IN VITRO ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF ISOLONGOIFOLENE FROM MURRAYA KOENIGII LEAVES

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ABSTRACT

Objective: The present research was undertaken to evaluate the invitro antioxidant activity of isolongifolene. Isolongifolene is a Sesquiterpene and it is present in Murraya koenigii leaves.

Methods: The antioxidant activity of isolongifolene was determined by various methods like DPPH, ABTS, hydroxyl radical, nitric oxide, hydrogen peroxide, super oxide radical scavenging, FRAP, TRAP and Reducing Power

Results: our results showed that isolongifolene exhibited the potent scavenging activity. The EC50 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 respectively. Isolongifolene showed EC50 with minimum concentration and more effective in scavenging activities.

Conclusion: Hence based on the results we suggest that Isolongifolene compound can be recommended as a potent antioxidant. It indicates that isolongifolene is a better source of antioxidant which might be helpful to prevent the progress of oxidative stress.

Keywords: Sesquiterpene, Murraya koenigii, Antioxidant, Radical scavenging activity, Oxidative stress.

INTRODUCTION

Many human diseases are caused by oxidative stress that results from imbalance between the formation and neutralization of pro-oxidants [1]. Oxidative stress is initiated by free radical, 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 [2, 3]. 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 [4]. 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 [5, 6, 7, 8], antioxidant, antimicrobial [9, 10, 11, 12], anti-inflammatory [13], hepatoprotective [14], anti hypercholesterolemic [15, 16], as well as efficient against colon carcinogenesis. So far, thirteen compounds were identified in Murraya koenigii leaves. The prevailing compounds were 1-Methyl -pyrroline-2-carboxylic acid (69.00%), isolongifolene (Fig.1), 4,5-dehydro - (36.8%), c-himachalene (2.88%), 1,2-Ethanediolmonooacetate (2.79%) 1,2- Benzenedicarboxylic acid, diisocyl ester (2.55%) [17].

Before the commencement of this work, 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-pikeryl hydrazyl radical (DPPH), Ferrous Sulphate, 2, 4, 6-tripyrdyl-triazine (TPTZ), Ammonium Per Sulphate, 2,2’-Azino-Bis(3-Ethylbenzthiazoline-6-Sulfonic Acid) (ABTS), 2,7 Dichloro Flurcein Diacetate, 2-amidine propene dihydrochloride (AAPH), Deoxyribose, phenyl hydrazine, Tri Carboxylic Acid (TCA), Thiobarbituric 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, Naphthyl ethylene diamine di Hcl, Potassium ferric cyanide, Ferric chloride, Hydrogen Peroxide, Butylated Hydroxy Toluene, Xylenol Orange, Ammonium ferrous sulphate, Folin ciocălteau reagent, Sodium bi carbonate, GaBic acid, Ascorbic acid, NaNo3, AlCl3, Sodium acetate, Rutin were of analytical grade and obtained from Sigma, SRL, Rankem and Merk.

DPPH Assay

DPPH was assayed as described by Elizabeth and Rao., [18]. The reaction mixture contained 1.0 ml of 0.3mM DPPH in 50 ml of methanol was added to 100 µl of Isolongifolene with concentrations ranging from 20-100 µg/ml. 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. Scavenging activity (%) = [Acontrol – A(sample) / Acontrol] × 100.

