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### Antibacterial Activity of Extracts of *Piper longum*

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**Abstract:** Dry roots of the plant *Piper longum* were extracted with n-hexane. The constituents were isolated and purified by column chromatography. The structures of the isolated constituents were confirmed by spectral analysis. The isolated constituents and n-hexane extract were found to show varying degree of antibacterial activity against all the tested bacteria. However, the aqueous extract did not show antibacterial activity against the tested bacteria. The isolated constituents were found to show better activity profile than the n-hexane extract, which indicates that the isolated constituents might be responsible for the antibacterial activity. The Minimum Inhibitory Concentration (MIC) value of piperine against *Bacillus cereus* and *Escherichia coli* was found to be 12.5 mg mL<sup>-1</sup>.

**Key words:** Antibacterial activity, n-hexane extract, minimum inhibitory concentration, *Piper longum*

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### INTRODUCTION

It is well documented from ancient times that the active principles from plant origin have been used as medicines for various diseases and microbial infections. These active principles from plant origin have provided numerous crucial molecules in the search of new drug medicine (Borris, 1996). The search of natural products has revolutionized the drug discovery programme. Many plant derived molecules have shown a promising effect in therapeutics. The diverse behavior of bacteria has always presented challenge in the treatment of their infections. Very few antibiotics are effective against *Pseudomonas* including fluoroquinolones, gentamycin and imipenem. Even these antibiotics are not effective against all strains. The children (below 5 years age) who are susceptible to *Escherichia coli* infection show symptoms of hemolytic uremic syndrome, in which the red blood cells are destroyed and the kidney fails, about 2-7% cases show these types of complications (Quiroga *et al.*, 2001). The resistance of bacteria against the traditional antibiotics needs urgent attention and thus necessitates for the development of the new drug molecules. The uses of medicinal plants for the development of the new drug molecule against bacterial infections show bright future (Baker *et al.*, 1995). A wide variety of medicinal plants used traditionally have not yet been systematically investigated against various microbial pathogens (Farrukh and Iqbal, 2003).

Plant derived products have shown their beneficial role in the treatment of chronic as well as infectious diseases (Ozlem, 2002). Several antibacterial drugs such as ciprofloxacin are available for the treatment of bacterial diseases. However their use is limited for many reasons such as poor solubility, low potency, emergence of resistant strains and the toxicity. Hence, it is necessary to develop new and more effective antibacterial agents (Penna *et al.*, 2001).

Since ancient times dried fruits and roots of the plant *Piper longum* (Piperaceae) have been used as thermogenic, stomachic, aphrodisiac, carminative, expectorant, laxative, digestive, emollient, anti-giardiasis, antiamoebic, anti-asthmatic, antiseptic and also active against bacterial diseases (Kirtikar and Basu, 1984; Warriar *et al.*, 1995).

Chemical investigation of the plant showed the presence of piperlongumine, piperlonguminine, piperine, sesamin, 3,4,5-trimethoxycinnamate,  $\beta$ -sitosterol, piperidine, aristrolactams, pipartine and hexacosanoic acid isobutyl amide (Desai *et al.*, 1989). Piperine (1-[5-[1,3-benzodioxol-5yl]1-oxo-2,4-pentadienyl]piperidine) is the major constituent of *Piper longum*. Piperine and the other constituents have been found to play an important role in bioavailability of various structurally and therapeutically diverse drugs (Agarwal *et al.*, 1997).

Amides isolated from *Piper longum* show diverse pharmacological and biochemical properties (Dahanukar and Karandikar, 1984). *Piper longum* (Piperaceae) is also widely used as a folk medicine to cure diseases such as leprosy and tuberculosis (Srinivasa *et al.*, 2001). The other species having antimicrobial activity are *Piper aduncum* (Orjala *et al.*, 1993) and *Piper betle* (Nair and Bruke, 1990).

Since the plant *Piper longum* has been used traditionally in medicine and the fruits of the plant have been used as food material, the biological evaluation of plants may lead to development of more safe therapeutic agents (Catalano *et al.*, 1998). The data shows that antibacterial profile of the roots of the plant has not been studied, the detail investigation of the antibacterial profile may lead to a development of more potential antibacterial agents with less resistance and toxicity. Hence the aim of the present study was to isolate various constituents of roots of *Piper longum* and to study their antibacterial activity.

