and solution without extract served as control. The mixture of methanol, DPPH and standard (ascorbic acid) served as positive control. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula. More significantly the IC_{50} of the extracts were also calculated.

2.3.2 Assay of superoxide radical (O_2^{*-}) scavenging activity

The assay was based on the capacity of the sample extract to inhibit formazan formation by scavenging superoxide radicals generated in riboflavin- light-NBT system (Beauchamp and Fridovich, 1971). Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 2.33µg riboflavin and 12 mM EDTA, and 11.55 g NBT. Reaction was started by illuminating the reaction mixture with of sample extracts (100 µL) for 90 seconds. Reaction mixture with extract kept in dark served as negative control while the mixture without extract was taken as blank. Immediately after illumination, the absorbance was measure at 590 nm. The activity was compared to ascorbic acid. The percentage inhibition of superoxide anion generation was calculated using the following formula:
Percentage of inhibition = (Control OD – Sample OD / Control OD) × 100

2.3.3 Reducing power assay

The reducing power of plant in different solvent extracts of was determined by the method reported by (Oyaizu, 1986). 100 µL of extract was taken in 2.5 mL of 0.2M phosphate buffer (pH 6.6) was added. To this, 2.5 mL of 1% Potassium ferricyanide solution was added and the mixture was incubated at 50 ºC for 20 minutes. After the incubation, 2.5 mL of 10 % TCA was added. The content was centrifuged at 3000 rpm for 10 minutes. The upper layer of the supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1 % ferric chloride. The absorbance of the reaction mixture was measured spectrophotometer at 700 nm.
% reducing power = (Control OD – Sample OD / Control OD) × 100

2.3.4 Phosphomolybdenum assay

The antioxidant power of the extracts has been assessed with the phosphomolybdenum reduction assay according to (Prieto *et al*., 1999). The assay was based on the reduction of the extract and subsequent formation of a complex (Green colour). 0.5 mL of extract combined with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was incubated at 95 ºC for 90 minutes. The absorbance of the mixture was measured at 695 nm using spectrophotometer, it was against the blank. The results were calculated in ascorbic acid equivalents/100g extract.
Percentage of Phosphomolybdenum = (Control OD – Sample OD / Control OD) ×100

2.3.5 Metal chelating activity

Iron II chelating activity was measured by the inhibition of the formation of Iron-(II)-ferrozine complex after pre-incubation of the sample. The Fe^{+} was monitored by measuring the formation of ferrous iron–ferrozine complex against methanol blank at 562nm.The chelating of ferrous ions by various extracts in plant was estimated by the method of Dinis *et al*., (1994). The chelating of ferrous ions by various extracts of *A. monophylla* was estimated. Initially, about 100µl the extract samples were added to 50µl of 2 mM $Fecl_2$ solution. Then the reaction was initiated by the addition of 200µl of 5mM ferrozine and the test tubes were vortexes well and left standing at room temperature for 10 minutes. The reaction mixture containing deionized water in place of sample was considered as the negative control absorbance of the solution was then measured spectrophotometrically at 562 nm against the blank (deionized water). EDTA was against the standard metal chelating agent and the results were expressed as mg EDTA equivalents/g extract chelate the ferrous ion was calculated by,
Percentage chelation=[1-(ABS sample/ABS control)] ×100
Ec_{50} value (mg extract / mL) is the effective concentration at which ferrous ions were chelated 50% by the extract.

2.3.6 FRAP -Ferric reducing antioxidant power

The antioxidant capacities of phenolic extracts of samples were estimated according to the procedure described by (Pulido *et al*,. 2000). FRAP reagent (2.7 mL), prepared freshly and incubated at 37 ºC, was mixed with 270 µL of

distilled water and 50 µL of test sample or methanol (for the reagent blank). The test samples and reagent blank were incubated at 37ºC for 30 minutes in a water bath. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 mL of 20 mM/L TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM/L HCl plus 2.5 mL of 20 mM/L $FeCl_3 \cdot 6H_2O$ and 25 mL of 0.3 m/L acetate buffer (pH 3.6) described by Siddhuraju and Becker, (2003). At the end of incubation, the absorbance readings were taken immediately at 593 nm, using a spectrophotometer. Methanolic solution of known Fe (II) concentration, ranging from 100 to 2000 µM ($FeSo_4$ $7H_2O$) were used for blank. Results were calculated in ferrous sulphate equivalents.

