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Original Research

Characterization of plumbagin from *Plumbgo zeylanica* L and screening of its impact on human intestinal micro flora

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ABSTRACT:

The present study was carried out to investigate the antibacterial activity of plumbagin from *Plumbago zeylanica* against various human intestinal micro flora including *Helicobacter pylori*. Plumbagin, a bioactive compound was isolated from the root bark of *P. zeylanica* by fractional method using soxhlet apparatus, column chromatography and Thin Layer chromatography (TLC). The purity of the compound was further analyzed by subjecting the compound to HPLC studies. The minimum inhibitory concentration of active compound was tested against *Staphylococcus aureus. Proteus vulgaris, Psedomanas aeruginosa, Escherichia coli* and *H. pylori*. Almost all cases of peptic ulcers are caused by either *H. pylori* or the use of anti-inflammatory medication. The results showed that higher activity of plumbagin against human intestinal micro flora while compared to standard drugs. No growth was observed against probiotics, a friendly bacteria.

Keywords:

Plumbagin, Human micro flora activity, *H. pylori*, Natural antibiotic.

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INTRODUCTION

The bacteria of the human gastrointestinal tract establish an complex ecosystem. Gastric acid and bile in the stomach destroys most organisms that are swallowed (Pietroiusti, 2005). Bacteria like bactericides, clostridium, *Peptostreptococcus, E. coli*, *H. pylori* are the prime cause of infection in the peritoneal cavity. The main cause of true gastritis is easily diagnosed through the use of the urea breath test (Nisha *et al*., 2002).

According to the National Institute of Diabetes and Digestive and Kidney diseases (NIDDK), one in every 10 Americans will develop a peptic ulcer at some time in their lives. Different types of peptic ulcers include duodenal ulcers, gastric ulcers, esophageal ulcers, stress ulcers and marginal ulcers (Mastroeni, 2002). World Health Organization (WHO) recommends global programme to reduce the antibiotics in animals plants and fist for promoting livestock growth and in human medicine and recommends increased efforts to prevent diseases through natural drugs and reduce over prescription and misuse of antibiotics (Davies, 1994).

MATERIALS AND METHODS

Roots of *Plumbago zeylanica* collected from at the grounds of Government Siddha Medical College, Palayamkottai, Tamil Nadu, were used and voucher of this plant was stored at the college herbarium under the number 2210. Extraction and fractionation: Extraction in the soxhlet apparatus were carried out at different duration (2 h, 5 h and 10 h).

The methanol extract was removed from the apparatus and the solvent was evaporated to dryness under reduced pressure. Evaporation of the solvent under reduced pressure gave crude extract. Extraction yields percentage of the crude methanol extract from the root of *Plumbago zeylamica* obtained by different process duration (Karamani *et al*., 2003).

In column chromatography column size of 90 cm X2 cm length and diameter column preparation wet packing technique and elution isocratic elution technique were used for separation of plumbagin. By elution process the yellow pigment plumbagin was collected time wire. The recovered yellow fraction was detected by TLC. Standard solution of plumbagin (Sigma Aldrich-Bangalore) was prepared by dissolving 1 mg of plumbagin in 1 mL of benzene.

Likewise 1 mg of plant extract was dissolved in 1 mL of benzene. After the development of TLC plates a yellow spot was visualized. The active principle was identified by HPLC and qualitative and quantitative were analyzed (Wang and Huang, 2005).

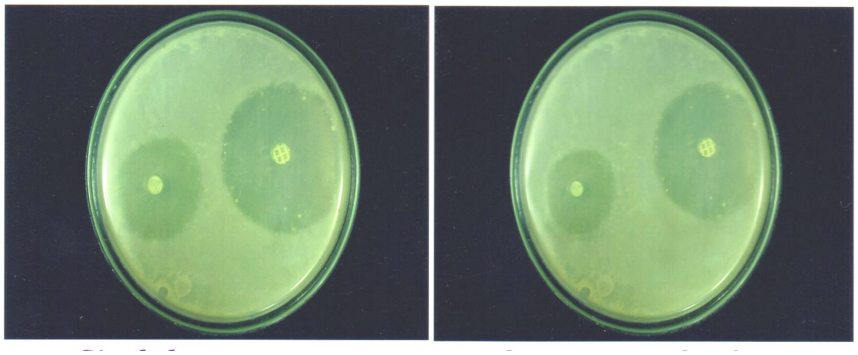
Zone of inhibition of plumbagin on intestinal bacteria

