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Geedhu Daniel
Department of Biochemistry, Kongunadu Arts and Science College (Autonomous), Coimbatore, Tamilnadu, India.

S Krishnakumari
Department of Biochemistry, Kongunadu Arts and Science College (Autonomous), Coimbatore, Tamilnadu, India.

Correspondence
S Krishnakumari
Department of Biochemistry, Kongunadu Arts and Science College (Autonomous), Coimbatore, Tamilnadu, India.

In vitro free radical scavenging activity of aqueous extract of *Eugenia uniflora* (L.) leaves

Geedhu Daniel and S Krishnakumari

Abstract
Medicinal plants form a major source of raw materials for drugs for the prevention and treatment of ailments. Antioxidants play an important role in protecting against damage by reactive oxygen species. The present study was designed to evaluate the potential of aqueous leaf extract of *Eugenia uniflora* as an antioxidant lead by using various in vitro models like superoxide radical scavenging assay, hydroxyl radical scavenging assay and nitric oxide radical scavenging assay using standard procedures. IC50 values were calculated respectively. In all these studies, a significant correlation existed between concentrations of the extract and percentage inhibition of free radicals. These results clearly indicated that leaf extract of *Eugenia uniflora* could be a potential source of natural antioxidant and effective against free radical mediated diseases.

Keywords: Antioxidant, Free radicals, Scavenging, Aqueous extract, *Eugenia uniflora*, IC50 (Inhibitory concentration)

Introduction
Plants are the basis of life on earth and are central to people's livelihoods [1]. In recent times, there is an increasing interest in the role of free radical- mediated damage in the etiology of human diseases. In normal metabolism, the levels of oxidants (i.e. free radicals) and antioxidants in humans are maintained in balance, for sustaining optimal physiological conditions [2]. Overproduction of free radicals in certain conditions can cause an imbalance, leading to oxidative damage to large biomolecules such as lipids, DNA, and proteins [3] and thus leads to a range of chronic diseases, such as cardiovascular disease, neuronal disease, cataracts, and several forms of cancer [4].
It is established that the intake of antioxidant substances reinforces defenses against free radicals. The use of synthetic antioxidants has been limited because of their toxicity [5]. Therefore, it is of great significant and necessity that research focuses on discovering potential natural, effective antioxidants to replace the synthetic ones.
There is extensive evidence to implicate free radicals in the development of degenerative diseases. Oxygen free radicals are formed in tissue cells by various endogenous and exogenous causes such as metabolism, chemicals, and ionizing radiation. Approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals.
All these radicals are known as reactive oxygen species (ROS), exert oxidative stress to the cells. When the generation of ROS overtakes the antioxidant defense of the cells the free radicals start attacking cellular proteins, lipids and carbohydrates leading to the pathogenesis of many disorders including arthritis and connective tissue disorders, liver disorders, neurodegenerative disorders, cardiovascular disorders, diabetes, chronic inflammation, mutagenesis, carcinogenesis and in the process of ageing [6, 7].
Antioxidants provide protection for living organisms from damage caused by uncontrolled production of reactive oxygen species (ROS) and the concomitant lipid peroxidation, protein damage and DNA strand breaking [8]. Current interest is focused on the potential role of antioxidants and antioxidant enzymes in the treatment and prevention of atherosclerosis, heart failure, neurodegenerative disorders, aging, cancer, diabetes mellitus and several others diseases [9]. Antioxidants are added to a variety of foods to prevent or deter free radical induced lipid peroxidation, which is responsible for the development of off-flavors and the undesirable chemical compounds in food [10].

These ROS cause destructive and irreversible damage to the components of a cell, such as lipids, proteins and DNA [11]. Although normal cells possess antioxidant defense systems against ROS in the cells induces diseases such as cancer and aging [12]. ROS are formed and degraded by all aerobic organisms. ROS can readily react with most biomolecules including proteins, lipids, lipoproteins and DNA. Exogenous chemical and endogenous metabolic processes in the human body or in the food system might produce highly reactive oxygen species, which are capable of oxidizing biomolecules, resulting in tissue damage and cell death [13].

When the mechanism of antioxidant protection becomes unbalanced by exogenous and endogenous factors, it results in inflammation, diabetes, genotoxicity, cancer and accelerating aging [14]. Antioxidant supplements or foods containing antioxidants may be used to help the human body reduce oxidative damage. Traditional medicine worldwide is being reevaluated by extensive research on different plant species and their therapeutic principles. Plants produce antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity.

Superoxide anion (O_2), the one-electron reduced form of molecular oxygen, is one of the most representative free radicals. It is a precursor to active free radicals that have the potential of reacting with biological macromolecules and thereby inducing tissue damage [15-17]. In cellular oxidation reactions, superoxide radicals are normally formed first, and their effects can be magnified because they produce other kinds of cell-damaging free radicals and oxidizing agents [15, 16].

