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In vitro Antioxidant and Free Radical Scavenging Activity of Isolongifolene

Kowsalya Rangasamy and Elangovan Namasivayam
Department of Biotechnology, Periyar University, Salem-11, Tamil Nadu, India

Corresponding Author: Elangovan Namasivayam, Department of Biotechnology, Periyar University, Salem-11, Tamil Nadu, India Tel: 9789097142

ABSTRACT

Herbs contain large amount of antioxidant such as secondary metabolites, which plays an important role in absorbing and neutralizing free radicals. A new Sesquiterpene, Isolongifolene is present in *Murraya koenigii* leaves. *Murraya koenigii* is a herbal species used in traditional medicine in Eastern Asia. Many diseases are associated with oxidative stress caused by free radicals. In this study, antioxidant activity of Isolongifolene was evaluated in a series of *in vitro* assay involving free radicals and EC_{50} values were determined. The EC_{50} value for *in vitro* antioxidant assays namely DPPH, ABTS, hydroxyl radical, nitric oxide, hydrogen peroxide, super oxide radical scavenging, FRAP,TRAP and Reducing Power, were 77.34, 40.9, 16.27, 238.3, 25.01, 16.79, 1.311, 6.701, 0.418 μg mL^{-1}, respectively. Isolongifolene showed EC_{50} with minimum concentration and more effective in scavenging activities. Hence, based on the results we suggest that Isolongifolene compound can be recommended as a potent antioxidant to the patients suffering from various oxidative degenerative diseases like cancer.

Key words: Sesquiterpene, *Murraya koenigii*, free radicals, radical scavenging activity, antioxidant assay

INTRODUCTION

Many human diseases are caused by oxidative stress that results from imbalance between the formation and neutralization of pro-oxidants (Hazra *et al*., 2008). Oxidative stress is initiated by free radicals, such as super oxide anions, hydrogen peroxide, hydroxyl, nitric oxide, play a vital role in damaging various cellular macromolecules such as proteins, lipids and DNA in healthy human cells (Braca *et al*., 2002; Maxwell, 1995). ROS have been implicated in several diseases like cancer, asthma, arthritis, etc. Production of reactive oxidants such as superoxide, hydroxyl radical and hydrogen peroxide in living cells is an inevitable process of normal oxygen metabolism (Droge, 2002). Recent investigations have shown that the antioxidants with radical scavenging properties of plant origins could have great importance as therapeutic agents in aging and free radical mediated diseases including neurodegeneration. Recently, much attention has been directed towards the development of ethno medicine with strong antioxidant properties but low cytotoxicity. *Murraya koenigii*. (Linn.) Spreng is a tropical tree of the family Rutaceae, which is native to India. The leaves used as a herb in ayurvedic medicine. Their properties include much value as an anti-diabetic (Arulselvan *et al*., 2006; Yadav *et al*., 2002; Vinuthan *et al*., 2004; Kesari *et al*., 2005), antioxidant, antimicrobial (Arulselvan and Subramanian, 2007; Baliga *et al*., 2003; Mathur *et al*., 2010; Muthumani *et al*., 2009), anti inflammatory (Arulmozhi *et al*., 2008), hepatoprotective

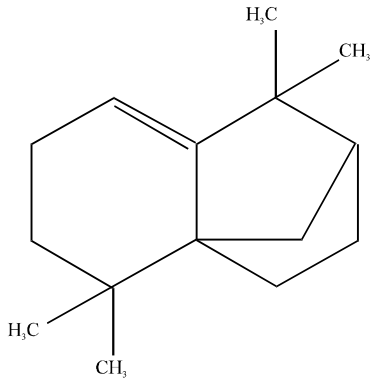

Fig. 1: Structure of Isolongifolene

(Pande *et al.*, 2009), anti hypercholesterolemic (Iyer and Mani, 1990; Khan *et al.*, 1996), as well as efficient against colon carcinogenesis. So far, thirteen compounds were identified in *Murraya koenigii* leaves. The prevailing compounds were 1-Methyl-pyrrolidine-2 carboxylic acid (69.00%), isolongifolene (Fig. 1), 4,5-dehydro-(3.68%), c-himachalene (2.88%), s1,2-Ethanediolmonoacetate (2.79%) 1,2-Benzenedicarboxylic acid, di isooctyl ester (2.55%) (Hema *et al.*, 2011).

