Qualitative Anti-bacterial assessment of "Piper longum. L":A Natural Antibiotic

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Abstract:

Piper longum L, long pepper or Pippali is a flowering wine belonging to the family Piperaceae,that is predominantly grown in southern parts of India. The fruits &dried roots of the plant have been used over the years as medicinal ingredient in Indian System of Medicine-Ayurveda, Siddha& Unani. The present study aims to explore the bio-active compounds through biochemical estimation followed byqualitative anti-microbial assay. The liquid extracts obtained from dry powder showed positive results for phytochemical constituents -tannins, saponins and alkaloids. These extracts were subsequently applied onto microbes through Disk Diffusion Assay (DDA) and their microbial inhibition was observed (visually) by the formation of cleared zones (in DDA).

Three Pippali extracts were prepared using ethanol, methanol,distilled water, ethyl acetate and acetic acid as solvents and piper longum (ground powder) as solute. Theextracts were tested against Actinomycetes,Bacillus including Lactobacillus andE.coli species.The piper longum extracts were found to be possessing antimicrobial activity against all the tested species. Acetic acid extractshave shown maximum zone of inhibition in DDA. It was also observed that the potency of the Pippali extracts was varying based on the solvent used for extract preparation.

The re-discovery of phyto-chemical, antimicrobial and pharmaceutical properties has led to its increasing demand in national and international markets. With proper cultivation technology & export mechanism our country can aim to rise as a major global market for long pepper and other herbal products. Besides, it may pave the way as a promising solution to overcome the adverse effects of synthetic (antimicrobial) agents, such as, hypersensitivity, cell toxicity, disturbance of normal gut microflora etc.

Key Points: Effectiveness of different solvent extracts, the potential market opportunities, Biochemical estimation and qualitative antimicrobial assays.

Key Words: Pippali Extract, Disc diffusion assay, DDA, anti-microbial activity, Phyto-chemicals, Plant Extracts

INTRODUCTION

Piper longum, also known as long pepper, is a flowering vine in the family Piperaceae, native to India, Sri Lanka, and Indonesia. It is a perennial plant that grows up to 1.5 meters in height and has long, slender spikes of tiny white flowers. The plant is best known for its fruit, which is a slender, cylindrical spike that is around 3-9 cm long and contains small, dark-brown seeds.It has been used in traditional medicine for thousands of years, particularly in Ayurveda, the traditional Indian system of medicine. The seeds of the plant are often used in Ayurvedic medicine to treat a range of conditions, including digestive disorders, respiratory problems, and inflammatory conditions.

Recent scientific studies have confirmed the potential medicinal properties of Piper longum, and the plant is now being investigated for its potential use in treating a variety of conditions, including cancer, diabetes, and Alzheimer's disease. Piper longum has been found to contain a number of bio-active compounds, including piperine, piperidine which has been shown to have antioxidant, anti-inflammatory, and anti-cancer properties.In addition to its medicinal properties, Piper longum is also used as a spice in cooking, particularly in Indian cuisine, where it is often used in spice blends and marinades. The plant is also used in the production of traditional alcoholic beverages, and the fruit sometimes used in the production of perfumes and fragrances. Overall, Piper longum is a versatile plant with multiple uses in medicine, food, and other industries.

Scientific name: Piper longum. L

Family: Piperaceae

Kingdom: Plantae

Order: Piperales

Genus: Piper

Species: P. longum

Piper longum contains a variety of bioactive compounds, including alkaloids, phenolic compounds, terpenes, flavonoids, lignans, and essential oils.1) Alkaloids-piperine, piperlongumine, piplartine, piperlonguminine, piperlongipinene, piperlongistipine, etc.2)Phenolic compounds: flavonoids, tannins, coumarins, etc.3)Terpenoids: sesquiterpenes, diterpenes, triterpenes, etc.4)Lignans: sesamin, sesamolin, etc.5)Essential oils: β-pinene, β-caryophyllene, limonene, α-terpineol, etc. Based on geographic origin, cultivation method and plant part, Piper longum was found to have varied levels of the above-mentionedbio-active compounds.

These compounds confer antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. Therefore, the phytochemical analysis of these composition is important for their potential use in various industries, including pharmaceuticals, nutraceuticals, and food additives.

MATERIALS AND METHODS:

Collection and Extraction of plant material: Dried fruits of P. longum were collected from local market, Hyderabad. They were groundto a fine powder. The powdered samples were sealed in airtight container to avoid the effect of humidity and then stored at 25 ° C for further analysis.The extracts of dried powder (P. longum) were madeusing five solvents - ethanol, methanol, ethyl acetate, glacial acetic acid and distilled water.

