



ANTIBACTERIAL ACTIVITY OF PLUMBAGIN AND ROOT EXTRACTS OF *PLUMBAGO ZEYLANICA* L.

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This work assesses the antibacterial activity of plumbagin (5-hydroxy-2-methylnaphthalene-1,4-dione) and of methanol, chloroform and aqueous extracts of *Plumbago zeylanica* L. root against various pathogenic bacteria, and the minimum inhibitory concentrations (MICs). Plumbagin and chloroform extracts of *Plumbago zeylanica* L. root showed antibacterial activity against *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus*. Inhibition against *Klebsiella pneumoniae*, *Serratia marcescens* and *Bacillus subtilis* was moderate, and lower against *Proteus vulgaris* and *Pseudomonas aeruginosa*. The methanolic extract exhibited moderate activity and the aqueous extract weak activity against the bacterial strains as assessed by disc diffusion assays. The bioactive compound plumbagin and extract of *Plumbago zeylanica* root show a wide spectrum of antibacterial activity. The compound shows promise as a new drug for various bacterial infectious diseases.

Key words: *Plumbago zeylanica*, Plumbaginaceae, antimicrobial screening, plumbagin, antibiotics, minimum inhibitory concentration (MIC).

INTRODUCTION

Over the past few decades there has been much interest in natural materials as sources of new antibacterial agents. Different extracts from traditional medicinal plants have been tested. Many reports show the effectiveness of traditional herbs against microorganisms; as a result, plants have become one of the bases of modern medicine (Evans et al., 2002). Plants have given the Western pharmacopoeia about 7,000 different pharmaceutically important compounds and a number of top-selling drugs of modern times, such as quinine, artemisinin, shikonin and camptothecin (Tshibangu et al., 2002). The acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led researchers to investigate the antimicrobial activity of medicinal plants (Bisignano et al., 1996; Lis-Balchin and Deans, 1996; Maoz and Neeman, 1998; Hammer et al., 1999).

Silver and Bostian (1993) have documented the use of natural products as new antibacterial drugs. There is an urgent need to identify novel substances active towards highly resistant pathogens (Recio, 1989; Cragg et al., 1997). In an effort to discover new compounds, many research groups screen plant extracts to detect secondary metabolites with the relevant biological activities. In this regard, several simple bioassays have been developed for screening purposes (Hostettmann, 1991).

It is thought that herbal remedies have the advantage of combining their active components with many other substances which appear to be inactive but which give the plant as a whole a level of safety and efficiency superior to that of its isolated, pure active components; moreover, in developing countries, synthetic drugs are presently too expensive and also are often adulterated (Shariff, 2001).

Plumbago zeylanica L. (Plumbaginaceae) is a tropical shrub. It grows wild as a garden plant in eastern, northern and southern India and Ceylon.

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The roots and leaves of *P. zeylanica* are widely used medicinally in India and China. Traditionally, *P. zeylanica* is believed to kill intestinal parasites, and it is used clinically to treat rheumatism, intestinal parasites, anemia due to "stagnant blood", external and internal trauma, toxic swelling and malignant furunculosis scabies (Jiangsu, 1979).

In India it is usually used to treat fever or malaria. Pharmacological studies have indicated that *P. zeylanica* extract has antiplasmodial (Simonsen et al., 2001), antimicrobial (Ahmad et al., 2000), antifungal (Mehmood et al., 1999), anti-inflammatory (Oyedapo, 1996), antihyperglycemic (Olagunju et al., 1999), hypolipidaemic and antiatherosclerotic activities (Sharma et al., 1991).

Plumbagin (5-hydroxy-2-methylnaphthalene-1,4-dione) is a naturally occurring yellow pigment produced by the members of *Plumbaginaceae*, accumulated mostly in root (van der Vijver, 1974). Plumbagin showed anticancer (Melo et al., 1974), antimicrobial and antibiotic (Brice, 1955; Durga et al., 1990), antibacterial and antifungal activities (Gujar, 1990). Five coumarins – seselin (Kostova et al. 2001), 5-methoxyseselin (Kofinas et al., 1998), suberosin (Uchiyama et al., 2002), xanthyletin and xanthoxyletin have been isolated from the roots of *Plumbago zeylanica* (Lin et al., 2003).

The aim of this study was to isolate plumbagin from *Plumbago zeylanica* root, to investigate the antimicrobial activity of plumbagin and crude extracts, and to compare it with that of standard antibiotic.

