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Phytochemical Characterization, Antioxidant and Antibacterial Activity Evaluation of Ethanolic extract of *Piper longum* Root

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ABSTRACT: Plant phytochemicals are found to be responsible for various pharmacological effects on the human health care system. *Piper longum* is one of the most commonly consumed spices, and its pungency is due to the presence of an alkaloid known as piperine. Piperine, the principle bio-molecular active compound of *Piper longum*, is found in black pepper (*Piper nigrum*), white pepper, and long pepper (*Piper longum), all* belonging to the family Piperaceae. Piperine is known as a bioavailability enhancer and elicits numerous pharmacological activities, including analgesic, anti-pyretic bioavailability enhancer, antioxidant, immune-stimulant, hepatoprotective, and many others. It is generally extracted or traditionally obtained from the fruits (pippali) and roots (pippalimula) of wild *Piper longum*, a medicinal plant of the family Piperaceae. The aim of this research paper will be to analyze the phytomolecules and evaluate the important activity of crude extract.

Keywords: *Piper longum,* HPLC, GC-MS, piperine, piperlongumine, antioxidant and antibacterial.

INTRODUCTION

Piper longum, commonly known as "long pepper", is an endangered medicinal plant belonging to the family Piperaceae. The native of this plant is considered to be South Asia and is found both wild and cultivated throughout the hotter parts of India, from the central to the north-eastern Himalayas. The herb also grows wild in Malaysia, Singapore, Bhutan, and Myanmar (Kirtikar and Basu 1993). The fruits of *Piper longum* (long pepper) have been widely used as household spices and also in various traditional systems of medicine. The Spikes and Roots of *P. longum* have been the primary sources of piperine since antiquity. There are several literatures on the piperine content of *P. longum* spikes, but only a few on the piperine content of the root parts (Hu *et al*., 1996; Santosh *et al*., 2005; Rajopadhye *et al*., 2012). But roots of long pepper also consist of piperine and piperlongumine alkaloids (3–5%), pungent resin (6.0%), volatile oil (1-2.5%), piperidine, and starch (about 30%) (Mukherjee, 2008; Kokate *et al*., 2010). Piperine and piperlongumine are major alkaloids found in long pepper that belong to the Piperaceae family and have anti-inflammatory (Bang *et al*., 2009; Sudjarwo, 2005) analgesic (Sudjarwo, 2005), antiarthritic, CNS-depressant, anticonvulsant (Evan, 1997). Properties, among other things. For the treatment of both acute and chronic bronchitis, a decoction made from dried fruits and *P. longum* roots is employed. It contains piperine and has somewhat higher piplartine (piperlongumine) content than *P. nigrum*. Asarinin, a chemical component found in the root of *Piper longum*, has been shown to have significant anticancer properties by causing apoptotic cell death through induction of apoptosis in human ovarian cancer cells (Jeong *et al*., 2019). In human colorectal cancer cells, the combination of piperlongumine and oxaliplatin has been shown to exhibit considerable anticancer activity (Chen *et al*., 2019). In addition to this, piperlongumine causes cell cycle arrest in human breast cancer by way of reactive oxygen species (ROS) (Jeong *et al*., 2019). The piperine and piperlongumine content in *Piper longum* roots was determined using thin-layer chromatography (TLC), GC-MS, and HPLC.

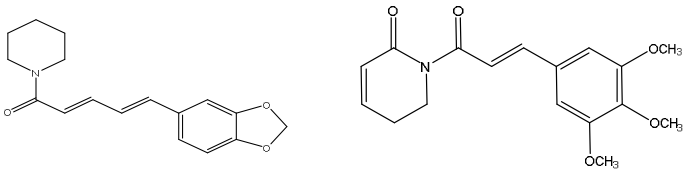
Piper longum is a plant that is usually recognised as pipli or long pepper in India. The plant is an aromatic, perennial, slim climber with woody roots. It is a creeping understory perennial shrub. The branches are erect with puffy nodes. The root is thin and curly, which originates from the nodes and helps when attached to the host plant. The leaves are alternate and ovate in shape, with an acute to acuminate top and an entire glabrous margin. The older leaves of this plant are dentate, brown in color, and heart-shaped. Anatomical studies have been done. *P. longum* is a dioecious plant, having male and female flowers on different plants. Floras have a similar morphology until the spike is formed. Male spears are tubular in shape