Screening for Phyto-chemical constituents

Qualitative tests for saponins, tannins, alkaloids were performed as described below

Test for tannins

500 mg of the sample was dissolved in 20 ml of distilled water boiled and filtered.A few drops of 0.1% Ferric chloride was added to the filtrate and observed for brownish or bluish black colour

Test for alkaloids (Meyer's test)

0.5 g of the dried powdered sample was boiled in 20 ml of water and filtered. To a few drops of the filtrate, a drop of Meyer's reagent was added by the side of the test tube. A creamy or white precipitate indicated a positive test

Test for saponins

200 mg of the powdered sample was dissolved in 20 ml of distilled water and boiled in a water bath. A stable froth was observed when 10 ml of the filtrate was added with 5 ml of distilled water followed by vigorous shaking. The obtained froth was finally added with 3 cc of olive oil to observe the formation of emulsion, which confirmed the presence of saponins.

Microbial Cultures for antibiotic assay

Four bacterial species belonging to both gram-positive and gram-negative strains were chosen for antibiotic assay. The strains chosen have similar characteristics as of pathogenic bacteria, so that the assay can emulate and assess the antibacterial activity without actually working with pathogenic strains.

Bacterial strains used in the experiment:

•Actinomycetes sps. (Gram-Positive, Filamentous) (Isolatedform Lab washes Area)

•Lactobacillus sps. from curd labelled as- Bacillus-1(Gram-Positive)

•Bacillus sps. From idly batter labelled as- Bacillus-2 (Gram-Positive)

•E.coli species- DH5α (Lab grown) (Gram Negative)

Culture source and Preparation

Actinomycetes species were isolated using random scraping of the material at wash area and dissolving it in 10 ml of sterile distilled water. 1 ml of curd and 1 ml of idly batter were taken as source material for bacillus-1 and bacillus-2 species. 1ml of LB broth containing DH5α cells were taken as source for E.coli cells.

Serial Dilution and obtaining pure cultures

1ml source material was dissolved in 9ml of sterile water for each sample and 10-folddilutionswere made. Subsequently the same method followed and 10^{-4} / fourth serial dilutionsample (100µl) wasplaced on freshly made LB-agar Plates. The plates were incubated for 24 hoursat 37^0C in an incubator. Well separated single colonies of each sample were picked up from the LB-Agar plates and inoculated in LB Broth. The cultures obtained in LB-Broth after overnight incubation were microscopically evaluated and treated as pure cultures. Obtained pure cultures were later used for Disk Diffusion Assay experiments.

Disk diffusion Assay procedure

Liquid Cultures were obtained by diluting 1 ml of pure culture in 9 ml of sterile distilled water.100 µl of culture was placed on LB-Agar plate using spread plate technique. Sterile filter disks dipped in different labeled solutions (sample nos. 1-6) of P. longum extracts were placed equidistant from the centre and were labeled (at the bottom of Petri plate accordingly). The plates were incubated and later observed for the formation of cleared zones.

Measurement of the Zone of Inhibition through Disk **Diffusion Assay**

After incubation, the diameter of the zones of complete inhibition staring from the centre of disk was measured to the nearest whole millimeter and recorded. The measurements were made with a ruler on the under surface of the plate in sterile environment The area of zone of inhibition caused due to the extract against standard reference (Synthetic antibiotic) was treated as qualitative MIC or disk diffusion assay

RESULTS

Interesting results were obtained with qualitative MIC/disc diffusion assay. Distinct cleared zones were observed in all the cultures. Standard control plate with no inhibitory agent was considered as positive control for bacteria. Discs with synthetic antibiotic labeled as sample -7 and 8 (negative controls) were placed in line with test disks, to compare and assess the potency against the test samples.

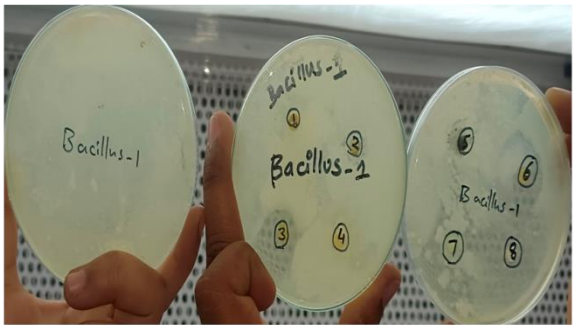

Fig.4 MIC in Bacillus -1

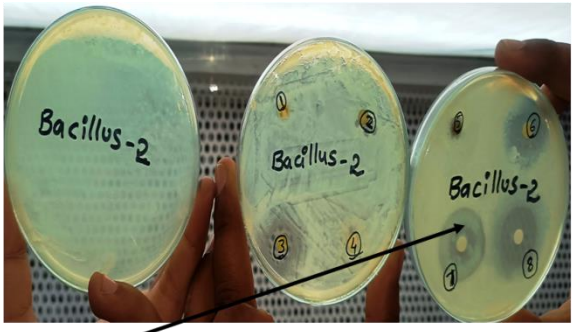

Fig.5 MIC in Bacillus -2

Cleared Zones

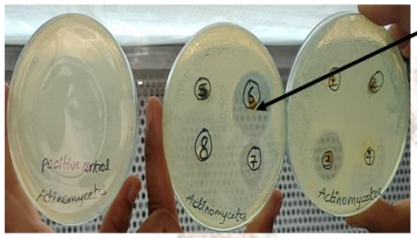

Fig.6 MIC in Actinomycetes -1

Fig.7a MIC in E.coli DH5α

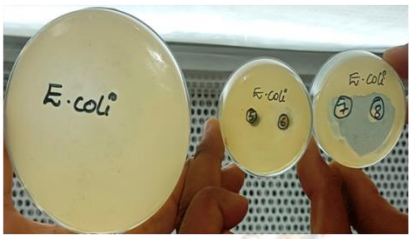

Fig.7b MIC in E.coli DH5α

Table.1 Measured MIC activity of Piper longum in different solvents against **different bacteria**