The isolated plumbagin from *Plumbago zeylanica* was tested against various intestinal microlora such as *staphylococcus aureus*, *Proteus valgaris, Psedomonas aeruginosa, Escherichia coli, Proteus vulgaris* access and *H. pylori. Helicobacter pylori* are strongly associated with gastric cancer and peptic ulcer. The biopsy were impregnated into sterile modified brain heart infusion agar plates and incubated under microaerophilic condition in a candle jar apparatus for 48 -72 h at room temperature. This bacteria isolates was characterized by various identification procedure. (Villavicencio *et al*., 1992)

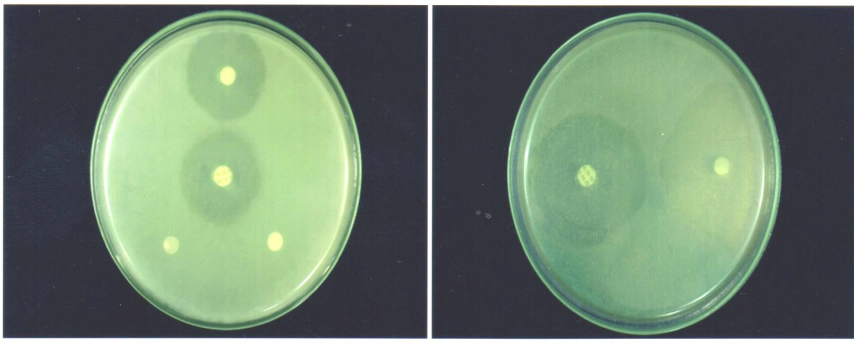
RESULTS AND DISCUSSION

Plumbago zeylanica belonging to Plumbaginaceae family is known because of its multiple biological uses. Plumbagin is a natural naphthoquinone extracted from *Plumbago zeylanica* roots**.** The efficiency of plumbagin extraction by different process duration were compared.

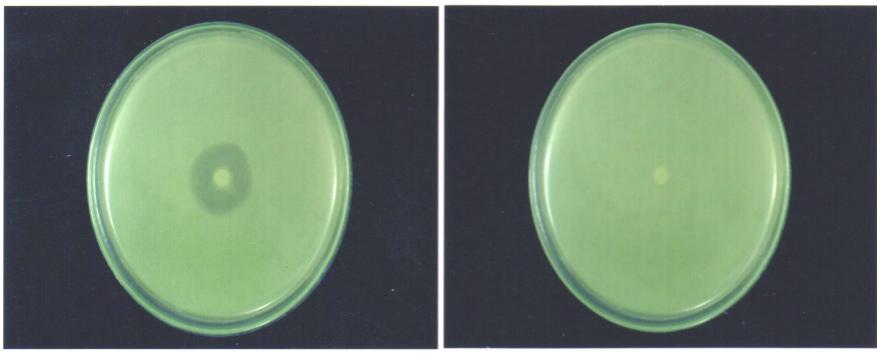
Extraction in soxhlet apparatus for 5 h demonstrated a high efficiency, yielding almost 50% over the extract obtained in 2 h of extraction. Comparatively 10 h of extraction yielded approximately 8.5% more than that obtained in 5 h (Hostettmannk *et al*., 1997).