## MATERIALS AND METHODS

### Plant Material

The dried roots of the *Piper longum* were obtained from local market of Pune, India. The plant material was authenticated by Botanical Survey of India, Pune (Specimen Voucher No. 2004/12.)

### Chemicals

n-Hexane, ethyl acetate, methanol, chloroform, benzene, toluene and dimethyl formamide were purchased from Merck India Ltd., Mumbai. Nutrient agar was purchased from Hi-media, Mumbai. Streptomycin (Nicholas, India) was purchased from the local market of Pune. Silica gel chromatographic grade for the separation was obtained from Rankem, India. All other chemicals used were of high purity.

### Bacterial Strains

*Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Serratia marcescens*, *Shigella dysenteriae* and *Staphylococcus aureus* were obtained from the Department of Microbiology, University of Pune, Pune. All the strains were maintained on nutrient agar medium.

### General Experimental Procedure

Melting points were determined in an open capillary (Gallenkamp melting point apparatus) and were uncorrected. IR spectra were recorded on Shimadzu 8400 FT Infrared spectrometer. <sup>1</sup>H NMR was recorded on Varian-Mercury (300 MHz) instrument with chemical shift data reported in ppm. Mass spectra were recorded on GC-MS Shimadzu (QP 5050) instrument.

### Extraction and Isolation

The dried and hard roots were first chopped into small pieces, crushed in mortar pestle. Crushed material was further grinded in blender to make fine powder. The root powder was subjected to Thin

Layer Chromatographic (TLC) analysis by using solvents like n-Hexane, ethyl acetate, methanol, chloroform, benzene, toluene, etc. n-Hexane has shown the presence of maximum spots on TLC plate and hence has been selected for the isolation. TLC plates were observed under UV light as well as in an iodine chamber.

The dried root powder (200 g) of *Piper longum* was extracted with n-hexane at room temperature for 48 h. This extract was then filtered and evaporated under reduced pressure to obtain a viscous mass (6 g). A small quantity (1 g) of n-hexane extract was preserved for antibacterial activity and remaining extract was chromatographed over silica gel using n-hexane as solvent (Wu *et al.*, 2004; Kambizi *et al.*, 2004). A total of twenty fractions were (50 mL each) collected. Major constituents were identified using spectral techniques like IR, NMR and GC-MS spectroscopy. The extraction of the plant resulted in the isolation of four known constituents. The isolated constituents were then subjected for the evaluation of their antibacterial activity.

#### **Aqueous Extract**

The aqueous extract of the root powder was prepared by refluxing the root powder (100 g) in 250 mL of distilled water for 16 h and then filtered through Whatman filter paper No. 1. The filtrate was concentrated so as to obtain a solid mass (5 g) (Deshpande *et al.*, 1999). The solid residue obtained was then preserved in tightly closed container in a refrigerator for further study.

#### **Antibacterial Activity**

Antibacterial activity of the isolated constituents and the crude extract was determined by the well diffusion method (Rios *et al.*, 1988; Mosquera *et al.*, 2004). The test constituents and the crude extract were dissolved in dimethyl formamide (DMF). The microbial cultures were grown at 37°C for 18 h and then approximately diluted by sterile saline (0.9% w/v) solution to obtain a cell suspension of  $10^5$  CFU mL<sup>-1</sup>. Diluted inoculum (0.2 mL,  $10^5$  CFU mL<sup>-1</sup>) of test organism was spread on nutrient agar plates. Wells of 6 mm diameter were punched into the agar medium and filled with 20 µL each of four isolated constituents (100 mg mL<sup>-1</sup>) n-hexane and aqueous extract (500 mg mL<sup>-1</sup>). The plates were incubated for 18-24 h at 37°C. The antibacterial activity was evaluated by measuring the Zone of Inhibition (ZOI). The antibiotic streptomycin at 100 µg mL<sup>-1</sup> was used in the test system as positive control. The average for the inhibition zone was obtained from three replicates for the isolated constituents as well as n-hexane and aqueous extracts.