and have tiny greenish yellow flowers. Female spears are thin and yellow in colour. The fruits of this plant are oval, green, or yellow, and are sunken in the fleshy spike. These are cylindrical and globular in shape; fruits turn black on ripening and possess a fragrant odour and pungent taste. The highest fruiting season is from November to February, although fruits continue to fruit and ripen in small quantities throughout the year. This plant's fruit contains about 120–130 small seeds. The dried, ripe fruits of the plant are known as pipli (Shivrajnan, 1994).

The wide-ranging usage of this shrub in different preparations is documented in ancient Ayurvedic manuscripts such as Charaka Samhita (Das and Sharma 2004) Susruta Samhita (Murthy, 2012). Vagbhata's Astangahrdayam recommends its importance in the traditional Indian medicinal system (Murthy, 2002; Londonkar and Kesralikar 2023). *P. longum* plant has gained pharmaceutical importance due to the presence of specific components or combinations of secondary metabolites. *P. longum is* most commonly used as a therapeutic agent in various diseases. The fruits and roots of *P. longum* are used to treat analgesia, bronchitis, and many other diseases. It is also most frequently used to treat bronchitis, anti-irritants, constipation, asthma, paralysis of the tongue, cholera, malaria, diarrhea, the spleen, cough, viral hepatitis, cancers, and tumors. Apart from the root and stem of *P. longum,* entire plants are also used for the conventional treatment of a variety of diseases. The roasted fruit powder of this plant is mixed with honey and used to cure rheumatic diseases. It is defined as a virtuous remedy for treating gonorrhea, menstrual pain, and sleeping problems. The fruits of this plant are also useful for tuberculosis, respiratory tract infections, pneumonia, gut-related pain, chest pain, and arthritis problems (Johri and Zutshi 1992).

The decoction of dried fruits and roots of *P. longum* is used in the form of a decoction in the treatment of acute and chronic bronchitis. The compounds of medicinal importance are present in the female spike of *P. longum.* Additional about 500 species belong to this genus, and *P. longum* is one of the best-known species there, including *Piper bettle* and *Piper nigrum. P. longum* forms an active component of the commonly using a poly-herbal Classical combination Trikatu (Tripathi *et al*., 1999). The fruit contains the alkaloids piperine (3-6%), piperidine, piperlongumine, brachyamide-A, retrofractamide A, pergumidiene, epipernonaline, asarinine, pellitorine, piperundecalidine, piperettine, piperlongumine, a dimer of N-isobutyldecadienamide, pipercide, tetrahydropiperine, brachystamide-B, and dehydropipernonaline. It has piperine and piplartine (piperlongumine) content is slightly greater than that in *P. nigrum.* The root of *Piper longum* has been stored with several chemical compounds, mainly asarinin, which has been used a induce female cervical cancer cells undergo apoptotic cell death by the activation of caspase (Jeong *et al*., 2019). The combination of piperlongumine and oxaliplatin has been shown to have significant anticancer activity in human colorectal cancer cells (Cheng *et al.,* 2019). Besides this, piperlongumine play important role in reactive oxygen species (ROS) generation for cell cycle arrest in human breast cancer (Jeong *et al*., 2019). Piperine also plays an important role in being used as a herbal booster to improve curcumin bioavailability. Piperine-based formulations have been administered by a variety of methods, including oral, topical, and intravenous (Pingale *et al*., 2023). Various amides (guineensine and pipericide) are also present as active constituents. Essential oils and resins are also present in the fruit of this plant. The essential oils of the fruits contain terpinolene, thujone, zingiberene, p-cymene, ethoxyacetophenone, dihydrocarveol, and vitamins E and A. Palmitic acid and tetrahydropiperic acid contain the most organic acids (Dutta and Gupta 1980). Roots of the plant have been known to contain piperine, pipelongumine, or piplartine, and dihydro-stigmasterol (Neelam *et al*., 2001). Recently researcher proposed *Piper longum* along with *Ocimum sanctum* phytoconstituents as ACE2 and TMRSS2 inhibitors: methods to combat COVID-19 (Jindal and Rani 2022).