3. Result And Discussion

3.1 DPPH radical scavenging activity

The free radical-scavenging activities in stem and leaf of the plants *A. monophylla*, samples along with standards such as rutin, quercelin, BHA and BHT were determined by the DPPH radical scavenging assay and the results and the results are shown in figure1. The decrease in absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation. The colour change from purple to yellow is visually evident. A lower value of IC_{50} (inhibitory concentration at 50%) indicates a higher antioxidant activity. Generally, the acetone and methanol extracts of all the parts showed significant reduction of DPPH radical. However *Atalantia monophylla* is the highest free radical scavenging activity was exerted by petroleum ether and chloroform extract of leaf and stem (IC_{50} value were 428.78µg/ml; 327.80µg/ml).

3.2 Phosphomolybdenum assay

The total antioxidant capacity of different solvent extracts of leaf and stem of *A. monophylla* was analysed and shown in figure 2. Among *A. monophylla* stem showed higher activity in most of its solvents compared to the extracts of leaf. Acetone extract of stem (174.1mg/g extract) have highest phosphomolybdenum reduction compared to other solvent extracts. The lower antioxidants capacities of the different parts of extracts were found to range from 16.33 to 149.6.11mg AAE/g extract.

3.3 Reducing power assay

The reducing power of solvent extracts of *A. monophylla*, leaf and stem extract samples are shown in figures 3 and 4 respectively. A strong reducing power was noted for the samples of *A. monophylla*, methanol extract of leaf and stem (39.1 and 31.2) more over for all the extracts. These observed a dose and time dependent activity which resulted in greater reducing ability.

3.4 Metal chelating activity

The Fe^{+} chelating capacity of different solvent extracts leaf and stem of *A. monophylla* were analysed and shown in figure 5. In *A. monophylla* maximum chelation were observed for the chloroform extract of leaf (3.340g EDTAE/100g) extract. Whereas the least in stem pet ether extract (1.32g EDTAE /100g) compare to leaf. And the leaf and stem extract of acetone (2.682 g ; 2.09g, EDTAE/100g extract).

3.5 Superoxide radical scavenging activity

The results of superoxide anion scavenging of different extracts of leaf and stem of *A. monophylla* was analysed and shown in figure 6, significant activity was shown by *A. monophylla* leaf acetone extract and (99.18%), the lower scavenging activity was noticeable in chloroform extract of stem (80.14%). These results were compared with natural (rutin) and synthetic (BHT) antioxidants.

3.6 FRAP assay

The FRAP assay is determined by the ferric reducing ability of plant crude extracts. The ferric reducing ability of different solvent extracts of leaf and stem of *A. monophylla* were analysed and shown in figure 7. The result of *A. monophylla* shows that the ferric reducing capacity of Acetone leaf extract was much higher (28478.93Mm/g) and least in pet ether (5052.72Mm/g). In stem chloroform extract was higher (15529.68 Mm/g) and least in pet ether (5679.40 Mm/g).