#### **Minimum Inhibitory Concentration Assay (MIC)**

The Minimum Inhibitory Concentration (MIC) values were determined only with those microorganisms that showed inhibitory zones greater than 20 mm (Demo *et al.*, 2005). The microorganisms selected for the study were *Bacillus cereus* and *Escherichia coli*. Piperine showed the maximum (>20 mm) zone, hence has been selected for MIC assay. The dilutions 100, 50, 25 and 12.5 mg mL<sup>-1</sup> were prepared in DMF so as to get effective concentration as 2, 1, 0.5 and 0.25 mg/well. The assay was carried out and zone of inhibition was measured. The MIC values were determined as the lowest concentration of the constituent which completely inhibited the growth (Mazzanti *et al.*, 2000).

## **RESULTS AND DISCUSSION**

Spectral data shows that the major constituents structurally resembled to that of piperine, piperlonguminine, piperlonguminine and pipartine. The isolated constituents of the plant showed moderate to high antibacterial activity with two Gram positive and six Gram negative bacteria. The n-hexane extract of the plant showed poor antibacterial activity against all the tested microorganisms.

Table 1: Antibacterial activity of extracts of *Piper longum*

Bacteria	Zone of inhibition (mm)				Extract	Standard
	Piperine	Piperlongumine	Piperlonguminine	Piplartine	(Pet ether)	(Streptomycin)
<i>Pseudomonas aeruginosa</i>	10	15	11	10	11	18
<i>Bacillus cereus</i>	24	12	10	14	14	27
<i>Serratia marcescens</i>	11	13	11	-	06	25
<i>Escherichia coli</i>	22	11	14	12	12	20
<i>Shigella dysenteriae</i>	11	10	12	-	-	21
<i>Salmonella typhi</i>	15	12	14	-	09	26
<i>Staphylococcus aureus</i>	15	14	10	-	06	20
<i>Klebsiella pneumoniae</i>	13	15	09	-	-	25

Maximum Inhibition >20 mm, Moderate Inhibition 16-20 mm, Poor Inhibition 10-15 mm, (All values are compared with standard Zone of Inhibition)

The n-hexane extract did not show the zone of inhibition against the Gram negative bacteria, *Shigella dysenteriae* and *Klebsiella pneumoniae*. The aqueous extract of the plant did not show any activity against all tested microorganisms (Table 1).

The isolated and purified constituents piperine, piperlongumine, piperlonguminine, piplartine inhibited the growth of microorganism at effective concentration of 2 mg/well while n-hexane extract shows same effect at 10 mg/well. Piperine, piperlongumine, piperlonguminine showed zone of inhibition against all the tested bacteria in a varied way. Piperine showed maximum zone of inhibition against *Bacillus cereus* and *Escherichia coli*. While it showed poor antibacterial activity against the remaining tested microorganisms. Piperlongumine and Piperlonguminine showed poor zone of inhibition against all the tested bacteria.

Piplartine was found to show poor inhibition against one Gram positive bacteria, *Bacillus cereus* and two Gram negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*. Piplartine did not show any antibacterial activity against remaining five bacteria. Table 1 indicates that n-hexane extract is comparatively less effective than the purified constituents. The tested constituents, n-hexane extract inhibitory zones were compared with the standard streptomycin zones.

The earlier studies (Srinivasa *et al.*, 2001) indicated the antibacterial activity of the fruits of *Piper longum* and no data is available on the antibacterial activity of the roots and its constituents. Since piperine showed better activity profile it has been subjected for MIC assay. The MIC values indicate that piperine is active at a low concentration of 12.5 mg mL<sup>-1</sup> when tested against *Bacillus cereus* and *Escherichia coli*.

## CONCLUSIONS

The data showed that the isolated constituents show antibacterial activity against all the tested microorganisms. The isolated constituents particularly piperine shows better activity profile. As the crude extract is mixture of all the constituents, the purity and the concentration of the isolated constituents exert better activity profile than crude extract. The basis of varying degree of sensitivity of test organism is due to the intrinsic tolerance of microorganism and the difference in chemical nature and structure of the constituents. On the basis of results obtained in present study, the detailed investigation of isolated constituents for their mode of action on the control of growth of microorganism is beneficial. Roots and fruits of the plant have been used as a food material in Asian countries, hence the isolated constituents are useful to develop the molecules against infectious diseases. Piperine has shown the better activity profile against Gram positive and Gram negative bacteria and hence it is a best target for further research for the development of broad spectrum antibacterial agents.

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