EXTRACTION, PURIFICATION AND IN VITRO ANTIOXIDANT ANALYSIS OF SEEDS OF *PIPER LONGUM LINN*

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**ABSTRACT**

*Piper longum* commonly known as “long pepper” is a well-known medicinal plant in Ayurveda. Different parts of this plant, such as root, seed, fruit, whole plant etc. are used traditionally in various ailments. Herbs are natural remedies for the disease with higher safety profile and efficacy. The country like India has got variety of climatic conditions and seasons favorable for growth of many species of plants. Amongst the large number of herbal drugs existing in India, very few have been studied systematically so far. The list of drugs is very meticulous and diversified hence extensive efforts are required for their correct recognition. *Piper longum* is a highly valuable drug and is one of the essential ingredients in most of the compound preparations. Hence, an attempt has been made to collect *Piper longum*, isolate protein and to purify using HPLC and MS MALDI techniques. Further in this paper invitro studies like Nitric oxide radical scavenging activity and Ferrous ion reducing activity invitro studies have also been carried out.

*Keywords:* *Piper longum*, medicinal plant, Protein, HPLC, MS MALDI, antioxidant activities.

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INTRODUCTION

Plants, as the source of medicine, have been playing an important role in the health services around the globe. The medicinal value of plants lies in some chemical substances that they contain. The most important of these bioactive compounds of plants are alkaloids, tannins and phenolic compounds. Spices and herbs are recognized as sources of natural antioxidants and thus play an important role in the chemoprevention of diseases and aging. Among the plants investigated to date, one showing enormous potential is the pepper family otherwise known as Piperaceae. *Piper longum* is a two-flowering vine in the family Piperaceae. *P. longum*, also known, as Long Pepper is one of the most widely used Ayurvedic herbs (1). It is used for the treatment of respiratory tract diseases like cough, bronchitis, asthma, cold, as counter-irritant and analgesic. It is applied locally for muscular pain and inflammation and used internally as a carminative in conditions such as loss of appetite and sleeplessness. It is said that the Roman emperors valued it even more highly than black pepper due to its high commercial and economical importance.

Vernacular names: English: Long pepper, Hindi: Pippali, Sanskrit: Pipali

Habitat: The native of plant is South Asia and is found both wild as well as cultivated, throughout the hotter parts of India from central to the north-eastern Himalayas. The herb also grows wild in Malaysia, Singapore, Bhutan, and Myanmar (2).


The alkaloids, of which some 5,500 are known, comprise the largest single class of secondary plant substance. Alkaloids are often toxic to man and many have dramatic physiological activities, hence their wide use in medicine. They are usually colorless, often optically active substances, most are crystalline but a few (e.g. nicotine) are liquids at room temperature. Piperine is the alkaloid responsible for the pungency of black pepper and long pepper, along with chavicine (an isomer of piperine) (3). It has also been used in some forms of traditional medicine and as an insecticide. In India, Sanskrit texts included cubeb in various remedies. Charaka and Sushruta prescribed a cubeb paste as a mouthwash, and the use of dried cubebs internally for oral and dental diseases, loss of voice, halitosis, fevers, and cough.

Antioxidants decreases oxidative stress and minimize the incidence of pathological conditions caused by the oxidants. The generation of oxidative stress is harmful to the body and may cause peroxidation of membrane lipids leading to loss of membrane integrity and cell death, denaturation of proteins including enzymes, ion channels and strand breakage in DNA (4). Thus, antioxidant-based drug formulations are instrumental in the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer’s disease and cancer (5).

In the present study an attempt was made to screen different multi solvent extracts prepared from dried fruits of *Piper longum* to study the antioxidant activity on basis of their phytochemical significance. Various extracts of the spice *Piper longum* seeds like Aqueous, Methanol, petroleum Ether and Acetone were prepared and concentrated to minimum volume. The extracts were analyzed for various phytochemical quantitative analyses such as Total phenols (Folin-Ciocalteu method), Flavonoids (Aluminum chloride method), total sugars (Dubois method) and Proteins (Bradford’s method).

MATERIALS AND METHODS

Collection of samples

*Piper longum* seeds were procured from authentic source in Mysore, Karnataka, India. The seeds were cleaned, washed in double distilled water to remove dust particles, shade dried and powdered (100mesh British Pharmacopea), stored in glass bottle for further studies.

Preparation of seed extract

The extracts were prepared like 10g of *Piper longum* seeds powder in 100ml of water, methanol, petroleum ether and Acetone. Vortexed for 2 hours at room temperature, filtered, and get it evaporated to concentrate the extract and stored in shaded area (6).

Chemicals

Standard reagents and chemicals of analytical grade were used. Bovine Serum Albumin (BSA), Quercetin, Gallic acid and DPPH were procured from Hi Media (Mumbai, India). Methanol, Acetone and Petroleum ether and other chemicals were procured from Merck and s.d. Fine chemicals India.
Purification
Protein separation by SDS-PAGE
SDS-PAGE was performed in 12% w/v acrylamide gel at 100V for 2 hr and 30 min. Sephadex G-50 column for Column chromatography.