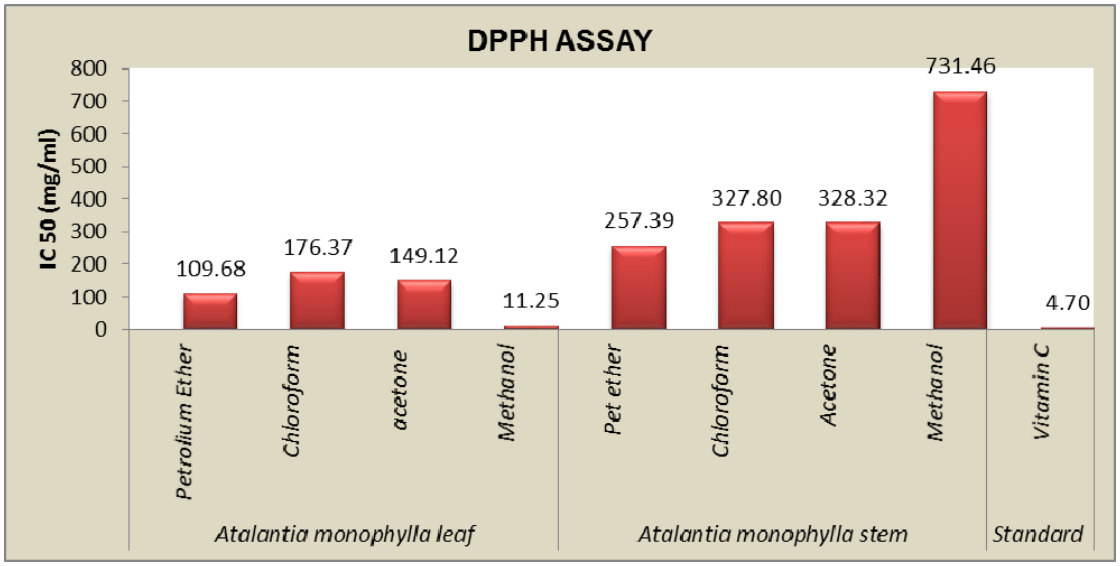

Fig 3.1: DPPH assay of leaf and stem extract of *Atalantia monophylla*.

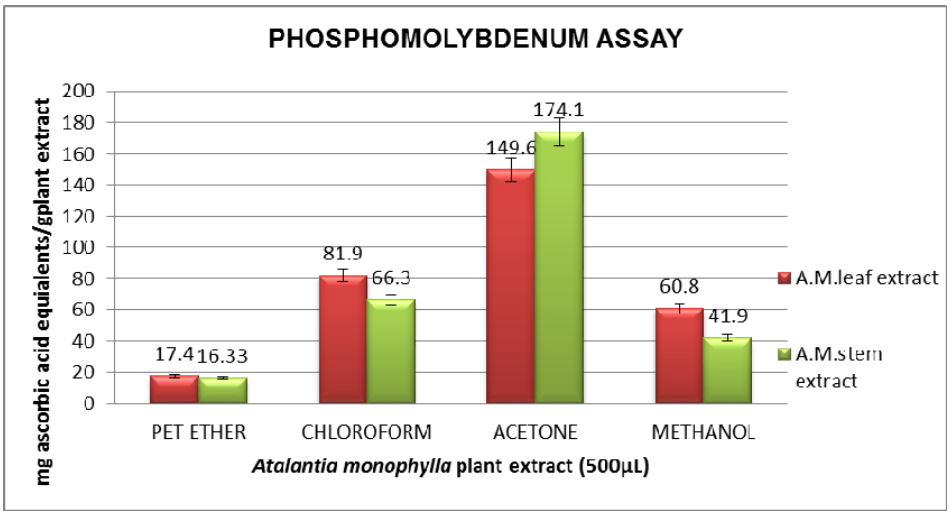

Fig 3.2: Phosphomolybdenum assay of leaf and stem extract of *Atalantia monophylla*.