Before the commencement of this study, there was no information in scientific literature on the free radical scavenging and antioxidant activity of the compound Isolongifolene both *in vivo* and *in vitro*. Therefore this study was aimed at providing information on the antioxidant activity of Isolongifolene.

MATERIALS AND METHODS

Chemicals: Isolongifolene was purchased from sigma, USA. Diphenyl 2-picryl hydrazyl radical (DPPH), Ferrous Sulphate, 2, 4, 6-tripyridyl-triazine (TPTZ), Ammonium Per Sulphate, 2,2'- Azino-Bis(3-Ethylbenzthiazoline-6-Sulfonic Acid) (ABTS), 2,7 Dichloro Flurecein Diacetate, 2-amidine propene dihydrochloride (AAPH), Deoxyribose, phenyl hydrazine, Tri Carboxylic Acid (TCA), Thio Barbituric Acid (TBA), Nicotinamide Adenine Dinucleotide (NADH), Nitroblue Tetrazolium, Phenazine Metho Sulphate (PMS), Phosphomolybdenum reagent, Sulphuric acid, Sodium phosphate, Ammonium molybdate, Sodium nitroprusside, sulphanilamide, O-phosphoric acid, Napthyl ethylene diamine di Hcl, Potassium ferric cyanide, Ferric chloride, Hydrogen Peroxide, Butylated Hydroxy Toluene, Xylenol Orange, Ammonium ferrous sulphate, Folin cio-calteau reagent , Sodium bi carbonate, Gallic acid, Ascorbic acid, $NaNo_2$, $AlCl_3$, Sodium acetate, Rutin were of analytical grade and obtained from Sigma, SRL, Rankem and Merk.

DPPH assay: DPPH was assayed as described by (Kunchandy and Rao, 1990). The reaction mixture contained 1.0 mL of 0.3 mM DPPH in 50 mL of methanol was added to 100 µL of Isolongifolene with concentrations ranging from 20-100 µg mL^{-1}. The mixture of DPPH in methanol used as positive control and methanol alone served as blank. When DPPH reacts with antioxidants in the sample, it was reduced and the colour changed from deep violet to light yellow and measured at 517 nm. Quercetin was used as a reference standard.

$$\text{Scavenging activity (\%)} = \frac{\text{A517 (control)-A517 (sample)}}{\text{A517 (control)}} \times 100$$

ABTS assay: The scavenging activity of the test sample was tested using $ABTS^+$ assay. The method was described by Re *et al.* (1999) with minor modifications. The $ABTS^+$ radical solution was prepared by mixing 14 mM ABTS stock solution with 4.9 mM ammonium per sulphate and incubating 16 h in the dark at room temperature until the reaction was stable. The absorbance of the $ABTS^+$ solution was equilibrated to 0.70±0.02 by diluting with ethanol at room temperature. To 1.0 mL of the $ABTS^+$ solution various concentration of the test sample (20-100 μg mL^{-1}) was added. The absorbance was measured at 734 nm after 6 min. The percentage inhibition of absorbance was calculated and plotted as a function of the concentration of standard and sample to determine the antioxidant concentration. Ascorbic acid was used as reference standard.

Hydroxyl radical scavenging activity: The Hydroxyl radical scavenging activity of the isolongifolene was done by the method described by Wenli *et al.* (2004) with major changes. Briefly, Reaction mixture contained 0.6 mL of 1.0 mM Deoxy ribose, 0.4 mL of 0.2 mM Phenyl hydrazine, 0.6 mL of 10 mM phosphate buffer (pH 7.4). The reaction mixture was incubated for one hour at room temperature. The 1.0 mL of 2% TCA, 1.0 mL of 1% TBA and 0.4 mL of isolongifolene at various concentrations were added. It was kept in water bath for 20 min. The absorbance of the mixture was measured at 532 nm. The hydroxyl radical scavenging activity was calculated. Mannitol was used a reference standard.

