

Evaluation of *in vitro* antioxidant activity of Ethanolic extracts from *Atalantia monophylla* leaves

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Abstract

Various methods employed in evaluating antioxidant activities of various samples gives varying results depending on the specificity of the free radical or oxidant used as a reactant. This study investigated the antioxidant /radical scavenging properties of the ethanolic extract of *Atalantia monophylla* leaves. Antioxidant capacity of *Atalantia monophylla* leaves was evaluated by measuring the DPPH photometric assay, Superoxide radical scavenging activity, Iron chelating activity, Hydroxyl radical scavenging activity, Nitric oxide radical scavenging activity, Total antioxidant activity, Ferric Reducing Ability Power (FRAP) assay, Total phenol content, Total flavonoids content.

The concentration was 125, 250, 500, 1000 µg/ml. The standard was used according to their methods, All values are expressed as mean ± SEM for three determinations with percentage of activity(±SEM) of IC50 values.the result revealed that DPPH as 461 µg/ml, Superoxide radical scavenging as 306 µg/ml, Iron chelating activity as 480 µg/ml, Hydroxyl radical scavenging as 414 µg/ml, Nitric oxide radical scavenging as 530 µg/ml, Total antioxidant as 399 µg/ml, FRAP as 339 µg/ml and the total phenolic, flavonoids content was 3.181±0.008 and 2.237±0.087. The findings from the study reveal that the antioxidative potentials of *Atalantia monophylla* leaves of ethanolic extract and could serve as free radical inhibitors, phenolic and flavonoids as possibly acting as principal antioxidants

Keywords: *Atalantia monophylla*, antioxidant, phenols, flavonoids, free radical.

INTRODUCTION

Medicinal plants typically contain several different pharmacologically active compounds that may act individually, additively or in synergy to improve health (Azaizeh et al., 2003; Gurib-Fakim, 2006). Bitters for example, are known to stimulate digestion while phenolic compounds could be responsible for anti-inflammatory and anti-oxidative activity of plant extracts (Torrás et al., 2005; Afolayan et al., 2007; Diouf et al., 2009). Medicinal plant parts are commonly rich in phenolic compounds, such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins. These compounds have multiple biological effects including antioxidant activity. In vitro experiments on antioxidant compounds in higher plants (Khalid Rahman., 2007). Free radicals of different forms are constantly generated for specific metabolic requirement and quenched by an efficient antioxidant network in the body. When the generation of these species exceeds the levels of antioxidant mechanism, it leads to oxidative damage of tissues and biomolecules, eventually leading to disease conditions, especially degenerative diseases.

Interest in the search for new natural antioxidants has grown over the past years because reactive oxygen species (ROS) production and oxidative stress have been shown to be linked to diseases such as cancer, cardiovascular disease, osteoporosis, and degenerative diseases. Natural antioxidant substances are believed to play a potential role in interfering with the oxidation process by reacting with free radicals, chelating catalytic metals and scavenging oxygen in biological systems (Halliwell B, & Gutteridge MC., 1984)

MATERIAL AND METHODS

Collection and Identification of *Atalantia monophylla*

Atalantia monophylla leaves were collected from Kanchipuram, Thirunelveli district of Tamil Nadu, India. Taxonomic identification was made from Botanical Survey of Medicinal Plants Unit Siddha, Government of India, Palayamkottai. The leaves parts of *Atalantia monophylla* were dried under shade, segregated, pulverized by a mechanical grinder and passed through a 40 mesh sieve. The powdered plant materials were stored in an airtight container.

Preparation of extracts from *Atalantia monophylla*

The above powdered materials were successively extracted with petroleum ether, benzene and ethanol by hot continuous percolation method in Soxhlet Apparatus for 24 hrs (Harborne, 1984). The extract was concentrated by using a rotary evaporator and subjected to freeze drying in a lyophilizer till dry powder was obtained and the ethanolic extract was taken for further studies.

DPPH photometric assay

A methanol solution of 0.5ml of DPPH (0.4mM) was added to 1ml of the different concentrations of plant extract and allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH in methanol without the extracts served as the negative control. After 30 min, the absorbance was measured at 518nm and converted into percentage radical scavenging activity as follows (Mensor et al., 2001).

$$\text{Scavenging activity (\%)} = \frac{A_{518} \text{ Control} - A_{518} \text{ Sample}}{A_{518} \text{ Control}} \times 100$$

Where A_{518} Control is the absorbance of DPPH radical with methanol; A_{518} Sample is the absorbance of DPPH radical with sample extract/ standard.

