

**INHIBITORY RESPONSE OF DRUG RESISTANT BACTERIA
TOWARDS METHANOL EXTRACT OF *PIPER LONGUM* L.
FRUIT**

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Summary

Plant extract could be a potential source of compounds usable against antibiotic resistant pathogenic bacteria because of structurally novelty from traditionally used microorganisms with high stability and activity in extreme conditions. The activity of different solvent extracts of *Piper longum* L. fruit on some selected pathogenic drug resistant bacterial strains were weight up. The fruit extracts were evaluated against four gram positive and four gram negative pathogenic bacteria by Disk diffusion method. Fruit extracted in methanol,

chloroform and hexane were effective against all tested bacterial strains however, methanol extract showed relatively better antibacterial activity against most of the tested bacterial strains. Methanol extract of *Piper longum* L. fruit also showed excellent antibacterial activity against hospital isolates *Pseudomonas aeruginosa*^R, *Salmonella typhi*^R and *Staphylococcus aureus*^R which were resistant to Ofloxacin. The present work will be a pioneer approach towards solving the most challenging problem of antibiotic therapy i.e. developing resistance in bacterial pathogens against commonly and currently used antibiotics.

Keywords: Medicinal spice, Drug resistant bacteria, *Piper longum*, Methanol extract.

Abbreviations:

(^R) Resistant strain
CFU Colony- forming Units
DMSO Dimethylsulfoxide

Introduction

Natural products are important source of innovative therapeutic agents for, infectious diseases, cancer, lipid disorders, and immunomodulation(1). Several experimental studies have contributed scientific evidence for the pharmacological effects of medicinal plants observed in folk medicine (2). Antimicrobial screening and phytochemical investigations of plants from diverse region of the world have established the presence of antimicrobial activities and novel antimicrobial compounds in many species (1, 3, 4, 5, 6, 7, 8). Pharmacological industries have produced a number of new antibiotics in the last three decades, however, developing resistance in bacterial pathogens against commonly and currently used antibiotics has necessitated a search for structurally novel antibacterial substances from plant sources other than the traditional microorganisms (9). The dried fruit of one such plant *Piper longum* Linn (Piperaceae), commonly known as Indian Long pepper, used as a spice and seasoning, is known to possess multitude of

pharmacological activities. The fruits and roots are attributed with numerous medicinal uses, and may be used for diseases of respiratory tract, viz. cough, bronchitis, asthma also as anti-irritant and analgesic. The fruits have been used as liver tonic, stomachic, emmenagogue, abortifacient, aphrodisiac and digestive (10). However, root and stem are used as an important drug in the Ayurvedic and Unani systems (11). Review of literature revealed that the all plant parts of *Piper longum* are very useful and the fact that few reports are available on the antibacterial activity of *Piper longum* fruit extract (12, 13,14).

However, there is no report regarding the antibacterial effect of *Piper longum* fruit extract against pathogenic bacteria which are resistant with antibiotics. Therefore the aim of present work was to weight up the antibacterial efficacy of different solvent extracts of *Piper longum* fruit on some selected pathogenic bacterial strains.

Material and methods

Plant materials: The fruits of *Piper longum* L. were bought from a local herbal market, and the identity of the fruits were authenticated at the Department of Dravyaguna, Faculty of Ayurveda, Institute of Medical Sciences, Banaras Hindu University (B.H.U.), Varanasi.

Preparation of extracts: The collected fruits were washed thrice with distilled water and dried on blotting paper in laboratory at $(37 \pm 1)^{\circ}\text{C}$ for 24 hours. After drying, the fruits were ground in grinding machine. Dry powdered plant fruits were separately extracted in methanol, chloroform, hexane, petroleum ether to obtain their extracts. Fifty gram of each sample was soaked in analytical grade organic solvents (2x200 mL) in stoppered flasks for 24 h at room temperature with occasional shaking. Extracts were filtered by filter paper (Whatmann No.1), concentrated and dried under reduced pressure in a rotary evaporator. All the dried extracts were stored in airtight vials at 4°C for subsequent use. To rule out the possibility of phototransformation of active principle(s), direct exposure to light was avoided during extraction.

Test organisms: The bacterial strains used during study were obtained from the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. The strains were sensitive to antibiotics were four Gram positive *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Bacillus*

megatarium, and four Gram negative *Pseudomonas aeruginosa*, *Vibrio cholera*, *Shigella dysenteriae*, *Salmonella typhi*. Three strains insensitive to Oflaxacin are *Pseudomonas aeruginosa*^R, *Staphylococcus aureus*^R and *Salmonella typhi*^R isolated from hospital.