HPLC analysis
Reagents and chemicals
Acetonitrile (HPLC grade from OmniSolv EM Science, Gibbstown, and NJ), ultrapure water (Milli-Q system, Millipore, Bedford, USA) and acetic acid (analytical grade, Merck, Darmstadt, Germany) were used for the mobile phase preparation. Methanol (HPLC grade from OmniSolv EM Science, Gibbstown, NJ) was used for samples preparation. The piperlongumine (2) was used as external standard and the piperovatine (1) were only used as reference to the corresponding peak in the sample extracts (7).

The GC-MS analysis of the plant
Extract was made in a (Agilent 789 A) instrument under computer control at 70 eV. About 1μL of the methanol extract was injected into the GC-MS using a micro syringe and the scanning was done for 45 min. As the compounds were separated, they eluted from the column and entered a detector which was capable of creating an electronic signal whenever a compound was detected. The greater the concentration in the sample, bigger was the signal obtained which was then processed by a computer. The time from when the injection was made (initial time) to when elution occurred is referred to as the retention time (RT). While the instrument was run, the computer generated a graph from the signal called chromatogram. Each of the peaks in the chromatogram represented the signal created when a compound eluted from the chromatographic column into the detector. The x-axis showed the RT and the y-axis measured the intensity of the signal to quantify the component in the sample injected. As individual compounds eluted from the gas chromatographic column, they entered the ionization (mass spectroscopy) detector, where they were bombarded with a stream of electrons causing them to break apart into fragments. The fragments obtained were actually charged ions with a certain mass 28. The M/Z (mass / charge) ratio obtained was calibrated from the graph obtained, which was called as the Mass spectrum graph which is the fingerprint of a molecule (8).

Antioxidant activity
Nitric Oxide radical Scavenging activity
Nitric oxide (NO) scavenging activity was measured following the method of Dinis and co-worker. Sodium nitroprusside (5 mmol) in phosphate buffered saline was mixed with different concentrations of the different extracts (10–500µg/ml) dissolved in ethanol and incubated at 25°C for 30 min. A control without the test compound but with an equivalent amount of ethanol was taken. After 30 min, 1.5 ml of the incubation solution was removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% naphthylethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphamidine and subsequent coupling with naphthylethylene diamine dihydrochloride was measured at 546 nm. The nitric oxide radical scavenging activity was expressed as the inhibition percentage (I %) and calculated as per the equation:

\[ I (\%) = (A_{blank} – A_{sample} / A_{blank}) \times 100, \text{where} A_{blank} \]

Is the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the test compound with all reagents. IC50 was calculated from the plot of inhibition (%) against extract concentration. All the tests were carried out in triplicate and average of the absorptions was noted. Ascorbic acid was used as positive control standard for this study (9)

Ferrous Ion Chelating Ability
The ferrous ion chelating activity was investigated according to the method of Dehpourand co-workers [20]. Different concentrations of the extracts (100-500µg/ml) were added to 0.1 ml solution of 2 mM ferrous chloride (FeCl2). The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. The mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured at 562 nm by spectrophotometer. The percentage of inhibition of ferrozine-Fe2+ complex formations was calculated according to the following equation:

\[ \text{Ferrous ion chelating ability (\%) = } [ (A_o – A_s) / A_o ] \times 100, \]

Where Ao is the absorbance of the control solution (containing all reagents except for the extract); as is the absorbance in the presence of the sample (plant extracts). All the tests were carried out in triplicate and the mean value was taken. EDTA was used as the standard (10).
RESULTS AND DISCUSSION

Reducing power assay and metal ion chelating activity

The ability of an antioxidant to donate an electron can be measured by the reducing power assay. This assay is based on reaction of samples with potassium ferricyanide (Fe$^{3+}$) to produce potassium ferrocyanide (Fe$^{2+}$), followed by reaction with ferric chloride (FeCl$_3$) to form ferric–ferrous complex that has an absorbance maximum at 700 nm. The reducing power of test samples increased by the concentration increase of sample. The maximum and minimum absorbances were respectively observed for seed extract with 2.33 and 0.92 at 500 μg/mL. Also, suitable linear relationship was observed in seed extract. Also, previous studies about reducing power of sample prove these results.

Ferrous-ion chelating ability of the different extracts of piper species in a dose-dependent manner. The ethanol extracts showed a higher ferrous-ion chelating ability than the water extracts. At 500 μg/ml, the scavenging activities of the ethanolic extracts of sample were 62.93 ± 0.06, 64.21 ± 0.17, 70.80 ± 0.18 and 62.06 ± 0.04% respectively, while that of the water extracts of different concentrations were 25.90 ± 0.02, 20.31 ± 0.04, 36.26 ± 0.33 and 19.00 ± 0.06%.