Reagents

- ✓ 0.4mM, DPPH.

Superoxide radical scavenging activity

Superoxide radical (O_2^-) was generated from the photo reduction of riboflavin and was detected by nitro blue tetrazolium dye (NBT) reduction method. The assay mixture contained sample with 0.1ml of Nitro blue tetrazolium (1.5 mM NBT) solution, 0.2 ml of EDTA (0.1M EDTA), 0.05 ml riboflavin (0.12 mM) and 2.55 ml of phosphate buffer (0.067 M phosphate buffer). The control tubes were also set up where in DMSO was added instead of sample. The reaction mixture was illuminated for 30 min and the absorbance at 560 nm was measured against the control samples. Ascorbate was used as the reference compound. All the tests were performed in triplicate and the results averaged. The percentage inhibition was calculated by comparing the results of control and test samples (Winterbourne *et al.*, 1975).

Reagents:

- ✓ 1.5mM, NBT
- ✓ .0.1M, EDTA.
- ✓ 0.12mM, Riboflavin.
- ✓ 0.067M, Phosphate buffer.

Iron chelating activity

The method of Benzie and strain (1996) was adopted for the assay. The principle is based on the formation of *O*-Phenanthroline- Fe^{2+} complex and its disruption in the presence of chelating agents. The reaction mixture containing 1 ml of 0.05% *O*-Phenanthroline in methanol, 2 ml ferric chloride (200 μ M) and 2 ml of various concentrations ranging from 10 to 1000 μ g was incubated at room temperature for 10 min and the absorbance of the same was measured at 510 nm. EDTA was used as a classical metal chelator (Benzie and Strain, 1996). The experiment was performed in triplicates.

Reagents

- ✓ *O*-Phenanthroline (0.05%).
- ✓ 200 μ M, Ferric chloride.

Hydroxyl radical scavenging activity

The assay is based on quantification of degradation product of

2-deoxy ribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+} -Ascorbate-EDTA- H_2O_2 system (Fenton reaction). The reaction mixture contained 0.1 ml deoxyribose (2.8mM), 0.1 ml EDTA (0.1 mM), 0.1 ml H_2O_2 (1mM), 0.1 ml Ascorbate (0.1mM), 0.1 ml KH_2PO_4 -KOH buffer, pH 7.4 (20mM) and various concentrations of plant extracts in a final volume of 1 ml. The reaction mixture was incubated for 1 hour at 37°C. Deoxyribose degradation was measured as TBARS and the percentage inhibition was calculated (Elizabeth K and Rao MNA, 1990).

Reagents

- ✓ 2.8mM, Deoxyribose.
- ✓ 01mM, EDTA.
- ✓ 1mM, Hydrogen peroxide.

Nitric oxide radical scavenging activity

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions. The reaction mixture (3ml) containing 2 ml of sodium nitroprusside (10mM), 0.5ml of phosphate buffer saline (1M) were incubated at 25°C for 150 mins. After incubation, 0.5ml of the reaction mixture containing nitrite was pipetted and mixed with 1 ml of sulphanic acid reagent (0.33%) and allowed to stand for 5 min for completing diazotization. Then 1ml of naphthylethylene diamine dihydrochloride (1% NEDA) was added, mixed and allowed to stand for 30mins. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions which can be estimated by the use of Griess Illosvery reaction at 540nm (Garrat, 1964).

Reagents

- ✓ 10mM, Sodium nitroprusside.
- ✓ 1M, Phosphate buffered saline.
- ✓ Sulphanilic acid reagent (0.33%).
- ✓ Naphthylethylene diamine dihydrochloride (1% NEDA).

Total antioxidant activity

The antioxidant activity of the sample was evaluated by the transformation of Mo (VI) to Mo (V) to form phosphomolybdenum complex (Prieto *et al.*, 1999). An aliquot of 0.4 ml of sample solution was combined in a vial with 4 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The vial were capped and incubated in a water bath at 95°C for 90 min. After the samples are cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The antioxidant activity expressed relative to that of ascorbic acid.