Screening for anti-bacterial activity: Sensitivity of bacterial strains to the commonly used antibiotics and extract (organic solvents) of fruits of *Piper longum* was assayed by using the modified Kirby Bauer Disk Diffusion susceptibility method (15). The bacterial strain (4-5 colonies) to be tested was suspended in 4ml of normal saline (0.85 %) and the density of suspension adjusted to approximately 10^8 CFU ml⁻¹ using a 0.5 M barium sulphate suspension as the turbidity standard. The surface of the sterile 3.8% Mueller Hinton Agar (Hi-Media) in Petri dishes was dried and the test strain was inoculated with a sterile swab to obtain a bacterial lawn. High potency bio-discs (Himedia) were placed on the agar to determine the inhibition zone created by different antibiotics used in the study. 10µl of fruit extracts was placed directly on the lawn. After incubation for 18 hours at 37⁰C the diameter of the inhibition zone was measured. DMSO was taken as control for organic extracts. The dissolution of different organic solvent extracts was aided by 1% (v/v) DMSO with water which did not affect the growth of microorganisms in accordance with our control experiments.

Results

To evaluate the antibacterial property, the results of different organic solvent extracts of *Piper longum* fruit were shown in table 1.

Table 1: Effect of fruit extract (500 mg/ml) of *Piper longum* extracted in different organic solvent

Test organisms	Inhibition zone diameter (mm)			
	Methanol extract	Chloroform extract	Hexane extract	Petroleum ether extract
Gram positive				
<i>S. aureus</i>	22	24	14	-
<i>E. faecalis</i>	24	22	18	-

<i>S. pneumoniae</i>	24	22	14	-
<i>B. megatarium</i>	22	20	16	-
Gram negative				
<i>P. aeruginosa</i>	30	24	16	-
<i>V. cholerae</i>	24	22	18	-
<i>S. dysenteriae</i>	22	18	14	-
<i>S. typhi</i>	18	22	16	-

(-) No inhibition

Here, each solvent extract was inhibitory (methanol and chloroform extract having more potent) to all tested gram positive and gram negative bacterial strain except petroleum ether.

Different concentrations of methanol extract were screened to evaluate the antibacterial potency against gram positive and gram negative bacterial strains (Table 2).

Table 2: Evaluation of methanol extract for inhibition zone at various concentrations against different bacterial strains

Test organisms	Inhibition zone (mm)					
	Concentrations of Methanol extract (mg/ml)					
	400	300	200	100	10	1
Gram positive						
<i>S. aureus</i>	20	16	15	13	10	-
<i>E. faecalis</i>	18	16	14	14	10	-
<i>S. pneumoniae</i>	20	18	16	12	-	-
<i>B. megatarium</i>	20	17	15	12	-	-
Gram negative						
<i>P. aeruginosa</i>	25	23	19	17	13	10
<i>V. cholerae</i>	22	20	17	14	8	-
<i>S. dysenteriae</i>	17	15	14	12	-	-
<i>S. typhi</i>	20	18	17	14	12	-

(-) No inhibition

Methanol extract (400mg/ml), was highly inhibitory against gram negative strains *Pseudomonas aeruginosa* followed by *Vibrio cholerae*, *Salmonella typhi* and *Shigella dysenteriae*, this concentration was also inhibitory against gram positive strain *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Bacillus megatarium* followed by *Enterococcus faecalis*. Methanol extract at lower concentration was inhibitory against *Pseudomonas aeruginosa* followed by *Salmonella typhi*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Vibrio cholerae*.

On the basis of above results, strains *Pseudomonas aeruginosa*^R, *Salmonella typhi*^R and *Staphylococcus aureus*^R were tested for sensitivity with methanol extract as well as with some commonly used antibiotics. Different concentrations of methanol extract (10mg/ml - 400mg/ml), Amikacin (30µg/ml) and Gentamycine (10µg/ml) were found effective against all resistant strains, however there was no effect of Oflaxacine was found on any resistant strain up to 20µg/ml as shown in figure1.