a. *Staphylococcus aureus* b. *Proteus vulgaris*



c. *Escherichia coli* d. *Pseudomonas aeruginosa*



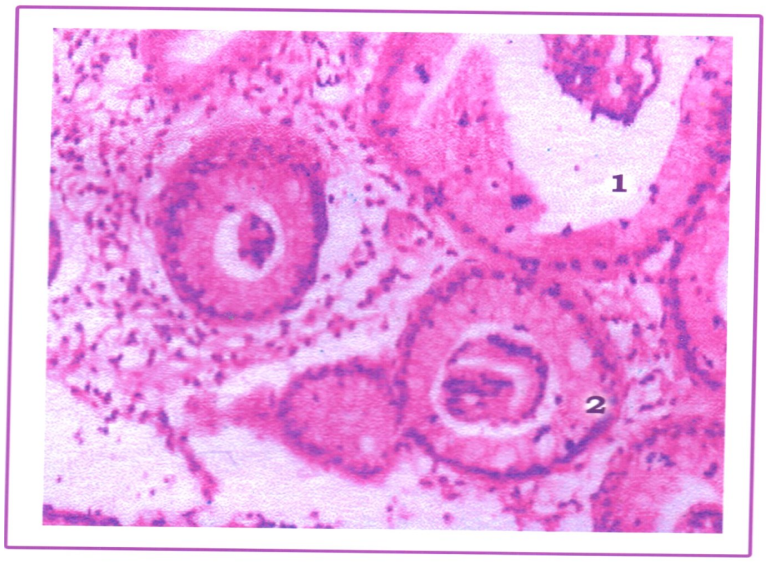
e. *Lactobacillus acidophillus*

Figure 1. Inhibition zone (diameter) of plumbagin against different intestinal bacteria

The results obtained in our extraction yield by soxhlet apparatus was 51% w/w for 5 h process duration. The results are described in Table 1. It could be concluded that the extraction in soxhlet apparatus was optimal to reach high percentage extraction of plumbagin, however the process duration should not be so long over 5 h in order to maintain the better yield of plumbagin and prolonged heating time promoted plumbagin degradation (Vijver, 1972).

A sample of extract solution in methanol was submitted to column chromatography and isocratic technique was followed to elute yellow pigment. The compound was identified by performing Thin Layer chromatography where Retardation Factor value (Rf value) of both standard plumbagin and extract were same (plumbagin standard 0.79 and plumbagin extract 0.78). Since the Rf value of both was same, the compound

Figure 2a. Histopathological examination of gastric antral specimen



1. Section studied shows gastric antral mulcosa with local epithellal erosion.

2. Section studied with warthin starrh stain is positive for *H. pylori*

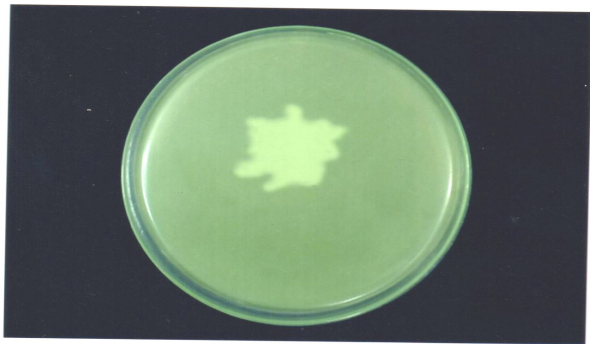


Figure 2b. Bacterial outgrowth from biopsy specimen after 72 h

plumbagin from the extract was identified by its standard.

The purity of the compound was analyzed by subjecting the compound to High performance Liquid Chromatography (HPLC) (Imlay and Fridovich, 1992). Under identical condition, the retention time of standard plumbagin and sample plumbagin and was 5.37 % 5.71 respectively. Presence of additional peaks in sample plumbagin revealed presence of impurities. The percentage of plumbagin in the extract was calculated.