MATERIALS AND METHODS

PLANT MATERIAL

Roots of the medicinal plant *Plumbago zeylanica* L. (*Plumbaginaceae*) were collected from the Kollu Hills, South India, in December 2005. The collected plant materials were identified at the Rapinat Herbarium, St. Joseph's College, Tiruchirappalli, South India. The roots were shade-dried at room temperature for 10 days.

PHYTOCHEMICAL STUDY

General procedure

Instrumentation consisted of an Agilent 6890N gas chromatograph equipped with a mass-selective detector on an HP-5MS capillary column (5% phenyl, 95% methyl siloxan), 30 m × 0.25 mm × 0.25 μm. ¹H NMR and ¹³C NMR experiments were performed with Bruker Unity 400 MHz spectrometers.

Vacuum liquid chromatography (VLC) employed silica gel (Merck; 43–63 μm) gradient elution with

chloroform solvent. TLC analyses were carried out on aluminum sheets precoated with silica gel 60 F254 (Merck, Darmstadt, Germany); the solvents for TLC were hexane:ethyl acetate and chloroform:methanol.

Extraction and separation of plumbagin

Shade-dried root (0.50 kg) was finely ground and extracted at room temperature with chloroform with a Soxhlet extractor for 48 h. The extracts were evaporated in a vacuum, yielding 3.6 g crude extract. Chloroform has previously been used for plumbagin extraction (Zhong et al., 1984; Pakulski and Budzianowski, 1996). The crude extract was eluted with chloroform solvent by vacuum liquid chromatography for removal of impurities. The eluted chloroform fractions yielded 3.6 g crude extract. The chloroform extract was subjected to TLC on silica gel preparative thin layer plates in hexane:ethyl acetate, chloroform:methanol (0–100%) with increased polarity. The eluted plumbagin compound was confirmed by spraying with 10% (w/v) ethanolic solution of KOH and heating.

ANTIMICROBIAL ASSAY

Extraction procedure

The dried and powdered plant material (100 g) was extracted successively with 600 ml water, chloroform or methanol with a Soxhlet extractor for 48 h at temperature not exceeding the boiling point of the solvent (Lin et al., 1999). The extracts were filtered through Whatman No. 1 filter paper and then concentrated in a vacuum at 40°C using a rotary evaporator. Each extract was transferred to glass vials and kept at 4°C before use.

Bacterial strains

Eight different bacterial strains were used: *Escherichia coli* (MTCC 1195), *Salmonella typhi* (MTCC 733), *Klebsiella pneumoniae* (MTCC 2405), *Serratia marcescens* (MTCC 2645), *Proteus vulgaris* (MTCC 1771) and *Pseudomonas aeruginosa* (MTCC 2642) (gram-negative), *Staphylococcus aureus* (MTCC 1430) and *Bacillus cereus* (MTCC 1272) (gram-positive). The bacterial strains were supplied by the Microbial Type Culture Collection & Gene Bank, Institute of Microbial Technology, Chandigarh, India.

Preparation of inoculum

Bacterial strains preserved in nutrient agar at 4°C were revived in nutrient broth (liquid medium) and incubated at 37 ± 1°C overnight, and the suspensions were checked to provide ~10⁵ cfu/ml.

MICROBIOLOGICAL TESTS OF PLANT EXTRACTS

The disc diffusion assay methods of Iennette (1985) as described by Rosoanaivo and Ratsimanaga-Urverg (1993) and Rabe and van Staden (1997), with modifications, were used to determine bacterial growth inhibition by plant extracts. Diluted bacterial culture (100 μ l) was spread over nutrient agar plates with a sterile glass L-rod. 100 μ l of the each extract and 20 μ g plumbagin were applied to each filter paper disc (Whatman No. 1, 6 mm diam.) and allowed to dry before being placed on the agar plate. Each extract was tested in triplicate (3 discs/plate) and the plates were inoculated at $37 \pm 1^\circ\text{C}$ for 24 h. After incubation, the diameter of the inhibition zones was measured with a caliper.