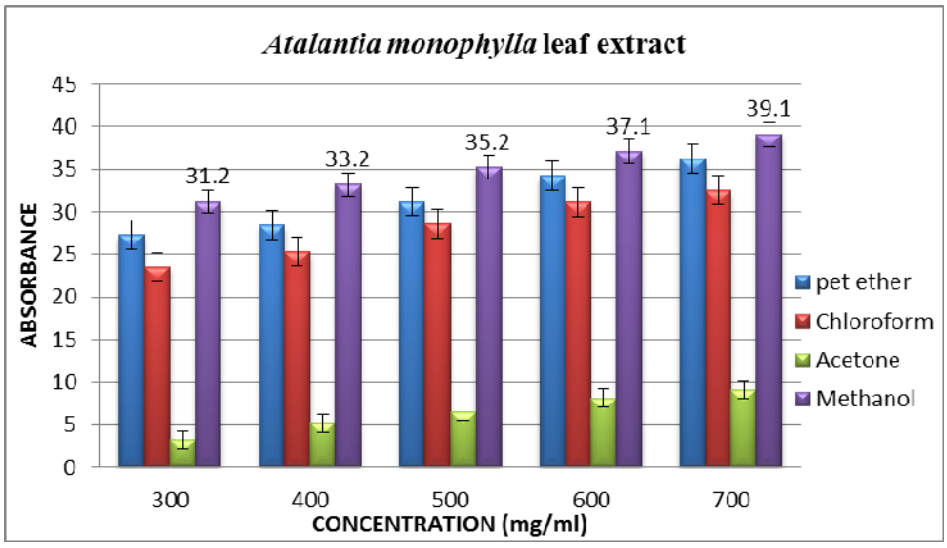

Fig 3.3: Reducing power assay of *Atalantia monophylla* leaf extract

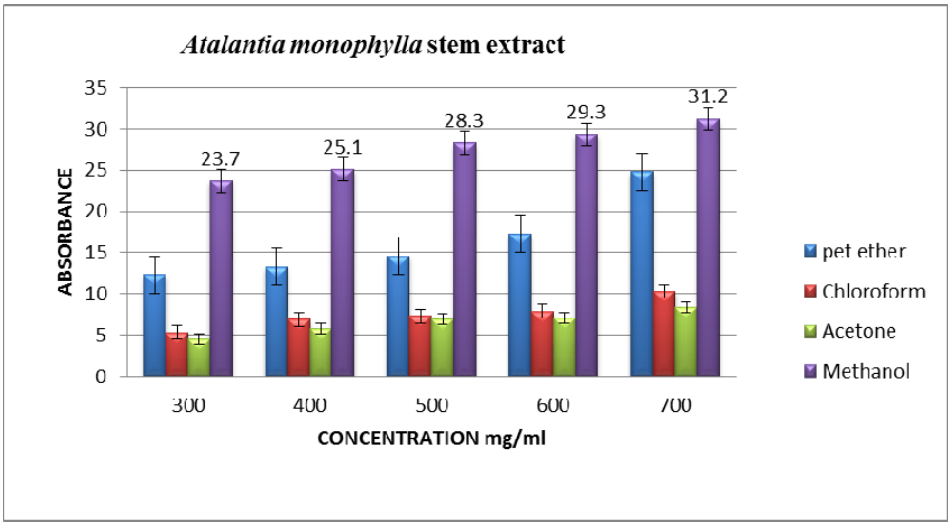

Fig 3.4: Reducing power assay of *Atalantia monophylla* stem extract

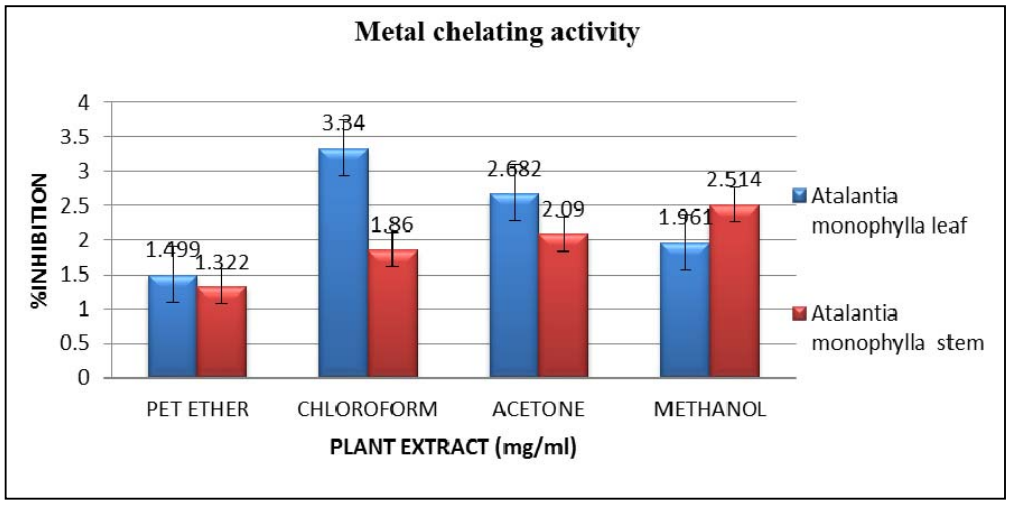

Fig 3.5: Metal chelating activity of leaf and stem extract of *Atalantia monophylla.*

