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Evaluation of *In-Vitro* Antioxidant Activity of Piperlongumine Isolated from *Piper Longum* Linn.

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ABSTRACT

Piper longum is widely used as a folk medicine to cure various ailments. Roots of *Piper longum* has many alkaloids, out of which Piperlongumine have been isolated. Piperlongumine has been evaluated for various antioxidant assays like DPPH, ferric Ion reducing Power, total antioxidant capacity and hydroxyl radical scavenging assay. The antioxidant activity of the Piperlongumine increased in a concentration-dependent manner. The compound showed significant reactive oxygen species (ROS) scavenging activity in all *in vitro* antioxidant assays. These results clearly indicate that Piperlongumine is effective against various diseases.
Keywords: alkaloid, ethnomedicine, reactive Oxygen reactive species, piperaceae, piperlongumine.

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INTRODUCTION

Oxygen-centered free radicals and other reactive oxygen species (ROS) can be generated as by products during oxidative progresses of living organisms [1,2] Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids, DNA and can initiate degenerative diseases. Antioxidant compounds like phenolic acids polyphenols and flavanoids scavenge free radicals and thus inhibit the oxidative mechanisms [3].

Medicinal plants used in Ayurveda can provide biologically active molecules and lead structures for the development of modified derivative with enhanced activity or reduced toxicity. On the other hand, the isolation and identification of the active principles and elucidation for the mechanism of action of a drug is of atmost important. Diseases like cancer cardiovascular and neurodegenerative diseases are linked to excessive amounts of the free radicals. Hence research for a potent compound from medicinal plant for trapping the free radicals in our body will be the main criteria for curing the complicated diseases. There is still a demand to find more information concerning the antioxidant potential of plant species as they are safe and bioactive.

Piper longum is a native of the Indo Malaya region belongs to family Piperaceae sparsely distributed in forests of the Western Ghats, India [4]. *Piper longum* is an aromatic climber with stout roots, jointed stems, and ovate leaves. It has been used as a therapeutic agent in the treatment of various pathological conditions. Fruits, roots and stem are used as an important drug Piplamul in the Arurvedic and Unani systems. Root of the plant is reported for the treatment of heart diseases in ancient literature of East Asia [5] and it is also used as stomachic, thermogenic, aphrodisiac, carminative, expectorant, laxative, digestive, emollient, antigiardias, anti-amoebic, antiasthmatic, laxative, antiseptic [6]. In the present study, the isolated molecules Piperlongumine were evaluated for various *invitro* antioxidant assays and thus justify their folklore use.

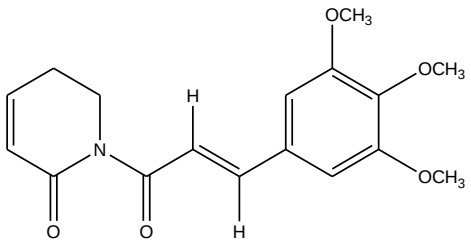

Figure 1: Piperlongumine

MATERIALS AND METHOD

Plant Material

The dried roots of *Piper longum* were purchased from local market of Bangalore, Karnataka, India. The plant material was authenticated by Dr. S Sundara Rajan, Professor, centre for Advanced studies in Biosciences, Jain University, Bangalore.(Voucher specimen number Bot. PL. 80).

Extraction and Isolation of Piperlongumine from *Piper longum*

1 kg of dried roots of *Piper longum* were taken in one liter capacity thimble of Soxhlet apparatus and refluxed with acetone (LR grade, Merck, India) until all soluble compounds had been extracted in 2 batches of 500 g each. The resultant extractive was concentrated in rotavapor. The net weight of the extractive was 100gms which was subjected to column chromatography. The column was packed with silica gel (60-120 mesh size) in hexane and eluted with 2% ethyl acetate in hexane and concentration of ethyl acetate was increased by 2% and finally at 14% of ethyl acetate in hexane. Pure compound was obtained and the compound was characterized with the help of NMR and Mass spectroscopy. A portion of the compound was subjected to screening for antioxidant activity.