Biological systems can produce hydrogen peroxide. Furthermore, hydrogen peroxide can be formed *in vivo* by many oxidizing enzymes such as superoxide dismutase. It can cross membranes and may slowly oxidize a number of compounds. It is used in the respiratory burst of activated phagocytes. It is known that H_2O_2 is toxic and induces cell death in vitro. Hydrogen peroxide can attack many cellular energy-producing systems. For instance, it deactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase.

Nitric oxide (NO) is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and antitumor activities. The toxicity of NO increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion (ONOO-). The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite.

Eugenia uniflora L. (Myrtaceae) is a tropical and subtropical shrub widely distributed in American countries [18]. It is commonly referred to as Pitanga cherry or Brazilian cherry. Regarding their effects on human health, both fruit and leaves are used as folk medicine to treat similar diseases, although the leaves show the advantage of being perennial and continuously available, while the fruit are available during a short period of the year [19]. The fresh or dried leaves have been used empirically as medicine, since the 15th century, for treating inflammatory and stomach diseases, rheumatism, fever, and hypertension [20, 21]. Some studies have confirmed that *Eugenia uniflora* possesses anti-inflammatory, antimicrobial, and antifungal properties [20, 22-24]. These benefits are usually attributed to the presence of many secondary metabolites present in the leaves, which includes many volatile terpenoid oils, flavonoids, and condensed and hydrolysable tannins, leucoanthocyanidins, and steroids and/or triterpenoids [25]. Flavonoids and phenolic compounds are widely distributed in plants which have been reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic etc [26]. In this view, present study is to evaluate radical scavenging activity of aqueous 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.

1) Superoxide radical scavenging assay

Superoxide scavenging was carried out using the standard method given by [27]. The auto oxidation of pyrogallol releases superoxide radicals. The inhibition of auto oxidation of pyrogallol is measured as a measure of scavenging of superoxide radicals. The reagents used are 10mM pyrogallol and 50mM Tris HCl (pH 8.2). Superoxide radical O_2^- scavenging capacity of leaf extract was examined by a pyrogallol autooxidation system. The reaction mixture contained 70µl 10mM pyrogallol, 4.5mL 50mM Tris Hcl (pH 8.2) and 0.5mL various concentrations of malt samples. The absorbance at 325nm was recorded immediately. Ascorbic acid was taken as standard antioxidant.

The scavenging activity of superoxide radical effect was calculated as follows,

$$\% \text{ of Scavenging} = \frac{A\text{control} - A\text{test}}{A_{control}} \times 100$$

Where:

$A_{control}$=Absorbance of the control in the absence of sample

A_{sample}=Absorbance of sample

2) Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of leaf extract was assayed by the method of [28]. The reaction mixture 3.0 ml contained 1.0 ml of 1.5 mM FeSO4, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate and varied concentrations of the extracts. After incubation for an hour at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm against blank sample and ascorbic acid is uded as positive control. The scavenging activity of hydroxyl radical effect was calculated as follows,

$$\% \text{ of Scavenging} = \frac{\text{Acontrol} - A_{test}}{A_{control}} \times 100$$

Where:

$A_{control}$=Absorbance of the control in the absence of sample

A_{sample}=Absorbance of sample

3) Nitric oxide scavenging activity

Nitric oxide radical scavenging activity of leaf extract was assayed by the method of[29]. Nitric oxide, generated by sodium nitrite, reacts with the Griess reagent to form a chromophore that is measured at 520nm. Absorbance is directly propotional to the amount of nitric oxide present. The reagents used are, 1 mM Sodium nitrite, 0.2 M Citrate buffer, pH 4.2, Griess reagent: 1% sulfanilamide, 1% naphthyl ethylene diamine dihydrochloride in methanol solution containing 30% acetic acid. Various concentrations of the extract were mixed with 1.0 ml of 1 mM sodium nitrite. Then the mixture was added to 8 ml of 0.2 M citrate buffer, pH 4.2. The mixture was incubated for 1 hour at 37 °C. 1.0 ml of the solution were withdrawn and added to
2.0 ml of 2% acetic acid and 0.4 ml of Griess reagent. The mixture was incubated at room temperature for 15 min and the absorbance was measured at 520 nm. Ascorbic was taken as standard antioxidant.

$$\% \text{ of Scavenging} = \frac{\text{Acontrol} - \text{Atest}}{A_{control}} \times 100$$

Where:

$A_{control}$=Absorbance of the control in the absence of sample

A_{sample}=Absorbance of sample

Statistical analysis

All assays were carried out in triplicates and results are expressed as mean ± SD.IC_{50} value was calculated and graph was plotted using MS Excel.

