International Journal of Biology Research
ISSN: 2455-6548; Impact Factor: RJIF 5.22
Received: 05-01-2019; Accepted: 09-02-2019
www.biologyjournal.in
Volume 4; Issue 1; January 2019; Page No. 42-45

Free radical scavenging potential of aqueous hot extract of *Eugenia uniflora* (L.) leaves

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Abstract
Indian medicinal herbs and plants are used since ancient times to treat different diseases and ailments as these natural products exert broad-spectrum actions. The present study was aimed to explore the free radical potential of aqueous hot extract of *Eugenia uniflora* leaves. In vitro assays like DPPH scavenging assay, DMPD scavenging assay, metal chelating ability, FRAP scavenging assay and anti hemolytic assay was carried out using standard procedures. In present investigation, aqueous extract of *Eugenia uniflora* leaves exhibited high antioxidant properties to scavenge free radicals thus protecting the biomolecules from damage. These results emphasized the benefit of the extract and thus augmented the urge of in vivo studies to further confirm the beneficial effect of these extract.

Keywords: antioxidant, free radicals, scavenging, aqueous extract, *Eugenia uniflora*, IC_{50} (Inhibitory concentration)

Introduction
Free radicals are molecules with unpaired electrons produced due to incomplete decomposition of oxygen inside our body. In low concentration these highly reactive oxygen species defense the body from infectious agents and play roles in cellular signaling. Over production of ROS decreases the function of lipids, proteins, DNA. Lipid peroxidation is the auto-oxidation process initiated by attack of free radicals in phospholipids or PUFA. Studies have reported that there are some speculation that generation of free radicals during some physiological conditions in the body is the major reason for the cellular changes and development of diseases. Studies have shown that plant derived antioxidants helps to scavenge these free radicals and modulate oxidative stress related degenerative diseases [1, 2].
Medicinal plants are widely used for the treatment of cellular and metabolic diseases like diabetes, obesity, cancer etc. Higher intake of antioxidant rich foods is associated with decreased risk of chronic diseases [3]. Antioxidants are free radical scavengers that neutralize free radical preventing them from causing cellular damage. Our body makes antioxidants called endogenous antioxidants and also body relies on antioxidants from external sources (exogenous antioxidants) primarily from diet. Dietary supplements supplies dietary antioxidants. Antioxidants have singlet oxygen quenching properties. The main characteristic of an antioxidant is its ability to trap free radicals. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases [4].
Eugenia uniflora is an evergreen shrub belongs to the family myrtaceae. It possesses spreading branches with aromatic foliage. Young stems are covered with red hairs. The leaves are yellowish brown in colour when young and turns to deep glossy green when matures. Flowers have white petals. Fruits of *Eugenia uniflora* are fleshy, orange red berries. The fruits of *Eugenia uniflora* can be eaten fresh after ripening. It is used for preparation of jams. Fruits are rich in Vit.C and pectin. One fruit of *Eugenia uniflora* a day provides all the Vit.C a body requires. The leaves of *Eugenia uniflora* are good substitute for tea. Leaves of *Eugenia uniflora* have cryoprotective effects. Phytochemicals present in leaves has antioxidant and chelating activities which has the potential to protect against the toxic effects of heavy metals like mercury. Study reports that essential oils from leaf extract of *Eugenia uniflora* has larvicidal activity against *Aedes aegypti* a vector of dengue and yellow fever. Essential oil exhibits antioxidant, antibacterial and antifungal properties. Essential oil also has antidepressant effect and also reduces lipid peroxidation.
On the basis of literature review, and considering the pharmacological properties of *Eugenia uniflora* leaves, present study aims to explore the radical scavenging potential of aqueous hot extract of *Eugenia uniflora* leaves

Materials and Methods
Plant Collection and Authentication
Fresh leaves of *Eugenia uniflora* (Linn), Family- Myrtaceae, were collected from Wayanad district, Kerala during the month of April 2014. Taxonomic authentication was done by Dr. V.S Ramachandran, Taxonomist, Department of Botany, Bharathiar University, Coimbatore, Tamil Nadu, India.