Fig. 1. (A) *Piper longum* plant (B) Plant root.



a. Piperine

b. Piperlonumine

Fig. 2. Structure of piperine and piperlongumine.

There are four isomeric forms of piperine, isopiperine, chavicine, and isochavicine. The geometrical isomers of piperine have no pungency compared to piperine.

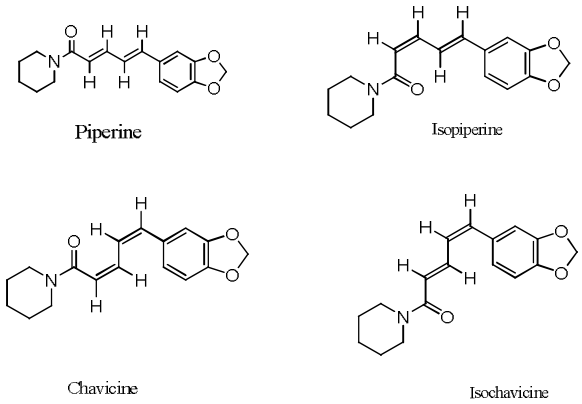


Fig. 3. Structures of piperine and its isomers.

MATERIAL AND METHODS

A. Materials

(i) Chemicals and reagents. The organic solvents used for extraction were of analytical grade from Fisher Scientific (UK) and Labscan (Thailand). Acetonitrile (HPLC grade, Fisher Scientific, UK), Orthophosphoric acid (analytical grade, BHD, England). Ultrapure water from Milli-Q system (Millipore, Bedford, USA) was used for the mobile phase preparation. The standard agents and melphalan were provided by Sigma-Aldrich Chemie GmbH (Germany).

(ii) Plant materials. *Piper longum* (long pepper) roots collected from CSIR-Central Institute of Medicinal and Aromatic Plant, Lucknow, India. All chemicals were provided by Fino Scientific House, Kanpur 208012.

B. Methods

(i) Standard Preparation. A standard stock solution of 1 mg/ml was prepared using standard piperine (SIGMA Aldrich, Germany) in methanol and kept at 4 °C for further use.

(ii) Extraction with ethanol. In a maceration extractor, 200 gm of *P. longum* root powder were extracted three times for 24 hours with 1000 ml of 95% ethanol. The solution was concentrated in a water bath at 40–50 °C after filtering through Whatman paper (125 mm).

(iii) Extraction with Methanol. In a maceration extractor, 200 gm of *P. longum* root powder were extracted three times for 24 hours with 1000 ml of 95% methanol. The solution was concentrated in a water bath at 40–50 °C after filtering through Whatman paper (125 mm).

(iv) Extraction with Diethyl Acetate. 200 gm of *P. longum* root powder were extracted with 1000 ml of distilled water in a maceration extractor for 24 hours, three times. The solution was concentrated in a water bath at 40–50 °C after filtering through Whatman paper (125 mm).

(v) Extraction with diethyl ether. 200 gm of *P. longum* root powder were extracted with 1000 ml of distilled water in a maceration extractor for 24 hours, three times. The solution was concentrated in a water bath at 40–50 °C after filtering through Whatman paper (125 mm).

(vi) Extraction with chloroform. In a maceration extractor with 1000 ml of distilled water, 200 gm of *P. longum* root powder was extracted three times for 24 hours. The solution was concentrated in a water bath at 40–50 °C after filtering through Whatman paper (125 mm).