Results

Free radicals are known to play a definite role in a wide variety of pathological conditions such as pain, inflammation, cancer, diabetes, Alzheimer, hepatic damage etc. Antioxidants scavenge free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or increasing the antioxidant defense mechanisms. Natural antioxidants that are present in medicinal plant extracts are responsible for inhibiting or preventing the deleterious consequences of oxidative stress. The free radical scavenging efficiency of the aqueous extract was evaluated by different *in vitro* methods. The results obtained are given below,

1) Superoxide radical scavenging assay

The result obtained for superoxide radical scavenging assay is given in Table 1. Superoxide scavenging activity of *Eugenia uniflora* leaves was increased markedly with increased concentrations of the extract. Thus, higher inhibitory effects of the extract on superoxide anion formation noted here in possibly renered its promising antioxidant potential. The half inhibition concentration IC_{50} value of leaf extract of Eugenia uniflora was 0.76±0.24 mg/ml (Figure 1). These results suggested that the leaf extract of *Eugenia uniflora* had a potent superoxide scavenging effects.

2) Hydroxyl Radical Scavenging Activity

Activity of the different concentrations of leaf extract of *Eugenia uniflora* on hydroxyl radical had been as shown in Table 2. The extract exhibited concentration dependent scavenging activity against generated hydroxyl radical. The IC_{50} value of extract was found to be 0.74 ± 0.55 mg/ml (Figure 2.). The observed dose dependent scavenging effect could be explained by understanding the nature and generation of radicals as well as studying different physical and chemical properties of the naturally occurring antioxidant.

3) Nitric Oxide Scavenging Activity

Leaf extract of *Eugenia uniflora* significantly inhibited nitric oxide in a dose dependent manner, Table 3, with the IC_{50} being 0.65 ± 0.35mg/ml (Figure 3.). The result indicated that the extract might contain compounds able to inhibit nitric oxide and offered scientific evidence for the use of the leaves in oxidative stress conditions.

Table 1: Superoxide radical scavenging assay

S.no	Concentration mg/ml	% Inhibition of leaf extract of Eugenia uniflora	IC_{50} Value (mg/ml)
1	0.2	17.02± 0.85	0.76 ± 0.24
2	0.4	33.64±0.58	
3	0.6	43.78±0.98	
4	0.8	52.65±0.32	
5	1	64.72±0.87	

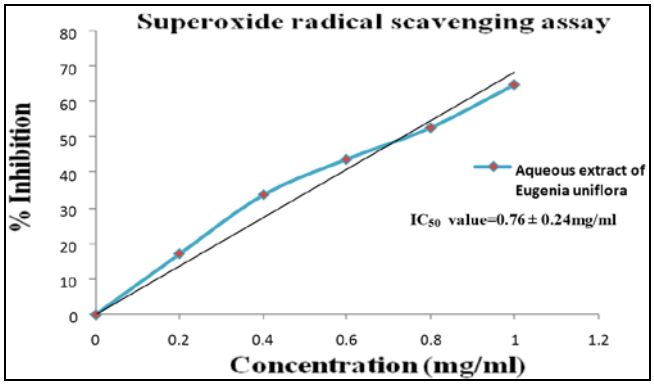

Fig 1: The superoxide radical scavenging capacity of leaf extract of *Eugenia uniflora*. Ascorbic acid was taken as Standard antioxidant. Values are means ± SD (n=3)

Table 2: Hydroxyl radical scavenging assay

S.no	Concentration mg/ml	% Inhibition of leaf extract of Eugenia uniflora	IC_{50} Value (mg/ml)
1	0.2	13.26± 0.32	0.74 ± 0.55
2	0.4	28.21±0.58	
3	0.6	43.12±0.45	
4	0.8	54.02±0.28	
5	1	64.12±0.87	

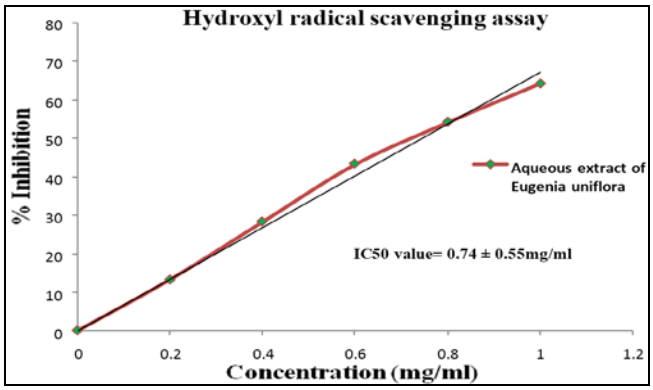

Fig 2: The hydroxyl radical scavenging capacity of leaf extract of *Eugenia uniflora*. Ascorbic acid was taken as Standard antioxidant. Values are means ± SD (n=3)