<table>
<thead>
<tr>
<th>Table 1: Fe$^{2+}$ Chelating activity of P. longum extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (μg/ml)</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>200</td>
</tr>
<tr>
<td>300</td>
</tr>
<tr>
<td>400</td>
</tr>
<tr>
<td>500</td>
</tr>
<tr>
<td>IC$_{50}$</td>
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</tbody>
</table>

Table 2: Reducing power assay of P. longum extracts

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>AA</th>
<th>PL-E</th>
<th>PL-W</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.78 ± 0.002</td>
<td>0.07 ± 0.003</td>
<td>0.06 ± 0.003</td>
</tr>
<tr>
<td>200</td>
<td>1.11 ± 0.003</td>
<td>0.10 ± 0.005</td>
<td>0.09 ± 0.008</td>
</tr>
<tr>
<td>300</td>
<td>1.52 ± 0.004</td>
<td>0.16 ± 0.004</td>
<td>0.13 ± 0.002</td>
</tr>
<tr>
<td>400</td>
<td>1.76 ± 0.003</td>
<td>0.22 ± 0.005</td>
<td>0.16 ± 0.003</td>
</tr>
<tr>
<td>500</td>
<td>1.99 ± 0.009</td>
<td>0.25 ± 0.008</td>
<td>0.19 ± 0.005</td>
</tr>
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</table>

Nitric oxide radical scavenging activity

The nitric oxide radical scavenging activity of the ethanol and water extracts of seed extract sample exhibited a dose-dependent relation in comparison to the standard, ascorbic acid. The NO scavenging activity of the water extracts was found to be higher than those of the ethanol extracts. At 500 μg/ml concentration, the scavenging activities of the ethanolic extracts of samples at different concentration were 17.14 ± 0.05, 13.20 ± 0.06, 11.30 ± 0.08 and 8.21 ± 0.08%, while that of the water extracts of different concentrations were 35.40 ± 0.09, 36.30 ± 0.20, 26.60 ± 0.18 and 21.60 ± 0.08%, respectively. The scavenging activity of AA, at the same concentration, was 73.90 ± 0.01%.

Table 3: Nitric oxide radical scavenging activity of P. longum extracts

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>NO radical scavenging activity (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>PL-E</td>
</tr>
<tr>
<td>100</td>
<td>45.33 ± 0.05</td>
</tr>
<tr>
<td>200</td>
<td>57.94 ± 0.09</td>
</tr>
<tr>
<td>300</td>
<td>68.11 ± 0.08</td>
</tr>
<tr>
<td>400</td>
<td>72.31 ± 0.04</td>
</tr>
<tr>
<td>500</td>
<td>77.92 ± 0.03</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>136 ± 0.08</td>
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</table>
HPLC
The HPLC chromatogram of standard piperine at an optimum wavelength of 345nm showed a mean area of 7328514.2 at a retention time of 23.733min. The recovery value of standard piperine was 99.4%. The HPLC chromatogram of second concentration of *Piper longum* corresponding to standard piperine was shown at a retention time of 23.616 min with an area of 25754634 at a wavelength of 345nm. As shown at a retention time of 23.723 min with an area of 9101266 at a wavelength of 345nm. Piperine showed good linearity in the concentration range of 0.005% - 0.1% with a correlation coefficient of 0.998.

MS-MALDI
The twelve different mass spectra presented a variation in the pattern of expressed proteins within the investigated range of mass values (1,500 to 15,000 Da). Nevertheless, some proteins have shown a constant presence in all analyzed samples: the proteins with masses around 4,500 Da and 5,100 Da. Posch and co-workers [4] performed a similar study with Capsicum annuum genotypes using 2-D electrophoresis for the detection of seed protein polymorphism. In their study 102 proteins were analyzed (including water-soluble and urea/detergent-soluble proteins). In comparison with 2-D electrophoresis analysis, MALDI-TOF represents a much faster and cheaper methodology, with the generation of results in only a few minutes. Even though the number of protein data sets in our study is likely to be underestimated, because the mass spectrometry analysis only allows a specified range of masses to be analyzed each time for the conditions used, further studies involving the analyses of a wider range of masses would assure a better general view of the functional proteome of the samples, and consequently a more reliable genetic diversity assessment.
CONCLUSION

However, role of aqueous extract of *Piper longum* proteins as an antioxidant has not been studied till date. In this study the protein concentration in aqueous extract of *Piper longum* is considerably higher than other solvent extracts. This is the basis for further *in vitro* studies to unfold the antioxidant activity of aqueous extract of *Piper longum* protein. Also, HPLC and MALDI MS analysis also reveal astounding properties of *Piper longum*. As MALDI MS analysis has been carried out for first time

REFERENCES