Sample Processing
The leaves were washed, shade dried at room temperature and powered in a mixer grinder. Hot Water Decoction: 10g of the powdered sample was dissolved in 100ml of distilled water which was boiled for one and half hours and filtered. The decoction was stored at 4 °C for further usage.

Chemicals
All chemicals used for the evaluation were in analytical grade and obtained from either Sigma–Aldrich or Merck.

Free radical scavenging assays in aqueous hot extract of *Eugenia uniflora* (L.) leaves
In present study radical scavenging assays were carried out in aqueous extract of *Eugenia uniflora* leaves based on standard procedures. The standard procedures used for free radical scavenging assay of aqueous extract of *Eugenia uniflora* leaves is presented in table 1.

Table 1: Radical scavenging assays in aqueous hot extract of *Eugenia uniflora* (L.) leaves

Parameters	References
DMPD scavenging assay	Fogliano *et al*., 1999 [5]
DPPH scavenging assay	Mensor *et al*., 2001[6]
Metal chelating ability	Dinis *et al*., 1994 [7]
FRAP scavenging assay	Benzie and Strain, 1996 [8]
Anti hemolytic assay	Naim *et al*., 1976 [9]

Percentage inhibition and IC_{50} value were calculated using standard formula. IC_{50} is the half maximal inhibitory concentration is the measure of effectiveness of a substance in inhibiting a specific biological function. Graphical representation was done for better understanding.

Statistical Analysis

All the analyses were performed in triplicate and the results were statistically analyzed and expressed as mean (n=3) ± standard deviation.

The scavenging activity was calculated as follows:

$$\% \text{ of Scavenging} = A_{control} - A_{test} / A_{control} \text{ X } 100$$

Where

A control=Absorbance of the control in the absence of sample

A sample=Absorbance of sample

Results and Discussion

1. DMPD scavenging assay

Aqueous extract of *Eugenia uniflora* leaves were analysed for the activity to scavenge DMPD (Dimethyl-4-phenylene diamine) radicals. Antioxidant compounds which are the hydrogen donors to DMPD quench the colour of DMPD solution. End point of the reaction is the measure of the efficacy of the antioxidant. This assay is more suitable for hydrophilic antioxidants. The decolourisation of DMPD solution was found to increase with the increase in concentration of the extract. The result obtained for the investigation is presented in table 2 and figure 1. The result was compared with standard rutin. The IC_{50} value of the extract was calculated as 50.03 ± 0.12 µg/ml.

Table 2: DMPD scavenging assay

Concentration(µg/ml)	% Inhibition of standard	% Inhibition of extract	IC_{50} value of the extract (µg/ml)
20	24.89 ± 0.53	18.90 ± 0.24	50.03 ± 0.12
40	42.10 ± 0.16	37.11 ± 0.45	
60	66.50 ± 0.23	57.98 ± 0.67	
80	79.81 ± 0.45	75.72 ± 0.56	
100	96.87 ± 0.98	94.20 ± 0.19	

Values are expressed as mean ± SD of three samples, Standard-Rutin, Extract- Aqueous extract of *Eugenia uniflora* leaves