Reagents

- ✓ 0.6M Sulfuric acid.
- ✓ 28mM Sodium phosphate.
- ✓ 4mM Ammonium molybdate.

Ferric Reducing Ability Power (FRAP) assay

A modified method of Benzie and Strain (1996) was adopted for the FRAP assay. The stock solutions included 300mM acetate buffer, pH 3.6, 10mM TPTZ (2, 4, 6-tripyridyl-S-triazine) solution in 40mM HCl and 20mM $FeCl_3 \cdot 6H_2O$. The fresh working solution was prepared by mixing 25ml acetate buffer, 2.5ml TPTZ and 2.5ml $FeCl_3 \cdot 6H_2O$. The temperature of the solution raised to 37°C before using. Plant extracts (0.15 ml) were allowed to react with 2.85 ml of FRAP solution for 30 min in the dark condition. Readings of the colored product (Ferrous tripyridyltriazine complex) were taken at 593nm. The standard curve was linear between 200 and 1000 μ M $FeSO_4$. Results are expressed in μ M (Fe (II) /g dry mass and compared with that of ascorbic acid (Benzie and Strain, 1996).

Reagents

- ✓ 300mM acetate buffer, pH 3.6.

- ✓ 10mM TPTZ (2, 4, 6-tripyridyl-S-triazine) solution.
- ✓ 40mM HCl.
- ✓ 20 mM FeCl₃.6H₂O.

Total phenol content

2.5ml of various concentration extracts added with 0.5 ml of Folin's phenol reagent and 2ml of sodium carbonate (20%). The reaction mixture was kept in boiling water bath for 1 min. the absorbance was measured at 650 nm in a spectrophotometer (Mallick and Singh, 1980).

Reagents

- ✓ 80% ethanol.
- ✓ Sodium carbonates (20%).

Total flavonoids content

0.5ml of various concentrations of extracts added with 4ml of the vanillin reagent (1% vanillin in 70% conc. H₂SO₄) and kept in a boiling water bath for 15 mins. The absorbance was read at 360 nm. A standard was run by using catechol (110 µg/ml) (Cameron et al., 1943).

Reagents

- ✓ 1% Vanillin in 70% conc. H₂SO₄.

RESULT AND DISCUSSION

DPPH scavenging activity

DPPH is a stable free radical at room temperature often used to evaluate the antioxidant activity of several natural compounds. The reduction capacity of DPPH radicals is determined by the decrease in its absorbance at 517nm, which is induced by antioxidants. The percentage of DPPH radical scavenging activity of ethanolic extract of *Atalantia monophylla* leaves is presented in Table 1 The ethanolic extract of *Atalantia monophylla* leaves exhibited a maximum DPPH scavenging activity of 65.80% at 1000 µg/ml whereas for ascorbate (standard) was found to be 70.13% at 1000 µg/ml. The IC₅₀ of the ethanolic extract of *Atalantia monophylla* leaves and ascorbate were found to be 461µg/ml and 463µg/ml respectively.

Table 1 Effect of ethanolic extract of *Atalantia monophylla* leaves on DPPH assay

S.No	Concentration (µg/ml)	% of activity(±SEM)*	
		Ethanol extract of	Standard
1	125	16.15±0.03	19.40±0.05
2	250	38.41±0.25	36.59±0.08
3	500	54.71±0.05	50.21±0.03
4	1000	65.80±0.09	70.13±0.05
		IC₅₀= 461µg/ml	IC₅₀=463µg/ml

*All values are expressed as mean ± SEM for three determinations

Superoxide anion scavenging activity: Superoxide is a highly reactive molecule that reacts with various substances produced through metabolic processes. Superoxide dismutase enzymes present in aerobic and anaerobic organisms catalyses the breakdown of superoxide radical (Shirwaikar and Punitha, 2007).