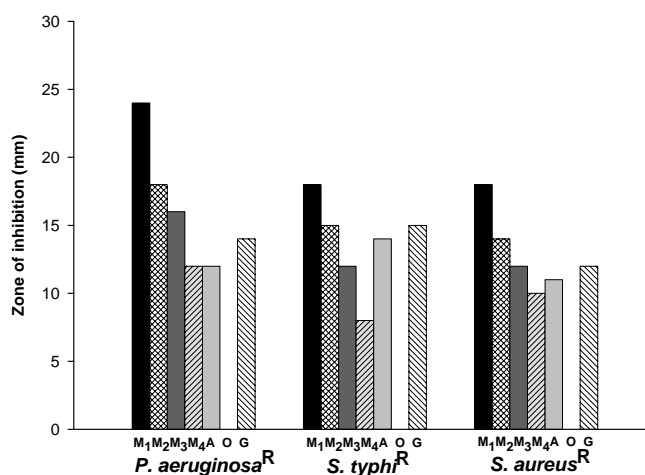


Figure1. Inhibition zone of different drug resistant bacteria against methanolic extract of 400 mg/ml (M₁), 200 mg/ml (M₂), 100 mg/ml (M₃) and 10 mg/ml (M₄) as well as different standard antibiotics such as 30µg/ml Amikacin (A), 20µg/ml Oflaxacine (O) and 10µg/ml Gentamycine (G).

Discussion

Four gram positive and four gram negative strains were screened for their sensitivity, out of these eight, six strains viz *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Bacillus megatarium*, *Pseudomonas aeruginosa*, *Vibrio cholerae* and *Shigella dysenteriae* were more sensitive with methanol extract, however *Staphylococcus aureus* and *Salmonella typhi* were sensitive with chloroform extract. Hexane extract was less effective in comparison to methanol and chloroform extract. These findings revealed that each solvent extract was inhibitory to all tested gram positive and gram negative bacterial strain except petroleum ether. Methanol extract and chloroform extract having potent inhibitory property against bacteria, support the view that most of the antimicrobial active compounds that have been identified were soluble in polar solvents such as methanol instead of water and thus organic solvent extracts have been found more potent (6, 16, 17). The most commonly used solvents for investigations of antimicrobial activity in plants are methanol, ethanol, Chloroform and water (12, 18, 19, 20, 21, 22,).

In this orientation methanol extracts were found effective from lower to higher concentration against bacterial strains and zone of inhibition increased with increasing concentration of methanol extract. Results concluded that gram negative strains were more sensitive than gram positive strains from lower to higher concentration of methanol extract. The activity of methanol extracts against both Gram-positive and Gram-negative bacteria indicates the presence of broad-spectrum antibacterial compound(s) in the methanol extract of *Piper longum* fruits. *Pseudomonas aeruginosa* was observed as most sensitive in comparison to other tested bacterial strains. However, gram-negative bacteria have more complex cell wall structure as compared to that of Gram-positive bacteria. The variations in the level of antibacterial activity of methanol extracts indicate that concentration of particular active constituent increased with increasing concentration of extract. Thus, fractionation of methanol extract is needful to purify the active constituent, to reduce the therapeutic doses. Pure antimicrobial compounds of plant origin generally possess considerably greater efficacy than that of source crude extracts from which they are isolated (4, 23, 24).

Strains *Pseudomonas aeruginosa*^R, *Salmonella typhi*^R and *Staphylococcus aureus*^R were tested for sensitivity with methanol extract and different antibiotics because bacterial species which was sensitive at lower concentration of methanol extract, considered as a base to select the resistant (R) strains. Amikacin caused inhibition of 50%, 77% and 61% against *Pseudomonas aeruginosa*^R, *Salmonella typhi*^R and *Staphylococcus aureus*^R respectively in comparison to methanol extract at 400mg/ml. Similarly, inhibition zone by Gentamycine was 58%, 83% and 66% against *Pseudomonas aeruginosa*^R, *Salmonella typhi*^R and *Staphylococcus aureus*^R respectively in comparison to methanol extract at 400mg/ml. However there was no effect of Oflaxacine was found on any resistant strains. These results indicate that methanol extract was found more effective than Amikacin, Gentamycine and Oflaxacine.

It concluded, from the above investigations that methanol extract of *Piper longum* fruit was effective against some pathogenic drug resistant bacteria thus may be used as drug to treat the disease caused by specific bacteria, which are resistant to commonly used antibiotics, beside their use as traditional spice. However, further and specific studies are needed to better evaluate the potential effectiveness of the crude extracts as the antimicrobial agents.

Acknowledgments

We thank University Grant Commission for financial support in the form of research fellowship. Financial assertion from Department of Biotechnology and Indian Council of Medical Research for infrastructure is also acknowledged.

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