Weight of sample injected - 0.02 mg

Weight of standard injected - 0.02 mg

Area of the peak of sample - 241269771

Area of the peak of standard - 367602385

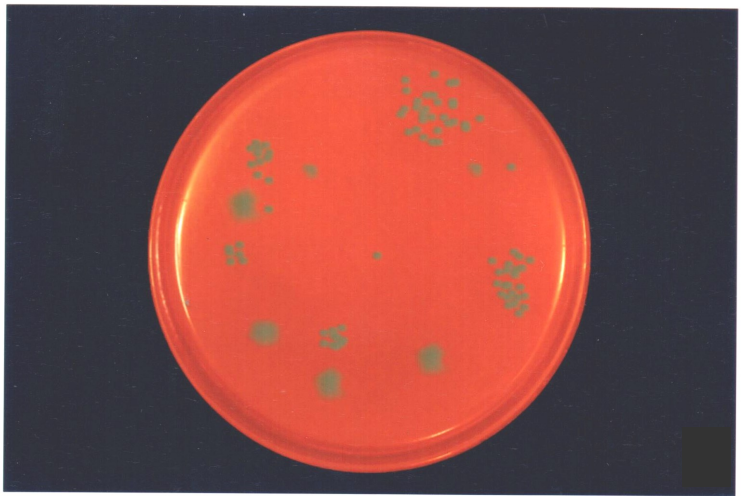
% of plumbagin

In the extract = 61.49 %

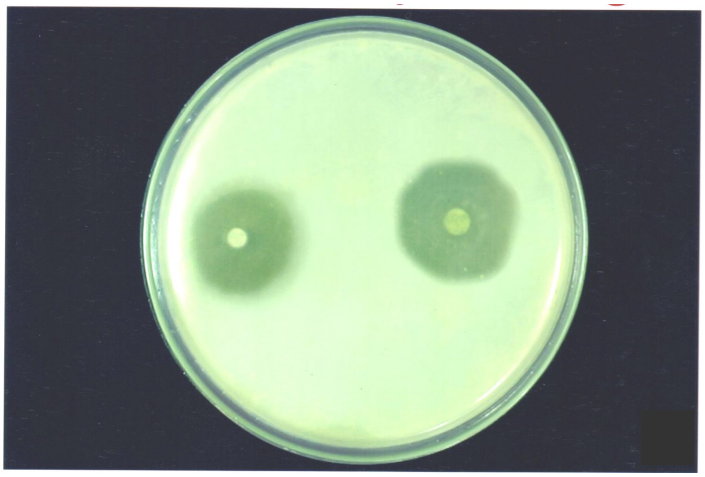
$$\frac{0.02 \times 241269771}{0.02 \times 367602385} \times 93.69$$

The isolated plumbagin from root of *Plumbago zeylanica* was tested (zone of inhibition) against various intestinal bacteria (Selma *et al*., 2003) results. Plumbagin has greater effect on *Staphylococcus aureus* and *Proteus vulgaris* whereas no effect against *E. coli.* The effect of plumbagin on *Staphylococcus aureus* and *Proteus vulgaris* were high but no effect on *Escherichia coli* due to its resistance against plumbagin *Pseudomonas*

Triphenyl tetrazolium chloride test



Antibacterial activity of plumbagin



Helicobacter pylori

Figure 3. Greyish brown colour colonies on sterile blood agar medium containing 0.04 % TTC

aeruginosa had moderate antibacterial activity. No growth was seen when *Escherichia coli* was inoculated into the antibiotic (streptomycin) medium, due to development of resistance in some of the cells. However, the growth was completely prevented when *E. coli* was grown in the medium containing antibiotic and plumbagin (Streptomycin 10 µg and plumbagin 10 µg./ disc) together. Broad spectrum antibiotic Amikacin 10 µg/ disc were used as control.

Streptomycin resistance *E. coli* has resistance against both streptomycin and plumbagin (Durga *et al*., 1990). It is sensitive with the combination of plumbagin and streptomycin 10 µg/disc respectively. Plumbagin (10 pg/disc) did not interfere with the growth of lactic acid producing bacteria *Lactobacillus acidophilus*. But chloramphenicol (10 µg/disc.) which affected the normal growth of *Lactobacillus acidophilus*. The results are presented in Figure 1.

Plumbagin does not affect *Lacidophilus* in contrast chloramphenicol has moderate effect on growth of it (Arina and Ahmad, 2000). To determine the minimum inhibitory concentration of plumbagin specific

concentration was prepared by means of a twofold serial dilution technique in an enriched broth medium (Desta 1993). For *Staphylococcus aureus,* the MIC was 1.95 pg/ mL, whereas in *Pseudomonas aeruginosa,* it was 0.97 pg/mL. The MIC of combination drug (Streptomycin and plumbagibn) against *E. coli* was 31.25 µg./mL. The minimum inhibitory concentration of plumbagin is compared with standard amikacin (Esquenazi *et al*., 2002).

The bacterial outgrowth from biopsy specimen was observed (Figure 2a). The biopsy specimen was stained with warthin starry stain which gave positive results for *H. pylori* (Figure 2b). For the identification of *H. pylori* broth culture was subjected to gram straining procedure and it was identified as gram negative bacteria. Appearance of gas bubble in catalase activity test gave a positive result.

The presence of urease activity was confirmed by the change in color of medium from red to deep pink (Henry, 2005). This color change was due to the activity of urease which splits urea into ammonia and its products. This leads to a shift of medium pH to alkalinity which was indicated by the conversion of phenol red to pink.

Greyish brown color colonies were observed on a sterile blood agar medium containing 0.04% Triphenyl Tetrazolium Chloride (TTC). The observation was shown in Figure 3. After the isolation of *H. pylori* from the biopsy specimen zone of inhibition of plumbagin aginst *H. pylori* was recorded. Plumbagin shows similar effect of standard cephalothin 30 µg/disc. The minimum inhibitory concentration of plumbagin against *H. pylori* was 0.97 µg /mL.