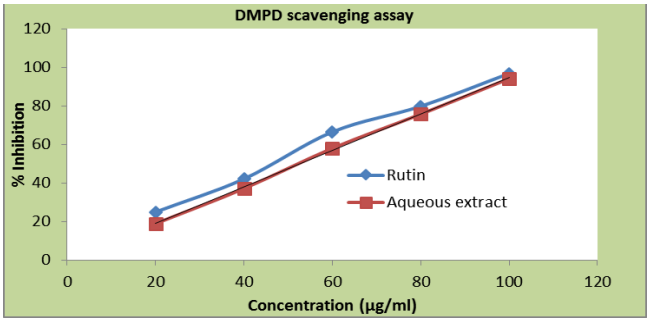

Fig 1: DMPD scavenging assay

2. DPPH scavenging assay

2, 2-diphenyl-1-picryl hydrazyl is one of the stable free radical to determine the radical scavenging potential of natural compounds. DPPH scavenging assay is very simple and used as screening of antioxidant properties in botanical extracts. The advantage of this method is broad solvent compatibility with aqueous plar and non-polar solvents. Usually DPPH is absorbed at 517 nm. Upon reduction with an antioxidant, the absorption decreases due to the formation of non radical form. Thus the radical scavenging activity of an antioxidant can be monitored by the decrease in the absorbance of DPPH solution.

The result obtained for DPPH radical scavenging activity of aqueous extract of *Eugenia uniflora* is presented in table 3 and figure 2. The result was compared with standard ascorbic acid. The IC_{50} value of the extract was calculated as 50.10 ± 0.89 µg/ml.

Table 3: DPPH scavenging assay

Concentration (µg/ml)	% Inhibition of standard	% Inhibition of extract	IC_{50} value of the extract (µg/ml)
20	14.67 ± 0.22	10.50 ± 0.20	50.10 ± 0.89
40	28.18 ± 0.32	21.76 ± 0.55	
60	36.87 ± 0.34	32.12 ± 0.67	
80	47.21 ± 0.78	43.70 ± 0.32	
100	59.21 ± 0.45	53.45 ± 0.11	

Values are expressed as mean ± SD of three samples, Standard-Ascorbic acid, Extract- Aqueous extract of *Eugenia uniflora* leaves

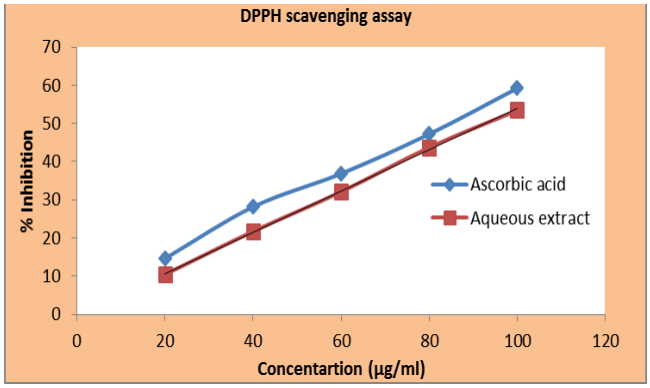

Fig 2: DPPH scavenging assay

3. Metal chelating ability of aqueous extract

Iron is one of the essential mineral normally. Excess of iron may result in injury of cells. If iron undergoes fenton reaction, it may form reactive hydroxyl radical and result in oxidative stress. The most important antioxidant activity of antioxidant is the ability to chelate transition metals which has the ability to catalyze H_2O_2 decomposition and fenton type reactions. So it is important to screen the iron chelating ability of the extract. Aqueous extract of *Eugenia uniflora* leaves showed maximum chelating ability. The result obtained is presented in table 4 and figure 3. The result is compared with standard ascorbic acid. The IC_{50} value of the extract was calculated as 50.73 ± 0.66 µg/ml. From the result it is clear that aqueous extract is able to play protective role against oxidative damage by eliminating Fe (II) ion that may catalyze fenton type reaction or metal catalyzed hydroperoxide decomposition reaction.