Nitric oxide radical scavenging activity: Nitric oxide radical scavenging activity was assayed according to the method reported by Garrat (1964). Nitric oxide was generated from sodium nitro prusside in aqueous solution at physiological pH, which interacts with oxygen to produce nitric ions, which may be determined by the Griess Illosvoy reaction. The 2.0 mL of 10 mM sodium nitro prusside, 0.5 mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of sample at different concentrations and the mixture was incubated at 25°C for 150 min. From the incubated mixture 1.5 mL was taken out and added into 1.0 mL of griess reagent (1% sulphanilamide, 2% O-phosphoric acid, 1% napthyl ethylene diamine di HCl) and incubated at room temperature for 5 min. The absorbance of the mixture was measured at 546 nm.

Hydrogen peroxide scavenging assay: This activity was determined according to a previously described method by Floriano-Sanchez *et al.* (2006) with minor changes. An aliquot of 50 mM H_2O_2 and various concentrations (20-100 μg mL^{-1}) of samples were mixed (1:1 v/v) and incubated for 30 min at room temperature. After incubation, 90 μL of the H_2O_2-sample solution was mixed with 10 μL HPLC-grade methanol and 0.9 mL FOX reagent was added (4.4 mM BHT added in 9 volumes of Methanol and 1 volume of 1 mM xylenol Orange, 2.56 mM Ammonium ferrous sulphate in 0.25 M H_2SO_4). The reaction mixture was then vortexed and incubated at 37°C for 30 min. The absorbance of the ferric-xylenol orange complex was measured at 560 nm.

Superoxide radical scavenging activity: The measurement of superoxide scavenging activity is based on method as described by Liu *et al.* (1997) with slight modifications. Superoxide radical scavenging activity is generally based on the anion radical which is associated with PMS/NADH

system. They are generated within PMS/NADH systems by the oxidation of NADH and are assayed by the reduction of nitroblue tetrazolium. Phosphate buffer (100 μM, pH 7.4) containing 1.0 mL NBT (156 μM) solution, 1.0 mL NADH (468 μM) solution and Isolongifolene (20-100 μg mL^{-1}) in methanol were mixed. The reaction was started when 0.1 mL of PMS solution (60 μM) was added to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance was read at 560 nm against the corresponding blank samples. Sodium metabi sulphate was used as a reference standard. Decreased absorbance of the reaction mixture indicated increased superoxide anion-scavenging activity.

FRAP assay: A modified method of Benzie and Strain (1996) was adopted for the FRAP assay. The stock solutions included 300 mM acetate buffer, pH 3.6, 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3$. $6H_2O$. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL $FeCl_3$.$6H_2O$. The temperature of the solution was raised to 37°C before use. Isolongifolene (20-100 μg mL^{-1}) was allowed to react with 900 μL of FRAP solution and made upto 1.0 mL with methanol. After 4 min, reading of the colored product (Ferrous tripyridyltriazine complex) was measured at 593 nm. Ferrous sulphate was used as a reference standard. The standard curve was linear between 200 and 1000 μM $FeSo_4$.

Total reducing antioxidant potential: The reaction mixture contains 0.35 mL of 2,7-dichloro flurecein diacetate (1 mM in ethanol), 1.75 mL of 0.01N NaOH incubated for 20 min at room temperature. As 7.9 mL of 25 mM sodium phosphate buffer (pH 7.2) was added and the upper portion of the solution (150 μL) was taken out. To this different concentration of Isolongifolene was added (20- 100 μg mL^{-1}). Isolongifolene was made upto 0.5 mL with methanol and 25 μL of 56 mM AAPH was added to initiate the reaction. The absorbance was measured at 490 nm against the corresponding blank samples. Ascorbic acid was used as a reference standard.

Reducing power: The Fe_3^+-reducing power of Isolongifolene was determined by the method of (Oyaizu, 1986) with a slight modification. Different concentrations (20-100 μg mL^{-1}) of the Isolongifolene were mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium hexa cyanoferrate (1%), followed by incubation at 50°C in a water bath for 20 min. After incubation, 2.5 mL of TCA (10%) was added to terminate the reaction. The upper portion of the solution (2.5 mL) was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ solution (0.1%) was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. A higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as a reference standard.

Statistical analysis: All data were presented as Mean±Standard Error Mean (SEM) of three replications. Statistical analyses were performed using Graphpad Prism Ver 5.01 software package and EC_{50} value was calculated.