ABTS Assay

The scavenging activity of the test sample was tested using ABTS+ assay. The method was described by Re et al., [19] 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) was added. The absorbance was measured at 734 nm after 6 minutes. 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 method described by Yu et al. [20] with major changes. Briefly, Reaction mixture contained 0.6 ml of 1.0 mM Deyoxy ribose, 0.4 ml of 0.2 mM Phenol hydratine, 0.6 ml of 10 mM phosphate buffer (pH 7.4). The reaction mixture was incubated for one hour at room temperature. 1.0 ml of 2% TGA, 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 mins. 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 et al.[21]. 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 Ilosvoy reaction. 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 mins. From the incubated mixture 1.5 ml was taken out and added into 1.0 ml of gries reactant (1% sulphanilamide, 2% O-phosphoric acid, 1% naphthy ethylene diamine di HCl) and incubated at room temperature for 5 mins. 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 Flaviano et al. [22] with minor changes. An aliquot of 50 mM H₂O₂ and various concentrations (20-100 µg/ml) of samples were mixed (1:1 v/v) and incubated for 30 min at room temperature. After incubation, 90 µl of the H₂O₂-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 1mM xylene orange, 2.56 mM Ammonium ferrous sulphate in 0.25 M H₂SO₄). The reaction mixture was then vortexed and incubated at 37°C for 30 min. The absorbance of the ferri-xylene 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. [19] [23] 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) 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 meta bi 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 et al. [24] 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 FeCl₃, 6H₂O. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ and 2.5 ml FeCl₃. The temperature of the solution was raised to 37°C before use. Isolongifolene (20-100g/ml) was allowed to react with 900 µl of FRAP solution and made up to 1.0 ml with methanol. After 4 minutes, 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 FeSO₄.

Total Reducing Antioxidant Potential

The reaction mixture contains 0.35 ml of 2.7- dichloro flurecein diacetate (1mM in ethanol), 1.75 ml of 0.1N NaOH incubated for 20 minutes at room temperature. 17.9 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). Isolongifolene was made up to 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 Oyaztu et al. [25] with a slight modification. Different concentrations (20-100 µg/ml) 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 FeCl₃ 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 EC50 value was calculated.

RESULTS AND DISCUSSION

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

DPPH activity

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 dose-response curve of DPPH radical scavenging activity of Isolongifolene compared with quercetin. The EC50 value of Isolongifolene was found to be 77.34µg/ml where as quercetin showed 8.685µg/ml respectively. 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 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 [26, 27].

Fig. 2: DPPH radical scavenging activity of Isolongifolene at different concentrations. Each value represents mean ± SEM (n=3)
solongifolene is a potent antioxidant. Isolongifolene has potent NO scavenging activity. Isolongifolene inhibits the nitrite formation by directly competing with oxygen in the reaction with NO. This result proved that Isolongifolene has potent NO scavenging activity.

ABTS Assay
Isolongifolene was fast and effective scavenger of ABTS radicals as shown in figure 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 µg/ml and 160.5µg/ml respectively. 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.

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

Hydroxyl Radical Scavenging Activity
Hydroxyl Radical Scavenging activity of Isolongifolene was calculated, which was measured spectrophotometrically at 532 nm. The results are shown in Fig. 4. EC_{50} values of Isolongifolene and mannitol were 16.27µg/ml and 75.5µg/ml respectively. When compared to the standard an excellent hydroxyl scavenging activity was produced. This data suggest that Isolongifolene possess better hydroxyl radical power.

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

Nitric oxide Radical Scavenging Assay
Isolongifolene significantly inhibited nitric oxide in a dose dependent manner. The EC_{50} values of Isolongifolene and Ascorbic acid were 16.79 µg/ml and 14.53 µg/ml respectively. The result 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 [28]. The toxicity of NO increases greatly when it reacts with super oxide radical, forming the highly reactive peroxynitrite anion ([ONOOO^−]) [29]. 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.

Hydrogen Peroxide Scavenging
Hydrogen peroxide scavenging was assayed by the Fox reagent method. The EC_{50} values of Isolongifolene and Ascorbic acid were 1.311 µg/ml and 2.26 µg/ml. The result shows that Isolongifolene is a scavenger of hydrogen peroxide. H_2O_2 is highly important because of its ability to penetrate biological membranes [30]. 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.

Superoxide Radical 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 sulpha were 238.3 µg/ml and 114.2 µg/ml respectively. When compared to standard absorbance of the isolongifolene decreased gradually.

FRAP
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. Ferric reducing antioxidant activity of Isolongifolene was assayed. The EC_{50} value of the Isolongifolene and ferrous sulphate were 40.9 µg/ml and 62.46 µg/ml respectively. The EC_{50} values of ferric reducing assay shows that Isolongifolene possesses excellent antioxidant activity.

TrAP
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 µg/ml and 2.37µg/ml.

Reducing Power
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 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 0.418µg/ml and 38.65µg/ml respectively. 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 [31]. In this study the antioxidant property of Isolongifolene is based on the concentration.

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.

REFERENCES