Percentage scavenging of superoxide anion examined at different concentrations of ethanolic extract of *Atalantia*

monophylla leaves (125, 250, 500, 1000 µg/ml) is depicted in Table 2

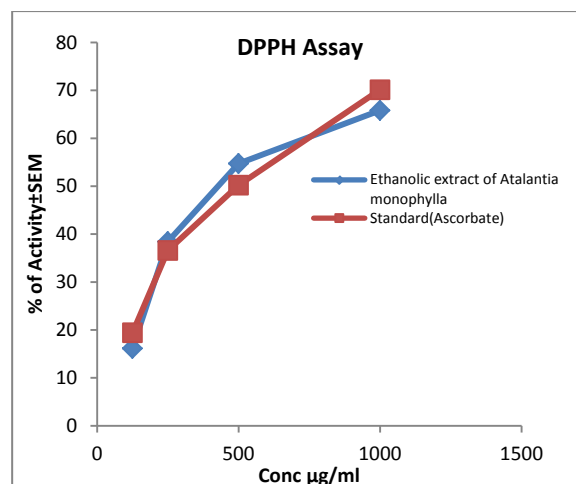


Figure 1 Effect of ethanolic extract of *Atalantia monophylla* leaves on DPPH assay

The percentage scavenging of superoxide radical surged with the enhanced concentration of plant extracts. The maximum scavenging activity of plant extract and Quercetin at 1000 µg/ml was found to be 68.37% and 63.55% respectively. Superoxide scavenging ability of plant extract might primarily be due to the presence of flavanoids.

The IC₅₀ value of plant extract and Quercetin was recorded as 306µg/ml and 313µg/ml respectively.

Table 2 Effect of ethanol extract of *Atalantia monophylla* leaves on Superoxide anion scavenging activity

S.No	Concentration (µg/ml)	% of activity(±SEM)*	
		Ethanol extract of	Standard
1	125	33.3±0.04	35.51±0.28
2	250	48.66±0.10	47.57±0.24
3	500	60.61±0.31	61.79±0.13
4	1000	68.37±0.17	63.55±0.11
		IC₅₀=306µg/ml	IC₅₀=313µg/ml

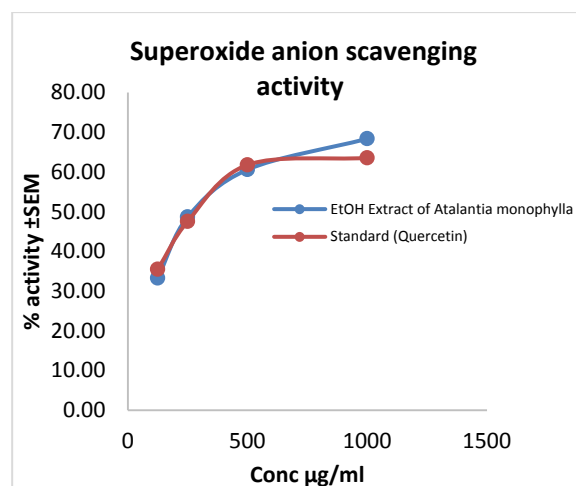


Figure 2 Effect of ethanol extract of *Atalantia monophylla* leaves on Superoxide anion scavenging activity

Iron chelating activity

Iron is essential for life because it is required for transportation of oxygen, respiration and activity of many enzymes. However, iron is an extremely reactive metal and catalyzes oxidative changes in lipids, proteins and other cellular components (St Angelo, 1992 and Smith *et al.*, 1992).

It causes lipid peroxidation through the Fenton and Haber-Weiss reaction (Halliwell and Gutteridge, 1990) and decomposes the lipid hydroxide into peroxy and alkoxy radicals that can perpetuate the chain reactions (Halliwell, 1991). Iron binding capacity of the ethanolic extract of *Atalantia monophylla* leaves and the metal chelator EDTA at various concentrations (125, 250, 500, 1000 µg/ml) was examined and the values are presented in Table 3

The results of the present study shows that the ethanolic extract of *Atalantia monophylla* leaves was found to be most effective and the plant extract possess iron binding capacity which might be due to the presence of polyphenols that averts the cell from free radical damage by reducing of transition metal ions (Duh *et al.*, 1999 and Gordon, 1990). Various plant extracts were proved to be good chelators (Ebrahimzadeh *et al.*, 2008) and correlation exists between phenols, flavonoids and chelating activity

Table 3 Effect of ethanol extract of *Atalantia monophylla* leaves on Iron-chelating method