Table 4: Metal chelating ability of aqueous extract

Concentration (µg/ml)	% Inhibition of standard	% Inhibition of extract	IC_{50} value of the extract (µg/ml)
20	22.78 ± 0.57	13.31 ± 0.11	50.73 ± 0.66
40	37.12 ± 0.14	27.09 ± 0.16	
60	45.51 ± 0.67	41.87 ± 0.76	
80	60.18 ± 0.25	55.78 ± 036	
100	79.32 ± 0.18	69.12 ± 0.29	

Values are expressed as mean ± SD of three samples, Standard- Ascorbic acid, Extract- Aqueous extract of *Eugenia uniflora* leaves

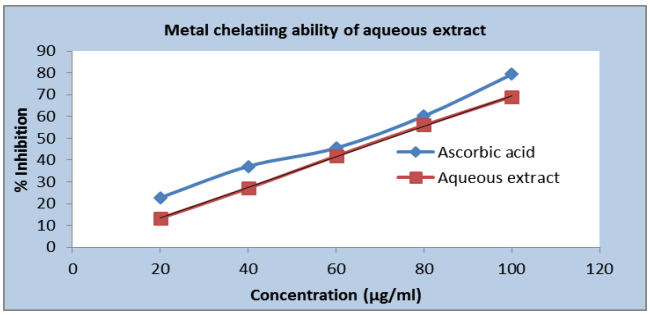

Fig 3: Metal chelating ability of aqueous extract

4. FRAP scavenging assay

Ferric reducing antioxidant power assay is used to determine the antioxidant activity since it is very quick and simple. It is related to the molar concentrations of the antioxidants. One of the disadvantage of this method is FRAP assay does not react with some antioxidants like glutathione. Schafer and Buettner, 2001 stated that FRAP assay can be used to assess antioxidant activity in plants as humans only absorb lonely limited amount of glutathione. High FRAP value indicates higher antioxidant capacity. Frap assay is based on reducing ferric ion. Reduction of ferric ions to ferrous ions causes change in colour which is measured spectrophotometrically.

The result obtained for the scavenging capacity of aqueous extract of *Eugenia uniflora* leaves is presented in table 5 and figure 4. The result was compared with standard ascorbic acid. The IC_{50} value of the extract was calculated as 66.38 ± 0.66 µg/ml.

Table 5: FRAP scavenging assay

Concentration (µg/ml)	% Inhibition of standard	% Inhibition of extract	IC_{50} value of the extract (µg/ml)
20	18.67 ± 0.56	14.87 ± 0.74	66.38 ± 0.66
40	40.09 ± 0.05	30.45 ± 0.33	
60	52.87 ± 0.17	45.08 ± 0.14	
80	62.98 ± 0.25	59.89 ± 0.37	
100	84.89 ± 0.18	75.67 ± 0.29	

Values are expressed as mean ± SD of three samples, Standard- Ascorbic acid, Extract- Aqueous extract of *Eugenia uniflora* leaves

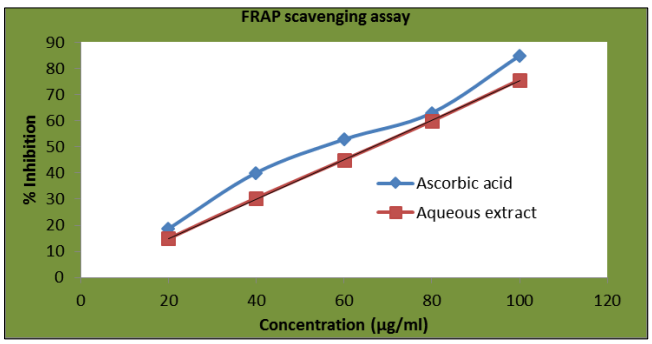

Fig 4: FRAP scavenging assay

5. Anti hemolytic assay

Anti hemolytic assay was assessed by spectrophotometric method. For this, 5 ml of blood was taken from healthy individuals and centrifuged for 2 minutes at 1500 rpm. Pellet of blood was washed in sterile phosphate buffered saline solution with pH 7.2. Then the pellet was re-suspended in normal 0.5% saline. Different concentration of extracts was added to 0.5 ml of cell suspension.