RESULTS

In vitro radical scavenging activity of Isolongifolene was assessed by its ability to scavenge DPPH, ABTS, Super Oxide, Nitric Oxide, Hydrogen Peroxide, Reductive Ability and Hydroxyl Radicals.

The stable radical DPPH has been used widely for the determination of primary antioxidant activity. Isolongifolene exhibits DPPH radical scavenging activity. Figure 2 shows the

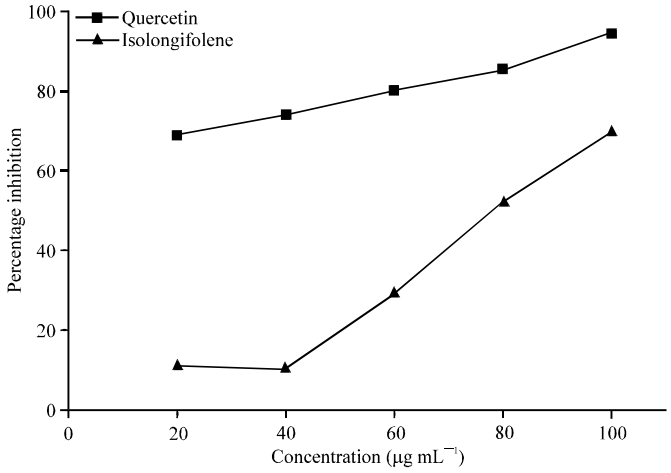

Fig. 2: DPPH radical scavenging activity of Isolongifolene at different concentrations. Each value represents Mean±SEM (n = 3)

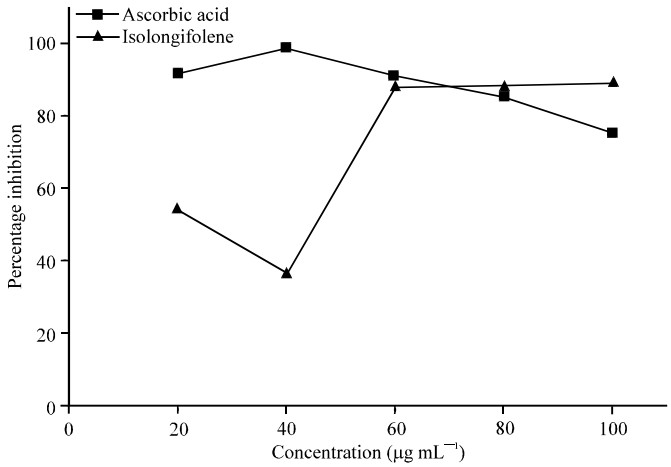

Fig. 3: ABTS activity of Isolongifolene at different concentrations. Each value represents Mean±SEM (n = 3)

dose-response curve of DPPH radical scavenging activity of Isolongifolene compared with quercetin. The EC_{50} value of Isolongifolene was found to be 77.34 µg mL^{-1} where as quercetin showed 8.685 µg mL^{-1} respectively.

Isolongifolene was fast and effective scavenger of ABTS radicals as shown in Fig. 3. The free radical scavenging activity of the Isolongifolene was tested through ABTS method. The EC_{50} values of Isolongifolene and Ascorbic acid were 25.01 and 160.5 µg mL^{-1}, respectively.

Hydroxyl Radical Scavenging activity of Isolongifolene was calculated, which was measured spectrophotometrically at 532 nm. EC_{50} values of Isolongifolene and mannitol were 16.27 and 75.5 µg mL^{-1}, respectively (Fig. 4).

Isolongifolene significantly inhibited nitric oxide in a dose dependent manner shown in Fig. 5. The EC_{50} values of Isolongifolene and Ascorbic acid were 16.79 and 14.53 µg mL^{-1}, respectively.