Table 3: Nitric oxide radical scavenging assay

S.no	Concentration mg/ml	% Inhibition of leaf extract of Eugenia uniflora	IC_{50} Value (mg/ml)
1	0.2	20.14± 0.96	0.62 ± 0.35
2	0.4	32.69±0.65	
3	0.6	51.62±058	
4	0.8	64.25±0.32	
5	1	77.56±0.57	

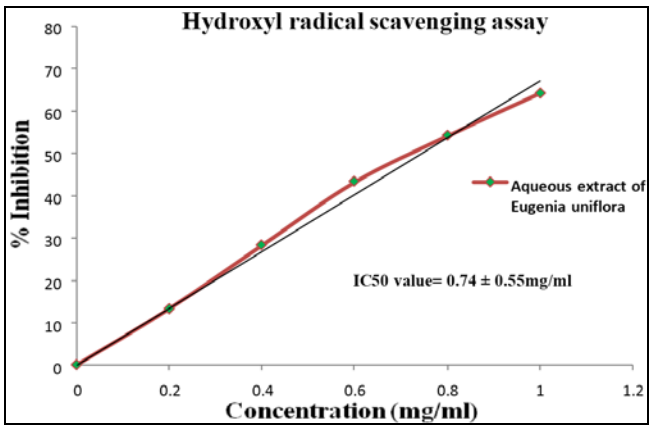

Fig 3. Nitric oxide radical scavenging capacity of leaf extract of *Eugenia uniflora*. Ascorbic acid was taken as Standard antioxidant. Values are means ± SD (n=3)

Discussion

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The propagation of free radical can brings about many adverse reactions leading to extensive tissue damage. Lipid, proteins, nucleic acids are all susceptible to attack by free radical. Many plant species with antioxidant activities act as protective agents against these radicals. In the present investigation potent antioxidant activity of *Eugenia uniflora* leaf extract to scavenge free radicals was observed using different methods. However the efficacy of extract to scavenge the different radicals differed in each method depending upon the mechanism of free radical scavenging and assay methodology

It is well known that superoxide anions damage biomolecules directly or indirectly by forming H2O2, •OH, peroxy nitrite or singlet oxygen during aging leading to pathological events such as ischemic reperfusion injury. Superoxide has also been observed to directly initiate lipid peroxidation[30]. The scavenging activity of this radical by the plant extract compared favorably with the standard reagent suggesting that the plant could also be a potent scavenger of superoxide radical. The probable mechanism of superoxide scavenging would be attributed to the inhibitory effects of *Eugenia uniflora* towards generation of superoxide in the *in vitro* reaction system.

Hydroxyl radical is highly reactive oxygen centred radical formed from the reaction of various hydroperoxides with transition metal ions. It attacks proteins, DNA, polyunsaturated fatty acid in membranes and most biological molecules it contacts[31] and is known to be capable of abstracting hydrogen atoms from membrane lipids[30] and brings about peroxidic reaction of lipids. In the present study a significant correlation existed between the concentration and hydroxyl radical scavenging ability of the extract.

Nitric oxide is an essential bioregulatory molecule required for several physiological processes like neural signal transmission, immune response, control of vasodialation and control of blood pressure[32, 33] etc. However, the elevation of the NO• results in several pathological conditions including cancer. Moreover in the pathological conditions, nitric oxide reacts with superoxide anion and form potentially cytotoxic molecules, peroxynitrite. Nitric oxide inhibitors have been shown to have beneficial effects on some aspects of inflammation and tissue damage seen in inflammatory diseases. The level of nitric oxide was significantly reduced in this study by the crude extract. Since NO• plays a crucial role in the pathogenesis of inflammation, this may explain the use of *Eugenia uniflora* for the treatment of inflammation.

Conclusion

Medicinal plants are of great importance for both individual and community health. It is well known that medicinal and aromatic plants production is challenging and involves a wide variety of issues, including agricultural, commercial, ecological, pharmacological, as well as social [34].

The results obtained in the present study indicated that *Eugenia uniflora* leaves extract exhibited free radical scavenging activity against hydroxyl, superoxide and nitric oxide. The overall antioxidant activity of *Eugenia uniflora* might be attributed to its flavonoid content and other phytochemical constituents. The findings of the present study suggested *Eugenia uniflora* leaves could be a potential source of natural antioxidant that would have great importance as therapeutic agents in preventing or slowing the progress of reactive oxygen species and associated oxidative stress related degenerative diseases.

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