In vitro antioxidant activity

DPPH Assay

The DPPH free radical scavenging activity was assessed according to [7] with slight modification. 300 ml of ethanolic solution of DPPH (0.05 mM) was added to 100 ml of Piperlongumine solution with different concentrations (100-500 µg/ml). The DPPH solution was freshly prepared and kept in the dark at 4 °C. Ethanol 96 % (2.7 ml) was added and the mixture was shaken vigorously. The mixture was left to stand for 5 min and the absorbance was measured using a spectrophotometer at 517 nm. Ethanol was used to zero the spectrophotometer. A blank sample containing the same amount of ethanol and DPPH was also prepared. All determinations were performed in duplicate. The radical scavenging activities of the tested samples, expressed as percentage of inhibition were calculated according to the following equation [8].

Percentage inhibition= [(AB-AT)/AB] × 100

Where AT and AB are the absorbance values of the test and of the blank sample, respectively.

Ferric Ion Reducing Power Assay

Antioxidant activity was determined by Ferric ion reducing antioxidant power assay (FRAP) as described by Oyaizu[9] 2ml of different concentrations of Piperlongumine solution (100 - 500 µg/ml) were mixed with phosphate buffer (2.5 ml, 0.2 m, pH 6.6) and 0.1 % potassium ferricyanide (2.5 ml). The mixture was incubated at 50 ºC for 20 min. Aliquots of 10 % trichloroacetic acid (2.5 ml) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm.

Phosphomolybdenum antioxidant assay

The total antioxidant property of the methanol extract was evaluated by the phosphomolybdenum assay method, [10] which is based on the reduction of Mo (VI) to Mo (V) by the compounds and subsequent formation of a green phosphate- MO (V) complex in acidic condition. A 0.3 ml (100-500 µg/ml) of Piperlongumine solution were combined with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and the reaction mixture was incubated at 95 °C for 90 min. Then the absorption of solution was measured at 695 nm using a UV visible spectrophotometer against blank after cooling to room temperature.

Hydroxyl radical scavenging assay

This was assayed as described by [11] with a slight modification. The assay is based on quantification of the degradation product of 2- deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe3+ -ascorbate-EDTA-H2O2 system (the Fenton reaction). The reaction mixture contained, in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM); KH2PO4 –KOH buffer (20 mM, pH 7.4); FeCl3 (100 ìM); EDTA (100 mM); H2O2 (1.0 mM); ascorbic acid (100 mM) and the Piperlongumine solution at concentrations in the range of 100-500 mg/ml). After incubation for 1 h at 37 °C, 0.5 ml of the reaction mixture was added to 1 ml 2.8 % TCA, then 1 ml 1% aqueous TBA was added and the mixture was incubated at 90 °C for 15 min to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed in duplicates. The percentage of hydroxyl radical scavenging was calculated as:

Percentage inhibition= [(AB-AT)/AB] × 100

Where AT and AB are the absorbance values of the test and of the blank sample, respectively.

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RESULTS AND DISCUSSION

Characterization of Isolated Compound

1H NMR and MASS spectral analysis confirmed the chemical structure of the compound. 1H NMR spectrum showed signals at 2.4 ppm integrated for one proton present in the ring appears as multiplet. –OCH3 proton of 15th carbon atom comes to resonate at _ 2.904ppm, –OCH3 proton of 16th carbon atom resonates at 3.760 as singlet and -OCH3 of 17th carbon atom resonate at 2.904 as singlet. The aliphatic proton of 7th and 8th carbon atom(C=C) resonates at 6.973 ppm as singlet represent two protons. Finally, the structure assigned as Piperlongmine, which was further supported by MASS spectral studies gave molecular ion peak at m/z 318 and confirms the structure Piperlongumine.

In vitro antioxidant activity

Percentage inhibition of DPPH was the parameter widely used to measure antioxidant/free radical scavenging power[12&13]. The extract showed potent free radical scavenging in a concentration dependent manner with the highest percentage inhibition of 51.29 at 500µg/ml (Figure 2). DPPH is a reactively stable free radical. The assay is based on the scavenging ability of antioxidants towards the stable radical DPPH. DPPH gives a strong absorption band at 517 nm in visible region. When the odd electron becomes paired off in the presence of a free radical scavenger, the absorption reduces and the DPPH solution is decolourised as the colour changes from deep violet to light yellow. The degree of reduction in absorbance measurement is indicative of the radical scavenging power of the isolated molecule. The Piperlongumine appeared to be potent with a maximum inhibition of 51.29 % at 500 µg/ml concentration. From the study, it may be postulated that the Piperlongumine of *Piper longum* reduces the free radical into corresponding hydrazine upon reaction with the hydrogen donors in the antioxidant principles.