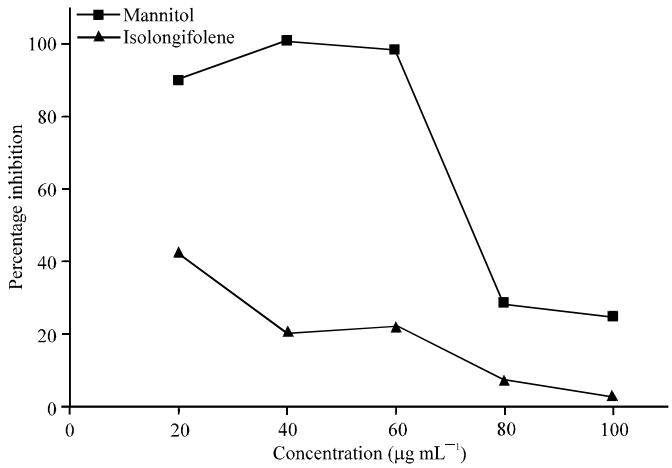

Fig. 4: Hydroxyl radical scavenging activity of Isolongifolene at different concentrations. Each value represents Mean±SEM (n = 3)

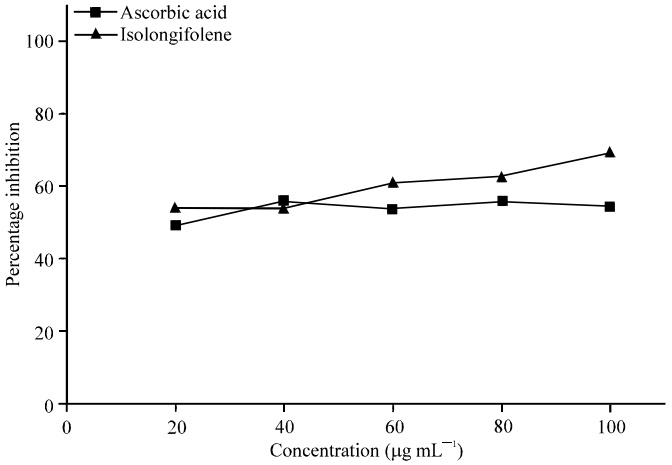

Fig. 5: Nitric oxide scavenging activity of Isolongifolene at different concentrations. Each value represents Mean±SEM (n = 3)

Hydrogen peroxide scavenging was assayed by the Fox reagent method. Figure 6 shows that Isolongifolene is a good scavenger of H_2O_2. The EC_{50} values of Isolongifolene and Ascorbic acid were 1.311 and 2.26 µg mL^{-1}. The result shows that Isolongifolene is a scavenger of hydrogen peroxide.

Isolongifolene and Ascorbic acid indicates their abilities to quench superoxide radicals in the reaction mixture. The EC_{50} value of Isolongifolene and sodium meta bi sulphate were 238.3 µg mL^{-1} and 114.2 µg mL^{-1}, respectively. Figure 7 shows decreased absorbance of isolongifolene. Ferric reducing antioxidant activity of Isolongifolene was assayed. The EC_{50} value of the Isolongifolene and ferrous sulphate were 40.9 and 62.46 µg mL^{-1}, respectively (Fig. 8).

Total reducing antioxidant property was done to analyze the antioxidant property of the Isolongifolene. The EC_{50} value of Isolongifolene and standard were 6.701 and 2.37 µg mL^{-1} (Fig. 9).

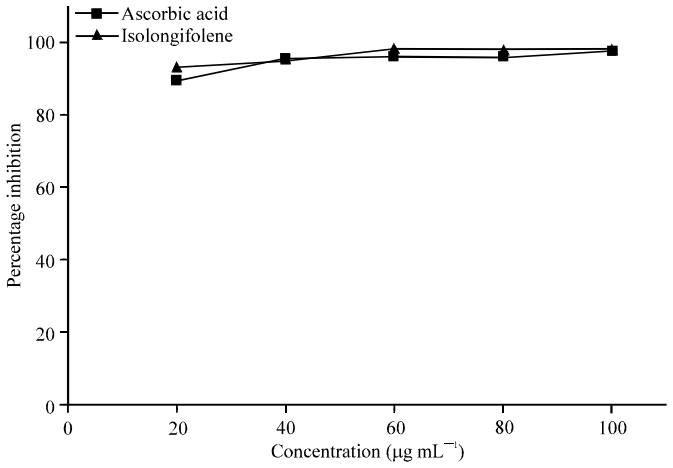

Fig. 6: Hydrogen peroxide scavenging activity of Isolongifolene at different concentrations. Each value represents Mean±SEM (n = 3)