S.No	Concentration (µg/ml)	% of activity(±SEM)*	
		Ethanol extract of	Standard
1	125	14.55±0.40	16.59±0.18
2	250	36.74±0.17	44.73±0.12
3	500	54.44±0.30	56.41±0.33
4	1000	65.54±0.11	64.38±0.23
		IC₅₀=480µg/ml	IC₅₀=423µg/ml

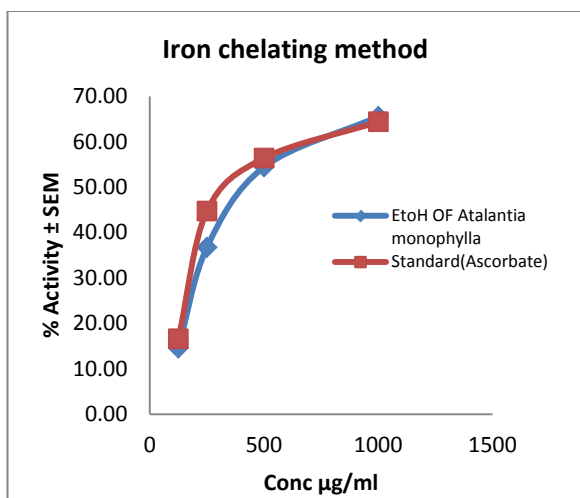


Figure 3 Effect of ethanol extract of *Atalantia monophylla* leaves on Iron-chelating method

Hydroxyl radical scavenging activity

The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins (Spencer *et al.*, 1994). The percentage of Hydroxyl radical scavenging activity of ethanolic extract of *Atalantia monophylla* leaves is presented in Table 4

The ethanolic extract of *Atalantia monophylla* leaves was exhibited a maximum Hydroxyl radical scavenging activity of 67.41 % at 1000 µg/ml whereas for ascorbate (standard) was found to be 58.46 % at 1000 µg/ml. The IC₅₀ values of the ethanolic extract of *Atalantia monophylla* leaves and ascorbate were found to be 414µg/ml and 474µg/ml respectively

Table 4 Effect of ethanol extract of *Atalantia monophylla* leaves on Hydroxyl radical scavenging activity

S.No	Concentration (µg/ml)	% of activity(±SEM)*	
		Ethanol extract of	Standard
1	125	14.72±0.27	24.58±0.44
2	250	36.59±0.29	30.63±0.18
3	500	64.39±0.34	60.60±0.28
4	1000	67.41±0.17	58.46±0.17
		IC₅₀=414µg/ml	IC₅₀=474µg/ml

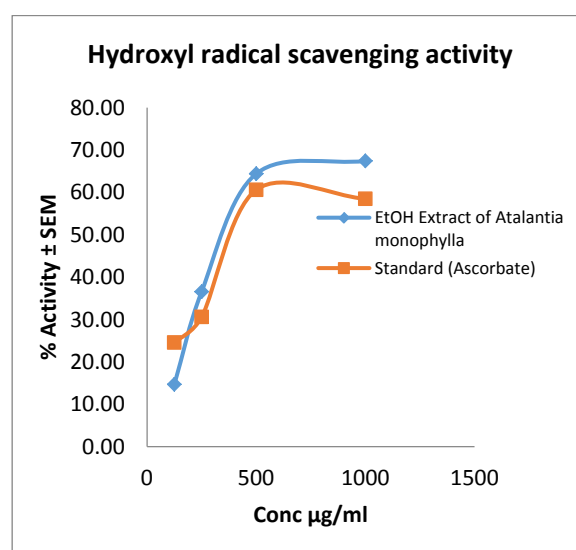


Figure 4 Effect of ethanol extract of *Atalantia monophylla* leaves on Hydroxyl radical scavenging activity

Nitric oxide scavenging activity

Nitric oxide is a diffusible free radical which is an important molecule in diverse biological systems (Hagerman *et al.*, 1998). The reduction of nitric oxide radical by the ethanolic extract of *Atalantia monophylla* leaves and ascorbate was noted to be concentration dependent and is illustrated in Table 5

The maximum scavenging activity of ethanolic extract of *Atalantia monophylla* leaves and ascorbate at 1000 µg/ml were found to be 64.58 % and 56.39% respectively. The IC₅₀ values of plant extract and ascorbate were recorded as 530µg/ml and 475µg/ml respectively.