(vii) Extraction with Hexane. 200 gm of *P. longum* root powder were extracted with 1000 ml of distilled water in a maceration extractor for 24 hours, three times. The solution was concentrated in a water bath at 40–50 °C after filtering through Whatman paper (125 mm).

(viii) Extraction with Acetone. 200 gm of *P. longum* root powder was extracted with 800 ml of distilled water in a maceration extractor for 24 hours, three times. The solution was concentrated in a water bath at 40–50 °C after filtering through Whatman paper (125 mm).

C. Isolation and purification by Column Chromatography

(i) Preparation of sample. Preparation of sample of Ethanolic extract (EE) for the column chromatography was done by adsorption of EE on activated Silica Gel (60-120) (105°C, 30 minutes) with ratio 1:10 respectively. It was kept for drying in an oven till free flowing material was formed.

(ii) Column specification and Solvent system. The dried prepared sample was subjected to column chromatography (CC), using a 38 × 4.5 cm glass column filled with silica gel 60 (mesh size: 60–120) in toluene: ethyl acetate (7:3). Prepared sample of EE extract was added to the free volume at the head of the column. After settling down of the material, Fractions (20 ml, 8 drops/minute) were collected, and the solvent was removed to reduce volume of fraction by evaporation in vacuum at 35°C. Fractions were monitored by TLC method with same solvent system and concentrated H_2SO_4 was used as spraying reagent.

Analysis of fractions by Thin Layer Chromatography (TLC). A precoated TLC plate (Silica Gel GF254 Plates, 20 cm × 10 cm, Merck) with a solvent system of chloroform, acetone, formic acid, and spray reagent was used to study various extracts. Anisaldehyde, water, acetone, and perchloric acid. The separation profile of PLRE on TLC was observed by putting the plate in an iodine chamber and confirmed with a concentrated H_2SO_4 solution. For every spot on the TLC Glass plate, Rf values were estimated. The genuine sample was additionally used to its RF value to separated piperine. The RF values for the fractions were nearly identical.

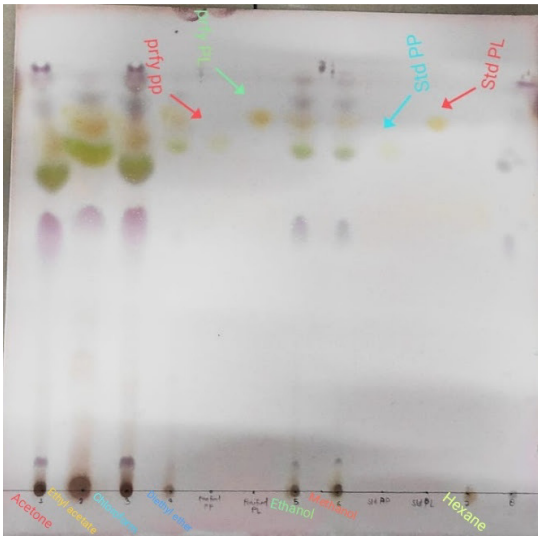


Fig. 4. Thin Layer Chromatography reveals presence of piperine and piperlonumine in various extract of *P. longum* root.

HPLC analysis. HPLC analysis was performed in HPLC system (Make-Waters) equipped with binary pump (Model-1525) and porous Silica with 5 µm diameter C 18 4.6 × 150 mm column. The mobile phase selected was consisted of a mixture of HPLC grade methanol and water, in a ratio of 60: 40, at a flow rate of 1 ml/min. The peaks were detected at 345 nm wavelengths and compared with authentic standard piperine sample for confirmation. The reproducibility of quantitative analysis was verified by carrying out ten replicate injections of standard and three replicate injections of each extract.