Erythrocytes are the major target of free radicals generated by redox reaction and high content of poly unsaturated fatty acids. Aqueous extract of *Eugenia uniflora* exhibited different hemolytic pattern at different concentrations towards human erythrocytes. Result indicated that the aqueous extract exhibits hemolytic activity showing the anti hemolytic behavior of the extract. Activity of the extract was found to increase with increase in concentration of the extract. Anti hemolytic activity of the extract is due to the presence of reported phytochemicals in the extract. From the report it is concluded that the antioxidants present in the extract interacted with the class of lipids present in the outer monolayer of human erythrocyte showing the protective effect of the extract.

The obtained result is presented in table 6 and figure 5. The result was compared with standard ascorbic acid. The IC_{50} value of the extract was calculated as 53.77 ± 0.22 µg/ml.

Table 6: Anti-hemolytic assay

Concentration (µg/ml)	% Inhibition of standard	% Inhibition of extract	IC_{50} value of the extract (µg/ml)
20	19.98 ± 0.27	16.87 ± 0.89	53.77 ± 0.22
40	37.67 ± 0.18	32.23 ± 0.56	
60	49.32 ± 0.14	48.91 ± 0.22	
80	68.12 ± 0.09	63.10 ± 0.18	
100	92.10 ± 0.47	89.98 ± 0.39	

Values are expressed as mean ± SD of three samples, Standard- Ascorbic acid, Extract- Aqueous extract of *Eugenia uniflora* leaves

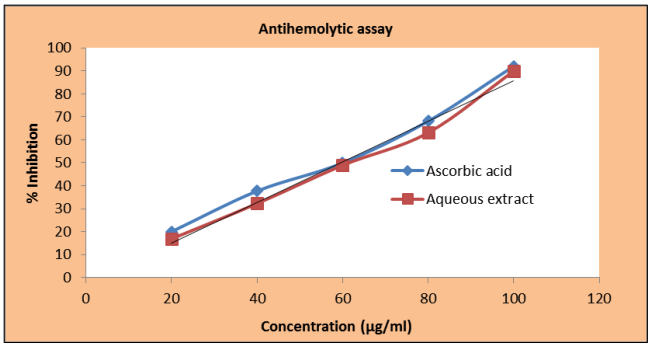

Fig 5: Anti hemolytic assay

Conclusion

Oxidation mechanism and role of free radicals have gained attention since few decades**.** Reactive oxygen species production takes place during normal cell metabolism. Excess of ROS leads to oxidative stress which results in oxidative DNA damage which is implicated in the pathogenesis of chronic diseases like cancer, diabetes, cardiovascular diseases, AIDS etc. Even though living species have efficient antioxidant defense system to quench the radicals, search for natural antioxidants are alternative to defense the free radicals. A diet rich in edible antioxidants like fresh vegetables and fruits is highly recommended to help humans to protect themselves against the attack of free radicals and oxidative stress.

In recent years herbal drugs which have free radical scavenging potential have gained importance in the treatment of several chronic diseases. Medicinal plants rich in phenols, flavonoids, tannins, coumarins etc. have multiple biological effects. The systemic literature collection pertaining to the current investigation indicates that the secondary metabolites especially flavonoids act as primary antioxidant to quench the free radicals.

In present investigation, aqueous extract of *Eugenia uniflora* leaves exhibited high antioxidant properties to scavenge free radicals thus protecting the biomolecules from damage. The most important activity of an antioxidant is the ability to chelate transition metals; present investigation clearly shows the capacity of the extract to chelate the transition metals thus protecting the cells from injury. As described before, erythrocytes are the major target of free radicals and the aqueous extract shows different hemolytic pattern suggesting the extract as an anti-hemolytic agent. Since natural antioxidants are more preferred as foods in present scenario because of its fewer side effects than synthetic medicines, aqueous extract of *Eugenia uniflora* leaves is recommended to be included in daily diet to protect the body against harmful diseases due to its scavenging potential to scavenge harmful free radicals.

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