Bacterial strains	**MIC Zone (calculated in millimeters) for each kind of Piper longum extract (sample)**							
	Sample -1	**Sample -2**	**Sample -3**	**Sample -4**	**Sample -5**	**Sample -6**	**Sample -7**	**Sample -8**
Actinomycetes	NCZ	2mm	12mm	NCZ	3mm	**14mm**	**10mm**	**NCZ**
Bacillus-1 (C)	NCZ	6mm	11mm	NCZ	2mm	**15mm**	**NCZ**	**>20mm**
Bacillus -2 (IB)	NCZ	6mm	**10mm**	NCZ	NCZ	9mm	**20mm**	**15mm**
E.coli	1mm	2mm	**11mm**	NCZ	NCZ	3mm	**6mm**	**>20mm**

1.**Sample -1-** Piper longum extract prepared in distilled water and stored at room temperature for 5 days (2.5 grams in 30 ml)

2.**Sample -2** -Piper longum extract prepared in ethyl acetate and stored at room temperature for 5 days (2.5 grams in 30 ml)

3.**Sample -3** -Piper longum extract prepared in acetic acid and stored at room temperature for 5 days (2.5 grams in 30 ml)

4.**Sample -4** -Piper longum extract freshly prepared in distilled water (2.5 grams in 30 ml)

5. **Sample -5** -Piper longum extract freshly prepared in ethyl acetate (2.5 grams in 30 ml)

6.**Sample -6** -Piper longum extract freshly prepared in acetic acid (2.5 grams in 30 ml)

7.**Sample -7** -Amoxycillin 50mg/ml

8. **Sample -8** -Azithromycin 50mg/ml

NCZ = **No Clearance Zone (observed)**.

IB = Idly batter

C = Curd

DISCUSSION

From Table.1, and Figures 4-7b, it is evident that Piper longum extract acting as a bactericidal agent against all the tested bacterial species. The area of zones of clearance / inhibition zones is variable. Piper longum extract obtained using acetic acid (samples 3 and 6) have given maximum clearance zones. Clearance zones obtained by natural extracts (sample 3 and 6) were comparable to the zones of clearance obtained synthetic standards (sample 7 and 8). Freshly prepared P. longum extract in acetic acid (sample 6) has shown maximum activity against actinomycetes and lactobacillus species. P. longum extract in acetic acid incubated for 5 days (sample 3) has shown maximum activity against Bacillus species from idly batter and E. coli species. From the above experiments, it has become clear that the potency of natural ingredients varies based on its processing (sample 3 and 6). Reasons for variable potency can be attributed to altered chemical constituents, physicochemical properties like dissolution/ dispersion of active ingredient, chelation, and sequestration and so on. This also asserts the fact that our ancestors / medicinal system has more insights into such intricacies. Their prescription of the same ingredient/ drug was recommended in different forms, after diverse processing, such that optimal dose of the active ingredient reaches the patient.

Due to its diversified ethno medicinal, ayurvedic and pharmaceutical applications, the market demand for Pippali is rapidly increasing both in national and international markets. By implementing appropriate cultivation techniques and export strategies, our nation has the potential to emerge as a significant player in the worldwide market for long pepper and other herbal-based products. It should be noted that though P. longum has extensive phytochemical content, playing role in pharmaceutical& medicinal industry, however,its bio-availability can vary depending on various factors, such as the method of preparation, dosage, and individual differences in metabolism and absorption. Apart from that, the bio-availabilityis also limited due to its low solubility in water and low permeability across cell membranes. In the present investigation, using alternate polar solvent such as acetic acid was found to show better anti-bacterial activity in comparison with distilled water as solvent. Based on above results, it was presumed that the same solvent (acetic acid) when attempted in *in-vivo* studies may produce same results with respect to potency and bio-availability and thus forms our future line of work.

Compliance with Ethical Standards

‘This article does not contain any studies with human participants or animals performed by any of the authors.”

Data Availability Statement

The data used to support the findings of this study are included within the article.

Authors Contribution

PP- Work design and information collection

(P Pushpalatha)

BM- Conducted Experimental Work

(Dr B Madhu)

NR- Collection and Preparation of Samples

(N Ramesh Kumar)

FA- Isolation and maintenance of Microbial strains

(Farheen Ayesha Birjees)

AR- Wrote the ManuscriptandCover Letter

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