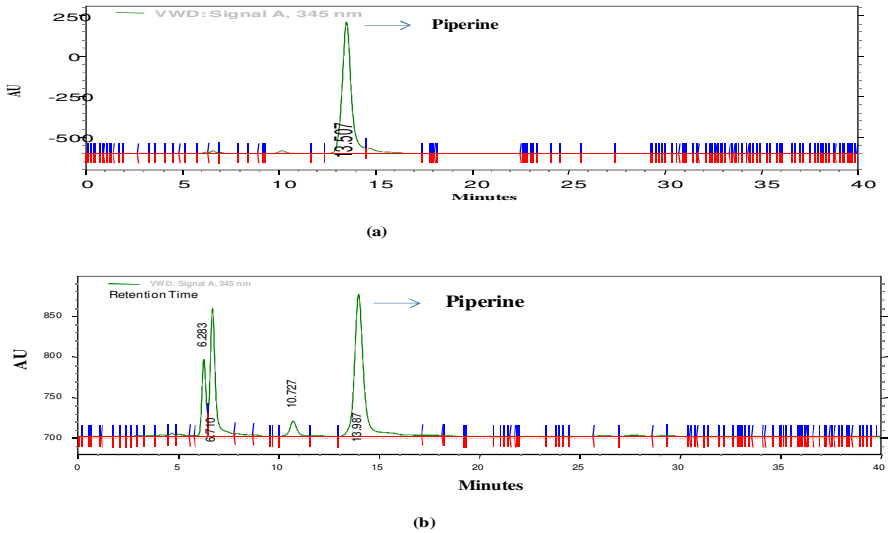


Fig. 5. (a) HPLC profile of Piperine standard (Rt =13.507 min) (b) HPLC profile of root extract of PL. The highest peak had a retention time (Rt) of 13.987 min which was similar to that of standard piperine (Rt= 13.507 min).

Table 1: Thin Layer Chromatography reveals presence of piperine and piperlonumine in various extract of *P. longum* roots.

Sr. No.	Solvent	Piperine	Piperlongumine
1.	Ethanol	+	+
2.	Chloroform	+	+
3.	Diethyl ether	+	+
4.	Methanol	+	+
5.	Ethyl acetate	+	+
6.	Hexane	-	-
7.	Acetone	+	+

+ present; - absent

Gas chromatography-mass spectroscopy (GC-MS). The solvent extraction was done (250 mg of sample kept in 1 ml of hexane for 1 hour in RT, and after moisture and debris removal, 2 µl from the same taken for analysis) and brought up for further analysis by GC-MS (Agilent Technologies 7980 A gas chromatograph system with the 5977 A mass selective detector). 2 µl of hexane extracted sample was injected for GC-MS analysis. The HP5-MS column with dimension 30 m × 320 µm having film thickness 0.25 µm was used for obtaining the peak separation in the chromatogram. Helium in a split ratio of 10:1 and flow rate of 1.5 ml/min was used as the carrier gas. The running condition for the sample is as following: 2 min initial hold at 50°C, increase up to 125°C at a rate of 7°C per min, 2 min hold at 125°C, increase up to 250°C at a rate of 3°C per min followed by a hold at this temperature for 5 minutes, finally increase up to 300°C at a ramp rate of 10°C per min followed by a hold at this temperature for 2 minutes. Mass spectrometry was conducted at 250 °C as a transfer line and ion source temperature while 150 °C as quadruple temperature, 70 eV ionization potential and 50 to 550 atomic mass units scan range. Data was processed by the MSD Chemstation F.01.01.2317 software. Version 2.0 g of NIST/EPA/NIH mass spectral library was used for compound identification (Agilent Technologies, Palo Alto, CA, USA).

Table 2: Results from GC–MS analysis of the essential oils in *P. longum* root.