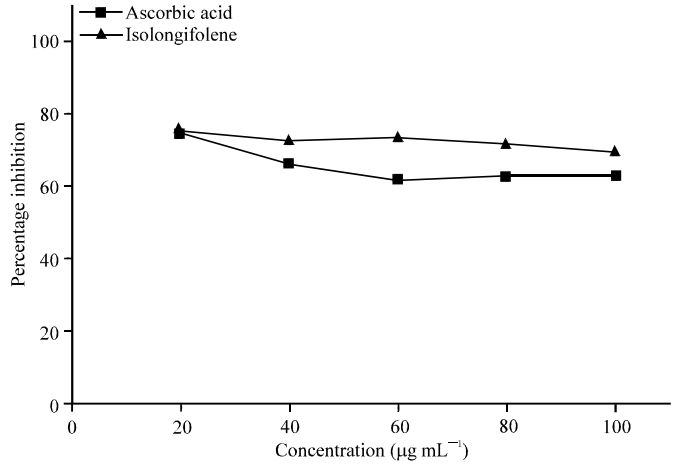

Fig. 7: Superoxide radical scavenging activity of Isolongifolene at different concentrations. Each value represents Mean±SEM (n = 3)

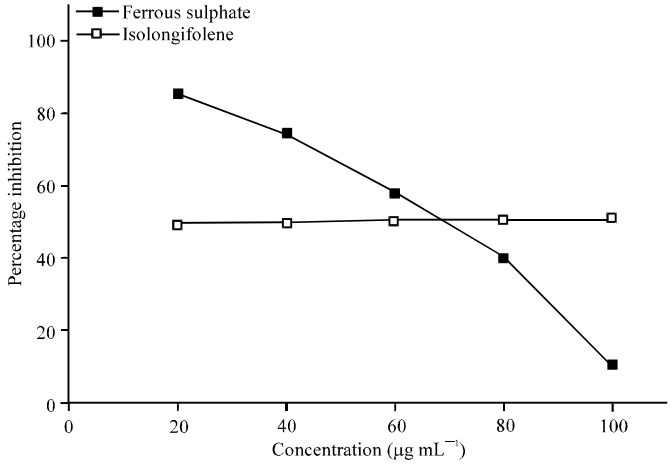

Fig. 8: Ferric reducing antioxidant property of Isolongifolene at different concentrations. Each value represents Mean±SEM (n = 3)

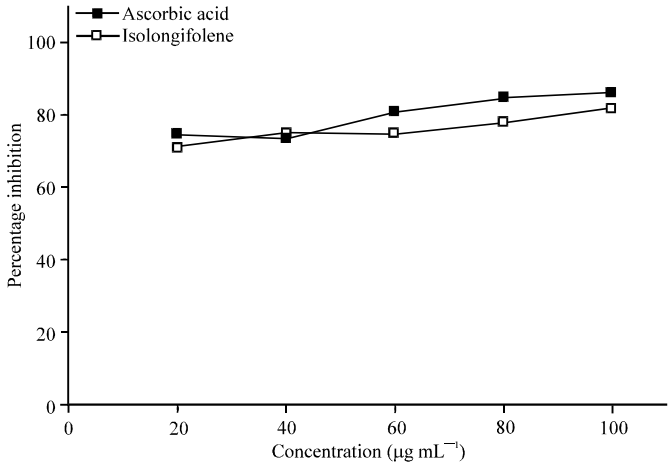

Fig. 9: TRAP activity of Isolongifolene at different concentrations. Each value represents Mean±SEM (n = 3)

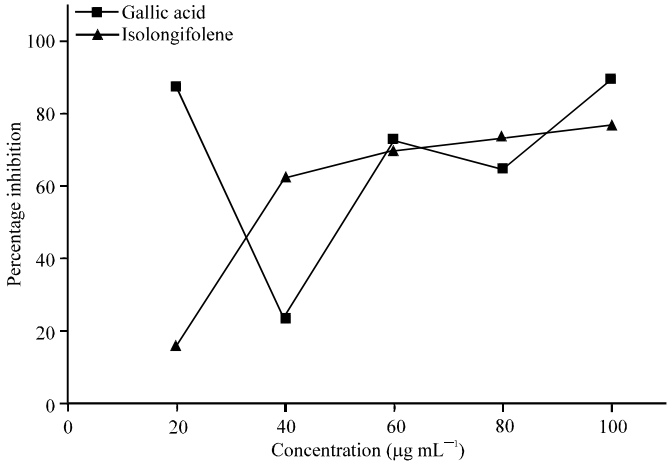

Fig. 10: Reductive ability of Isolongifolene at different concentrations. Each value represents Mean±SEM (n = 3)