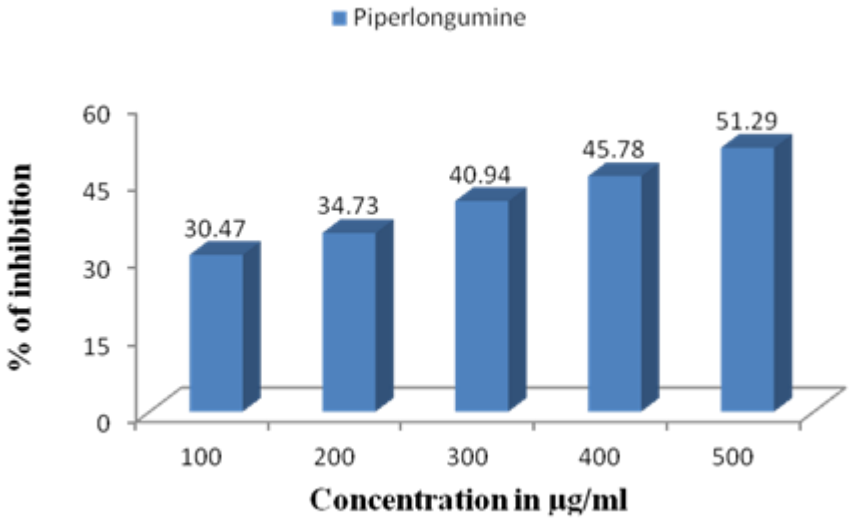

Figure 2: Free radical scavenging using DPPH radical

For the measurements of reducing ability, the transformation of Fe^{3+}-Fe^{2+} in the presence of isolated molecule was adopted (Figure-3) The reducing power of the extract increased with the concentration. However, the absorbance remained constant after 400 µg/ml concentration. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [14]. Fe^{3+} / Fe^{2+} transformation was investigated in the presence of Piperlongumine for the measurements of the reductive ability. In the present study effectiveness of the radical scavenging activity appeared to be more pronounced at the concentration of 400 µg/ml and remained constant.

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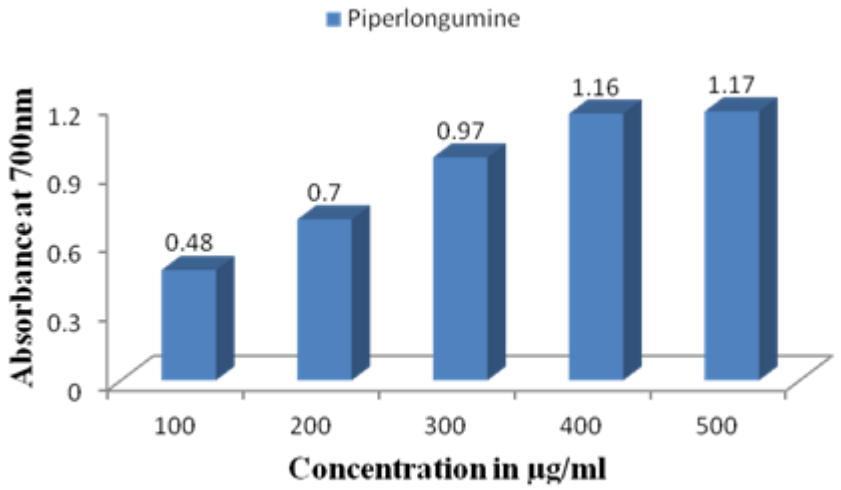

Figure 3: Ferric ion reducing power assay

The total antioxidant capacity of Piperlongumine of *Piper longum* was determined by phosphomolybdenum assay and the highest absorbance was recorded at 400 µg/ml. The absorbance decreased at 500 µg/ml concentration (Figure-4). The antioxidant capacity of the Piperlongumine was measured by phosphomolybdenum method, which is based on the reduction of Mo (IV) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/ Mo (V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of isolated molecule was found to increase with increase in concentration. However, it was noticed that there was a decrease in activity at 500 µg/ml which could be indicative of the functional saturation of the compound.