Sr. No.	Retention time	Compound name	Mol Weight (amu)	CAS Number
1.	5.568	Furan, tetrahydro-2,5-dimethyl-, cis-	100.089	002144-41-4
2.	5.969	2-Pentanol, 2-methyl-	102.104	000590-36-3
3.	6.37	3-Pentanol, 3-methyl-	102.104	000077-74-7
4.	6.742	Toluene	92.063	000108-88-3
5.	10.642	alpha.-Pinene	136.125	000080-56-8
6.	10.642	beta.-Pinene	136.125	000127-91-3
7.	10.791	2-Nonanone, 9-[(tetrahydro-2H-pyran-2-yl)oxy]-	242.188	054699-41-1
8.	11.972	Mesitylene	120.094	000108-67-8
9.	12.744	D-Limonene	136.125	005989-27-5
10.	13.287	Decane, 3,6-dimethyl-	170.203	017312-53-7
11.	13.948	o-Cymene	134.11	000527-84-4
12.	13.948	p-Cymene	134.11	000099-87-6
13.	14.587	Benzene, 1,2,3,4-tetramethyl-	134.11	000488-23-3
14.	16.02	Azulene	128.063	000275-51-4
15.	20.619	Caryophyllene	204.188	000087-44-5
16.	20.767	trans-.alpha.-Bergamotene	204.188	013474-59-4
17.	20.886	beta.-GURJUNENE	204.188	1000425-17-9
18.	21.265	Humulene	204.188	006753-98-6
19.	21.837	alpha.-Guaiene	204.188	003691-12-1
20.	21.837	Valerena-4,7(11)-diene	204.188	351222-66-7
21.	21.837	Longifolene	204.188	000475-20-7
22.	21.837	Aromandendrene	204.188	000489-39-4
23.	21.882	trans-Verbenol	152.12	001820-09-3
24.	21.963	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.beta.)]-	204.188	003691-11-0
25.	22.773	Nerolidol 2	222.198	1000285-43-6
26.	23.241	Diethyl Phthalate	222.089	000084-66-2
27.	23.791	Isospathulenol	220.183	088395-46-4
28.	26.844	Dibutyl phthalate	278.152	000084-74-2
29.	29.303	Retrofractamide-A	327.183	094079-67-1
30.	30.93	gamma.-Sitostenone	412.371	084924-96-9
31.	31.257	(E)-5-(Benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl)pent-2-en-1-one	287.152	023512-46-1
32.	32.163	Piperidine, 1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]-, (Z,Z)-	285.136	000495-91-0
33.	32.95	Piperlonguminine	285.136	000094-62-2
34.	33.508	Piperine	285.136	000094-62-2
35.	36.568	(+)-Sesamin	354.11	000607-80-7