The reducing power of the Isolongifolene increased with increasing concentration when compared with gallic acid it gives better result. The EC_{50} value of Isolongifolene and gallic acid were 0.418 and 38.65 µg mL^{-1}, respectively. The reductive ability of isolongifolene shown in Fig. 10.

DISCUSSION

DPPH radical scavenging is considered as a good *in vitro* model widely used to assess antioxidant efficacy within a very short time in its radical form, DPPH has disappear on reduction by an antioxidant Isolongifolene or a radical species to become a stable diagrammatic molecule resulting the colour changes from purple to yellow, which could be taken as an indication of the hydrogen donating ability of the tested sample (Marxen *et al*., 2007; Lee *et al*., 2007).

Isolongifolene was fast and effective scavenger of ABTS radicals. $ABTS^{+}$ is a blue chromophore produced by the reaction between ABTS and potassium persulfate. Addition of Isolongifolene to this pre-formed radical anion reduced it to ABTS in a concentration dependent manner. The result shows that Isolongifolene is a potent antioxidant.

Hydroxyl radical scavenging activity of Isolongifolene was calculated, EC_{50} values of Isolongifolene and mannitol were 16.27 and 75.5 μg mL^{-1}. When compared to the standard an excellent hydroxyl scavenging activity was produced. This data suggest that Isolongifolene possess better hydroxyl radical power.

Nitric oxide scavenging activity indicated that Isolongifolene is able to inhibit nitric oxide and offers scientific evidence. It is well known that nitric oxide has an important role in various inflammatory processes. Sustained levels of production of this radical are directly toxic to tissues and contribute to the vascular collapse associated with septic shock, where as chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions (Hazra *et al.*, 2008). The toxicity of NO increases greatly when it reacts with super oxide radical, forming the highly reactive peroxy nitrite anion ($ONOO^-$) (Huie and Padmaja, 1993). The nitric oxide generated from sodium nitro prusside reacts with oxygen to form nitrite. Isolongifolene inhibits the nitrite formation by directly competing with oxygen in the reaction with NO. This study proved that Isolongifolene has potent NO scavenging activity.

H_2O_2 is highly important because of its ability to penetrate biological membranes (Arulmozhi *et al.*, 2008). H_2O_2 itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. The results showed that Isolongifolene had an effective H_2O_2 scavenging activity.

The superoxide radicals generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT. The decrease in absorbance at 560 nm with Isolongifolene and Ascorbic acid indicates their abilities to quench superoxide radicals in the reaction mixture. The EC_{50} value of Isolongifolene and sodium meta bi sulphate were 238.3 and 114.2 μg mL^{-1}, respectively. Figure 7 shows decreased absorbance of isolongifolene which depicts that it possesses superoxide scavenging activity.

An antioxidant capable of donating a single electron to the ferric-TPTZ (Fe (III)-TPTZ) complex would cause the reduction of this complex into the blue ferrous-TPTZ (Fe(II)-TPTZ) complex which absorbs strongly at 593 nm.

The reductive ability of antioxidant and it is evaluated by the transformation of Fe (III) to Fe (II) in the presence of the sample. The reducing capacity of Isolongifolene may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1995). In this study the antioxidant property of isolongifolene is based on the concentration and it acts as good scavenger.

CONCLUSION

In conclusion, this is the first work describes *in vitro* antioxidant activity of Isolongifolene. Higher absorbance of the reaction mixture indicates higher reductive potential. These *in vitro* assays indicate that this Isolongifolene is a significant source of antioxidant, which might be helpful in preventing or slowing the progress of various oxidative stress-induced diseases. Isolongifolene was showed significant result in above mentioned assays. Literature reports are evident that the reducing power of bioactive Isolongifolene is associated with antioxidant activity. In future the ability of Isolongifolene to treat stress related diseases may be attributed to the observed result.

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