Table 5 Effect of ethanolic extract of *Atalantia monophylla* leaves on Nitric oxide radical scavenging activity

S.No	Concentration (µg/ml)	% of activity(±SEM)*	
		Ethanol extract of	Standard
1	125	23.70±0.27	25.67±0.21
2	250	29.70±0.12	30.53±0.27
3	500	48.40±0.25	61.52±0.27
4	1000	64.58±0.18	56.39±0.37
		IC₅₀=530µg/ml	IC₅₀=475µg/ml

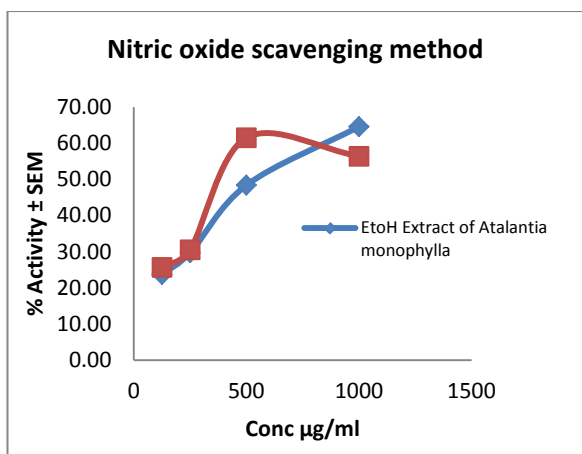


Figure 5 Effect of ethanolic extract of *Atalantia monophylla* leaves on Nitric oxide radical scavenging activity

Total antioxidant activity (Phosphomolybdic acid method)

The percentage of total antioxidant activity of ethanolic extract of *Atalantia monophylla* leaves is presented in **Table 6**. The ethanolic extract of *Atalantia monophylla* leaves exhibited a maximum total antioxidant activity of 60.44% at 1000 µg/ml, whereas for ascorbate (standard) was found to be 57.70% at 1000 µg/ml.

The IC₅₀ of the ethanolic extract of *Atalantia monophylla* leaves and ascorbate were found to be 399 µg/ml and 441 µg/ml respectively.

Table 6 Total antioxidant activity of Ethanol extract of *Atalantia monophylla* leaves

S.No	Concentration (µg/ml)	% of activity (±SEM)*	
		Ethanol extract	Standard
1	125	24.76 ± 0.22	27.55 ± 0.30
2	250	45.38 ± 0.28	32.59 ± 0.27
3	500	57.46 ± 0.22	62.56 ± 0.38
4	1000	60.44 ± 0.11	57.70 ± 0.22
		IC ₅₀ = 399 µg/ml	IC ₅₀ = 441 µg/ml

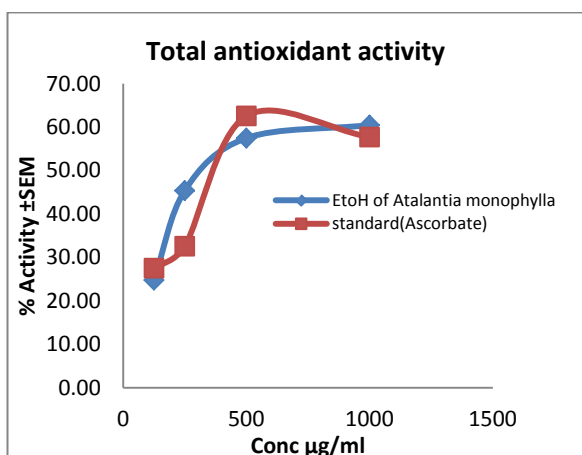


Figure 6 Effect of ethanolic extract of *Atalantia monophylla* leaves on total antioxidant activity

FRAP assay

The antioxidant potential of *Atalantia monophylla* leaves was ascertained from FRAP assay based on their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II). The

reducing ability of the ethanol extract of *Atalantia monophylla* leaves and ascorbate at various concentrations (125, 250, 500, 1000 µg/ml) was examined and the values are presented in **Table 7**.

The maximum reducing ability at 1000 µg/ml for ethanolic extract of *Atalantia monophylla* leaves and ascorbate were found to be 65.52% and 58.31% respectively.

The IC₅₀ values of ethanolic extract and ascorbate were recorded as 339 µg/ml and 450 µg/ml respectively.