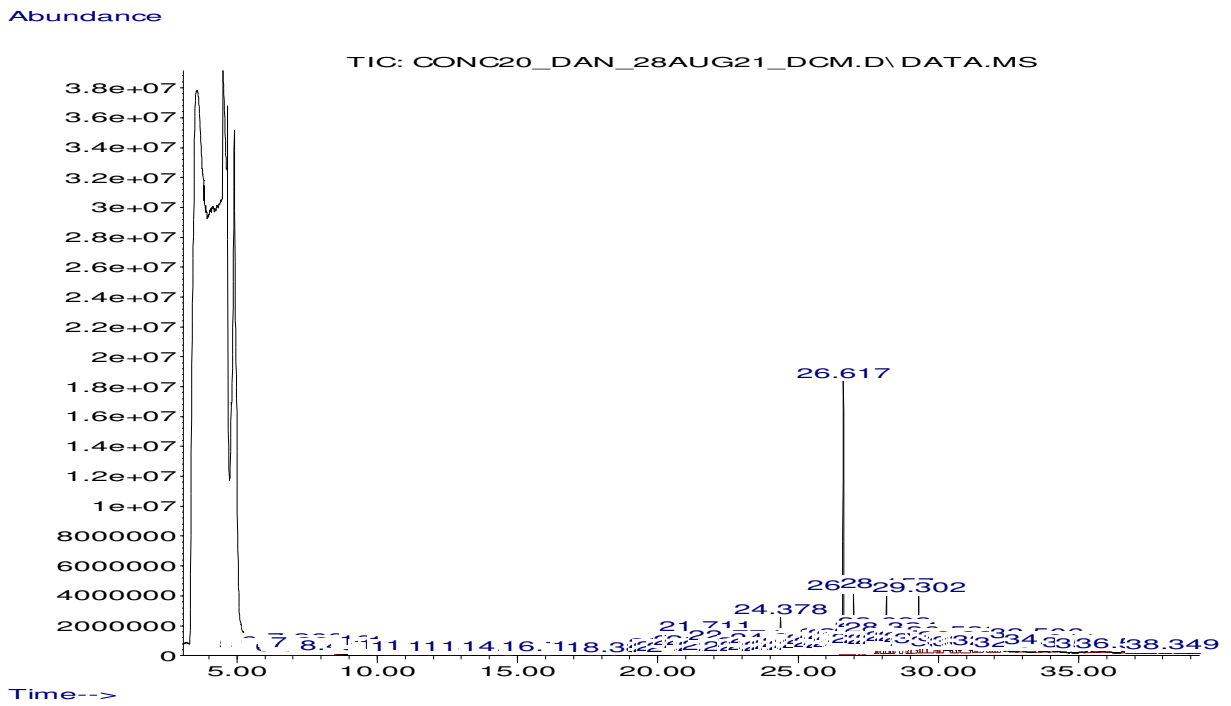


Fig. 6. GC-MS total ion analysis of hexane extract of *P. longum* root.

Approaches for Assessing Antioxidant Capacity

DPPH free radical scavenging activity. The DPPH radical scavenging activity was carried out according to Govindarajan's approach (Govindrajan *et al*., 2003). The stock solutions were serially diluted to produce concentrations of 1, 2, 4, 6, 8 and 10 mg/ml. The sample solution and the 0.1 mM DPPH solution were combined in equal parts. After being vortex, the mixture was left in the dark for 30 minutes. Using a double beam Analykjena UV/Visible spectrophotometer, the absorbance of the combination was read against a blank at 517 nm after incubation (Model 205, Jena, Germany). The dpph scavenging activity was expressed as the inhibition percentage (I %) and calculated as per the equation: I (%) = (A control– A sample/A control) × 100, where A control is the absorbance of the control (containing all the reagents except the testing compound), and A sample is the absorbance of the experimental sample with all reagents. The graph of inhibition (%) against the amount of the extract was used to determine the IC50 value, or the concentration of a sample needed to scavenge 50% of the DPPH radical. Each evaluation was made in three copies, and the average was recorded. Ascorbic acid served as the typical antioxidant standard.

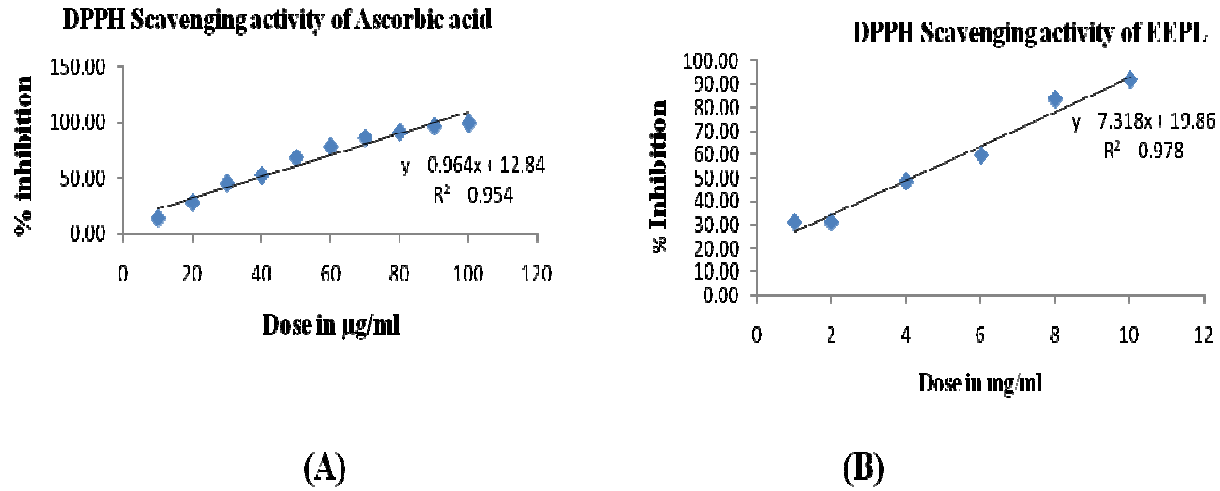


Fig. 7. DPPH Scavenging activity of (A) Ascorbic acid (standard) (B) Ethanolic extact of *piper longum* root.