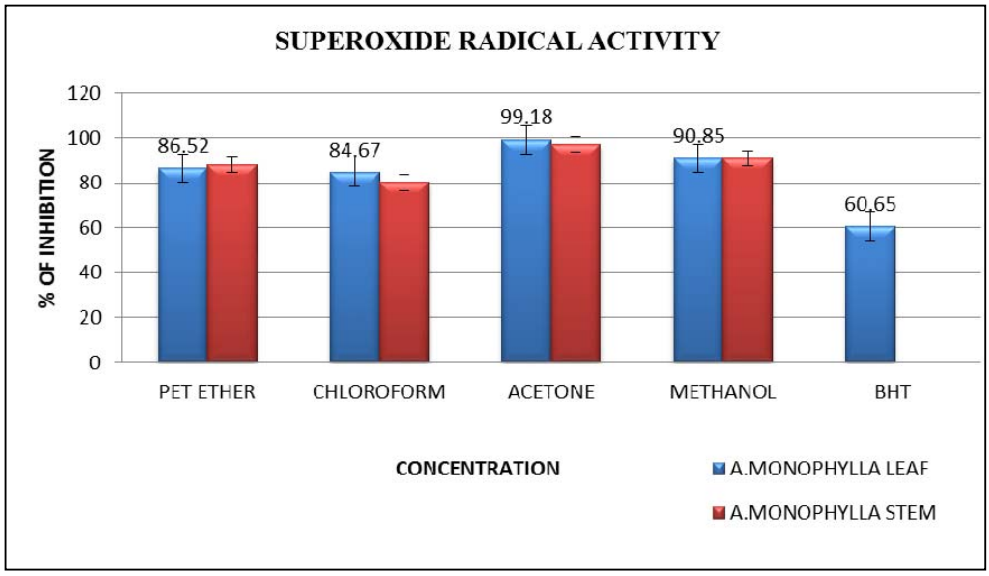

Fig 3.6: Superoxide radical activity of leaf and stem extract of *Atalantia monophylla.*

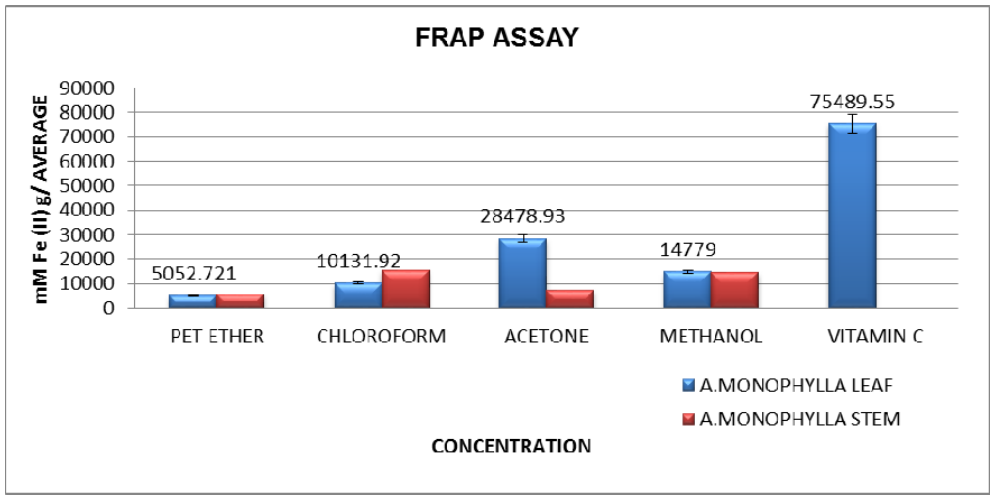

Fig 3.7: FRAP assay of leaf and stem extract of *Atalantia monophylla*,

Plants scavenge free radicals by donating electrons or transferring hydrogen atoms, there by neutralizing free radicals [11].

Prevention of radicals formed by oxidative stress is of utmost importance so as to maintain a healthy system. Several plants are being screened for potent anti-oxidative properties. Secondary metabolites of plants, differing in their structure, mechanism of action and biological properties, possess several medicinal attributes [12]. DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant

assay based on electron-transfer that produces a violet solution in ethanol [13].Reducing power is associated with antioxidant activity and may serve a significant reflection of the antioxidant activity [14]. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants [15]. FRAP assay treats the antioxidants in the sample as a reluctant in a redox-linked colorimetric reaction [16]. However, antioxidant supplements or foods containing antioxidants may be used to help the human body reduce oxidative damage [14].

4. Conclusion

The oil extract from the berries of *Atlantia monophylla* is used externally in chronic rheumatism and paralysis [18]. The oil extract from the leaves and from the barriers are antibacterial and antifungal. A decoction of the leaves is applied to cutaneous affections [18]. The presence of various bioactive compounds confirms the application of *A. monophylla* for various ailments by traditional practitioners. This study provides evidence that *A. monophylla* leaves and stem acetone and methanolic extract have high antioxidant properties including different free-radical scavenging properties like DPPH assay, reducing power assay, superoxide radical (O_2^{*-}) scavenging activity, phosphomolybdenum assay, metal chelating activity, superoxide radical (O_2^{*-}) scavenging activity, and ferric Reducing Antioxidant Power assay. Therefore, this plant *Atalantia monophylla* may have great relevance in the prevention and treatment of diseases in which oxidants or free radicals are implicated.

5. Acknowledgements

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