Table 7 FRAP assay of Ethanol extract of *Atalantia monophylla* leaves

S.No	Concentration (µg/ml)	% of activity (±SEM)*	
		Ethanol extract of	Standard
1	125	31.69 ± 0.27	26.60 ± 0.17
2	250	48.53 ± 0.17	32.32 ± 0.21
3	500	56.66 ± 0.29	61.48 ± 0.26
4	1000	65.52 ± 0.22	58.31 ± 0.17
		IC ₅₀ = 339 µg/ml	IC ₅₀ = 450 µg/ml

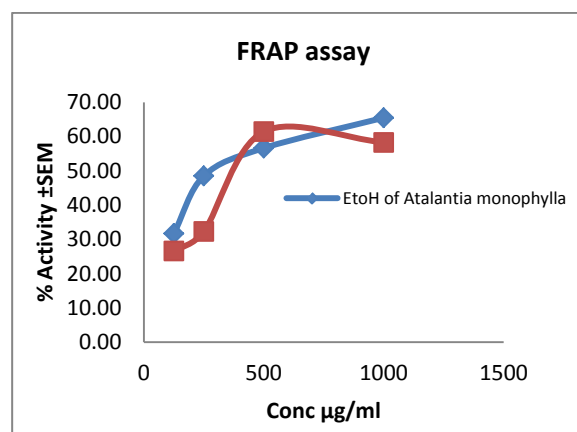


Figure 7 FRAP assay of Ethanol extract of *Atalantia monophylla* leaves

Total phenol content

Phenolic compounds are known as powerful chain breaking antioxidants. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups (Hatano, 1989). The total amount of phenolic content of ethanolic extract of *Atalantia monophylla* leaves is presented in **Table 8**.

Table 8 The total Phenolic content of ethanolic extract of *Atalantia monophylla* leaves

S.No	Extracts	Total phenol content
1	Ethanolic extract of	3.181 ± 0.008

*All values are expressed as mean ± SEM for three determinations

Total flavonoids content

Flavonoids present in food of plant origin are also potential antioxidants (Salah et al., 1995 and Van Acker et al., 1996). Most beneficial effects of flavonoids are attributed to their antioxidant and chelating abilities (Hassig, 1999). The total amount of flavonoids content of ethanolic extract of *Atalantia monophylla* leaves is presented in **Table 9**.

Table 9 Total Flavonoids content of ethanolic extract of *Atalantia monophylla* leaves

S.No	Extracts	Total flavonoids content
1.	Ethanolic extract of	2.231 ± 0.087

*All values are expressed as mean ± SEM for three determinations

CONCLUSION

The ethanolic extract of *Atalantia monophylla* leaves was able to reduce the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) to yellow colored diphenylpicrylhydrazine and showed better antioxidant activity with IC₅₀ value of 461 µg/ml. Superoxide anion scavenging activity methods shows activity with an IC₅₀ value of 306 µg/ml and in hydroxyl radical scavenging activity showed antioxidant activity with IC₅₀ value of 414 µg/ml. Iron chelating activity showed better antioxidant activity with IC₅₀ value of 480 µg/ml. Nitric oxide scavenging activity showed effective antioxidant activity with IC₅₀ value of 530 µg/ml. The total antioxidant activity of ethanol extract of *Atalantia monophylla* leaves was determined by phosphomolybdic acid method, it showed antioxidant activity with IC₅₀ value of 399 µg/ml. FRAP method radical potential showed effective antioxidant activity with IC₅₀ value of 339 µg/ml. Total phenol content was 3.181 ± 0.008 and respectively the total flavonoids content are 2.237 ± 0.087.

The present study has revealed that the ethanol extract of *Atalantia monophylla* leaves contains substantial amount of phenolics and flavonoids thus, can be inferred that these phenolics and flavonoids are responsible for its marked antioxidant activity as assayed through various *in vitro* models used in this study. This is consistent with several reports that have shown close relationship between total phenolic contents, flavonoids and antioxidative activity of fruits, plants and vegetables (Albayrak et al., 2010, Biju et al., 2014, Baba and Malik, 2015). On the basis of the results obtained in the present study, it was concluded that the ethanolic leaves extracts of this species possess significant antioxidant activity.

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