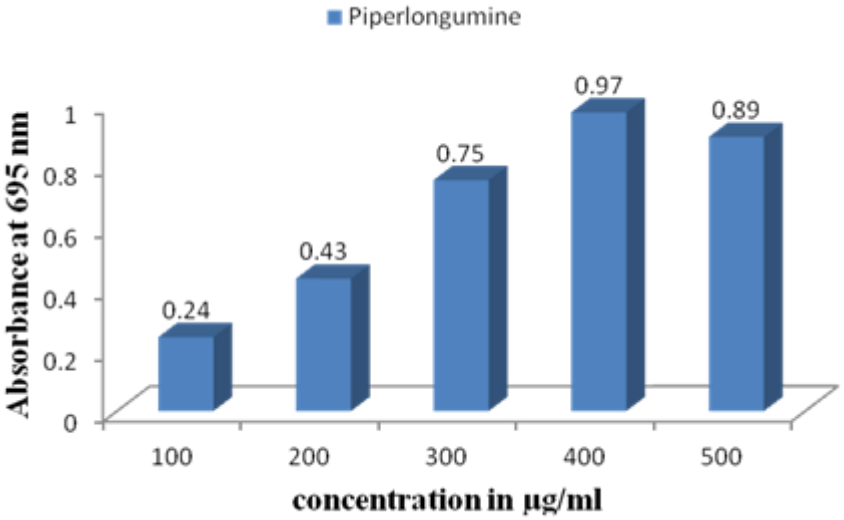

Figure 4: Phosphomolybdenum antioxidant assay

The compound was examined for its ability to act as $OH^{\cdot}$ radical scavenging agent. The hydroxyl radicals were detected by their ability to degrade 2- deoxy-2-ribose into fragments that on heating with TBA and low pH form a pink chromogen. The Piperlongumine at different concentrations exhibited moderate scavenging effect (Figure-5). The percentage inhibition of hydroxyl radical increased as the concentration increased and reached 49.89 % at the highest concentration (500µg/ml). In the present investigation, hydroxyl radical scavenging ability of the compound was determined by deoxyribose assay. The pentose sugar 2-deoxyribose is attacked by $OH^{\cdot}$ radicals to yield a mixture of products[15]. On heating with thiobarbituric acid at low pH, some or all of these products react to form a pink chromogen that can be measured by its

absorbance at 532 nm. The non site-specific scavengers would compete with deoxyribose for availability of hydroxyl radicals, resulting in the reduction of rate of reaction. On the other hand, site-specific scavengers would offer protection by chelating with ferrous ions. The compound moderately, decreased the degradation of deoxyribose by scavenging the hydroxyl radical.

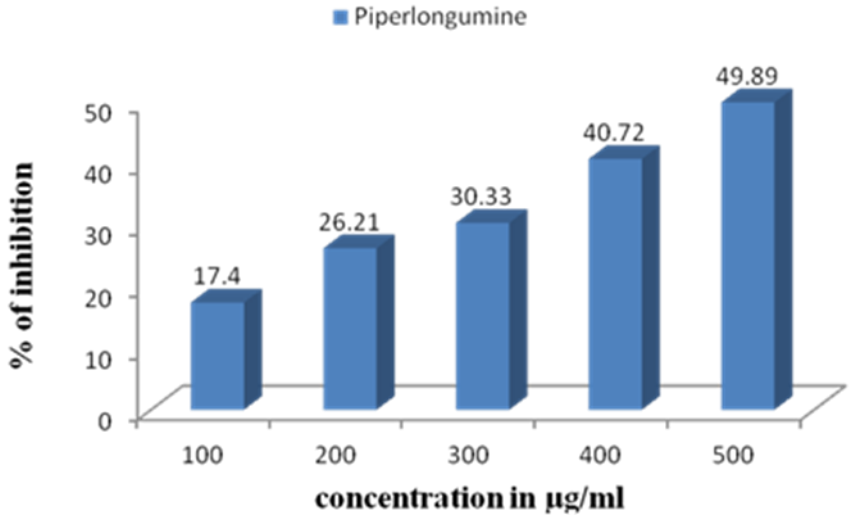

Figure 5: Scavenging effect of Piperlongumine on hydroxyl radical.

CONCLUSION

Our study demonstrates that Piperlongumine has potential antioxidant activity. Since isolates of plant extracts are quite safe and their toxicity is not a problem of concern, Piperlongumine can be exploited as antioxidant additives or as nutritional supplements.

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