Antibacterial activity. The in vitro antibacterial activity of PLRE was evaluated at concentrations of 100-500 mg/mL against *S. aureus* (Gram positive) and *E. coli* (Gram-negative) bacteria by disc diffusion method using Mueller- Hinton agar (MHA) medium as reported previously. Antibiotic linezolid and piperacillin tazobactam (PIT) were used as a positive control while 100% DMSO served as a negative control.

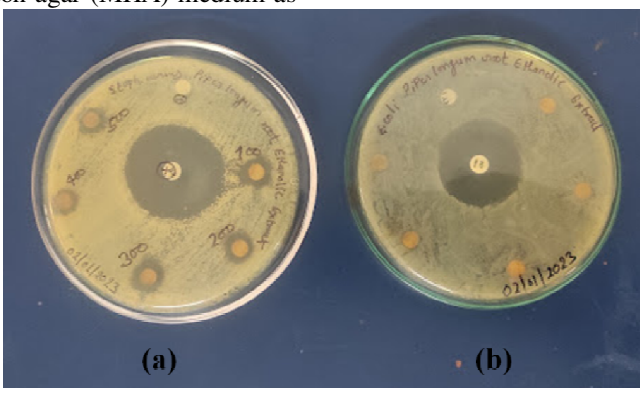


Fig. 8. Antibacterial activity of ethanolic extracts of *Piper longum* root against (a) *Staphylococcus aureus* and (b) *Escherichia coli.*

RESULT AND DISCUSSION

Piperlongumine and piperine content were assessed from the various root extracts of *P. longum* collected from CSIR-Central Institute of Medicinal and Aromatic Plant, Lucknow, Through TLC, GC-MS, and HPLC methods. In this experiment, a comparative analysis of seven types of extraction solvent was prepared. Piperlongumine and piperine content were found to be highest in the roots of plants grown from nodal vine cuttings in the experiment. However, the plants grown through petiolar and apical cuttings also showed the presence of piperine content in a visible quantity that can be utilised by pharmacists for drug formulation. Last but not least, the HPLC system is found to be the most precise and accurate method for quantitative estimation of piperine as compared to the crude spectrophotometric method. Antibacterial activity of ethanolic extract against *E. coli* and *S. aureus* (dose 100-500 mg/ml). However, such interpretations require further validation by unique in vitro and in vivo investigations and molecular dynamics simulation.

CONCLUSIONS

The conventional choice Indian medicinal herbs are thought to be a large basis for disease therapy. We studied the potential of certain phytocompounds derived from the most widely used medicinal plant, Piper longum, for this study. Several diseases and ailments, including as diabetes, inflammation, hepatotoxicity, depression, obesity, and cancer have been shown to be significantly improved by *P. longum.* The plant markedly improves microbial infections, cardiac disease, and protects against the effects of radiation. The specific effects of the plant make it more useful for animals and human beings. Furthermore, the plant appears to be nontoxic, as no deaths have been reported with the use of high doses of the plant's extracts. We conclude that this plant is safe and effective for use in various diseases. The plant is easily available, inexpensive, and free from adverse effects. Thus with the matter collected in this article we can scientifically work for various other pharmacological interrelated activities.

FUTURE SCOPE

The future of this research study is to provide a complete description of phytochemical constituents as well as a participatory scientific assessment of the important phytocompounds and their pharmacological action for the future creation of novel ethnomedicine.

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Conflict of Interest. None.

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