Traditionally, plumbagin from *Plumbago zeylanica* has been used to treat many diseases, some of them caused by bacteria (Park *et al*., 2005)**.** The results from the current study revealed that naphthoquione Plumbagin could be the main constituent from root bark responsible for its activity. Selma *et al*. (2003) detailed that aqueous and alcoholic extracts from *P. zeylanica* roots demonstrated antibacterial activities against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Bacillus subtilis* and against the yeast *Candida albicans***.**

The results showed that plumbagin exhibited relatively specific antibacterial activity against various intestinal bacteria. *S. aureus, P. aeruginosa* and *P. vulgaris*. Anyhow it was ineffectual against *E. coli* showing the specificity of plumbagin action. The growth of *E. coli* was completely prevented when *E. coli* was grown in the medium containing streptomycin and plumbagin together (Durga *et al*., 1990).

The results obtained for *Lactobacillus acidophilus* were interesting. According to *Park et al.* (2005), plumbagin, a naturally occurring selective growth inhibiting agent could be useful as new preventive agents against various diseases caused by harmful intestinal bacteria. No growth inhibition was observed against *L. acidophilus* at 10µg/disc while chloramphenicol 10µg/disc showed moderate growth inhibition *L. acidophilus***.**

Wang and Huang (2005) standard that plumbagin had most astounding inhibitory activity against Helicobacter pylori. The outcomes revealed that plumbagin from *Plumbago zeylanica* (root bark) has likewise a similar impact.

Minimum inhibitory concentration tests against *S. aureus*, *P. aeruginosa, E. coli* and *H. pylori* were performed with the naphthaquinone plumbagin in order to evaluate its potential. Comparing the obtained results to the control, plumbagin could be considered as a promising antimicrobial agent for gastro intestinal disease including peptic ulcer disease.

Phytochemical evaluation

The main emphasis in photochemistry is on the isolation of bioactive chemical (plumbagin) from root bark of *Plumbago eylancia*. The phytochemical analysis of *Plumbago zeylanica* root bank has brought forth

isolation of plumbagin from the extract of methanol. The plant

Extract was studied for its presence / absence of plumbagin, the antibacterial agent by column chromatography, thin layer chromatography and High Performance Liquid Chromatography. The HPLC Study revealed the confirmation of Plumbagin from the plant studied.

Microbial assay

An experiment was conducted using different human intestinal bacteria to study the antibacterial efficiency of plumbagin. The zone on inhibition of plumbagin on bacteria that cause GIT disorders including peptic ulcer disease was observed.

For the availability of *Helicobacter pylori* culture, gastric antral specimen was collected from fifty two year old male patient. After careful evaluation gastric antral biopsy was chosen as specimen. This biopsy specimen was taken for his to pathological examination. The microscopic, biochemical and cultural characteristics of bacterium isolated from the biopsy specimen was identified as *H. Pylori* which causes peptic ulcer disease.

There was a significant growth inhibition in antibiotic resistance bacteria by combination drug. Single drug has no effect on it. No growth inhibition was observed against *Lactobacillus acidophilus***.** The antibacterial efficiency of plumbagin was high on *Staphylococcus aureus, Proteus vulgaris and Helicobacter pylori.*

The minimum inhibitory concentration test against *Staphylococcus aureus, Pseudomonas, E. coli* and *H. Pylori* were performed with the napthoquinone plumbagin. This result indicates plumbagin as a potential antibiotic to be considered in this moment of great spread of *H. Pylori*. Based on our observation, plumbagin a potent natural antibiotic may be used to treat gastro intestinal disorder. Antibiotic resistance intestinal bacteria can be treated by plumbagin along with antibiotic. Plumbagin has not interacted with the growth of probiotics. This clearly suggests that plumbagin being a natural antibiotic can be used in place of synthetic antibiotics. Plumbagin is not only a potent antibiotic but a good hepato protective and antioxidant also.

WHO recommends global programmes to reduce over prescription and misuse of antibiotics and recommends increased efforts to prevent disease through natural drugs.

CONCLUSION

This herb is found all through India and develops wild as a greenhouse plant in East, North and Southern India and Ceylon. The rationale behind in selecting this taxa is the folklore claims of pharmaceutical potential attributed to it. This research dealt with two major aspects of study, *viz.* photochemistry and microbiology. In addition histopathology of gastric antral results is also presented.

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