The minimum inhibitory concentration (MIC) was determined for the plumbagin and the aqueous, chloroform and methanol extracts that the disc diffusion method showed to be highly effective against microorganisms. Plumbagin and the aqueous, chloroform and methanol root extracts (50 mg) obtained by infusion were diluted in the respective solvents and applied to filter paper discs at increasing concentrations (1 to 25 μ g/disc). MIC was measured at the end of incubation. The inhibition zones produced by the plumbagin and plant extracts were compared with the inhibition zones produced by a commercial antibiotic. Streptomycin (10 mg/disc) was used as the positive control, and the solvents (aqueous, chloroform and methanol) as the negative control.

STATISTICAL ANALYSIS

The experiments were carried in using a completely randomized design. Calculations were carried out in triplicate with their mean values and standard deviations by the formula given by Gupta (1977). Differences were considered significant at $p < 0.05$. The statistical analyses employed SPSS ver. 13.

RESULTS

ISOLATION AND CHARACTERIZATION OF PLUMBAGIN

The chloroform extract of *Plumbago zeylanica* L. was eluted with chloroform by vacuum liquid chromatography for further purification. The eluted chloroform fraction (3.6 g crude) was subject to TLC on silica gel thin layer plates in the solvent mixture of hexane/ethyl acetate, chloroform/methanol with increasing polarity. The chloroform/methanol (8:2) solvent system yields 4 bands. The R_f value of band I (0.91) gives 57 mg of plumbagin, band II (0.76) 39 mg, band III (0.58) 29 mg, and finally band IV (0.42) 21 mg. The plumbagin was detected by spraying with 10% (w/v) ethanolic solution of KOH, followed by heating at 100°C until the red color

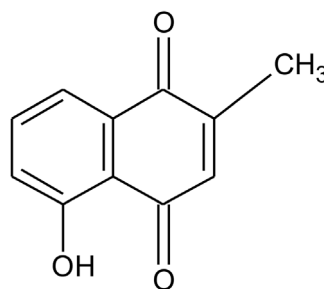


Fig. 1. Structure of plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) isolated from root of *Plumbago zeylanica* L.

appeared. The isolated plumbagin (Fig. 1) was confirmed by comparison of ^1H , ^{13}C NMR and GC-MS spectral data with values described in the literature for plumbagin (Sankaram et al., 1986; Carvalho, 1986; Zhong et al., 1984).

ANTIMICROBIAL ACTIVITY OF PLUMBAGIN

The in vitro antimicrobial activity of plumbagin isolated from the root of *Plumbago zeylanica* is shown in Table 1. Plumbagin (20 μ g/disc) showed good activity against *Escherichia coli* (25.6 ± 0.56 mm), *Salmonella typhi* (24.3 ± 0.23 mm), *Staphylococcus aureus* (21.6 ± 0.55 mm), *Klebsiella pneumoniae* (19.8 ± 0.76 mm), *Serratia marcescens* (17.6 ± 0.65 mm) and *Bacillus subtilis* (14.5 ± 0.76 mm). It exhibited moderate activity against the other organisms, *Proteus vulgaris* (10.1 ± 0.14 mm) and *Pseudomonas aeruginosa* (9.6 ± 0.67 mm). The activity of plumbagin against all tested bacterial strains was significantly higher than that of streptomycin (10 μ g/disc).

ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACT

Methanol, chloroform and aqueous extracts of *Plumbago zeylanica* L. root were tested against various gram-negative and gram-positive bacteria (Tab. 1). Among the extracts assayed, the chloroform root extracts of *Plumbago zeylanica* exhibited good activity against *Escherichia coli* (16.7 ± 0.14 mm), *Salmonella typhi* (14.3 ± 0.04 mm) and *Staphylococcus aureus* (12.0 ± 0.54 mm). Inhibition against *Klebsiella pneumoniae* (9.2 ± 0.73 mm), *Serratia marcescens* (8.6 ± 0.07 mm) and *Bacillus subtilis* (8.0 ± 0.61 mm) was moderate, and was low against *Proteus vulgaris* (5.9 ± 0.55 mm) and *Pseudomonas aeruginosa* (4.8 ± 0.87 mm). The methanol root extract exhibited significantly higher activity against *Escherichia coli* (11.0 ± 0.98 mm) and *Salmonella typhi* (10.0 ± 0.87 mm), and moderate activity against *Staphylococcus aureus* (9.8 ± 0.67 mm), *Klebsiella pneumoniae* (8.5 ± 0.56 mm) and

TABLE. 1 Antimicrobial sensitivity assay of crude extracts and plumbagin of *Plumbago zeylanica* L. root

Bacterial strain	Chloroform	Methanol	Aqueous	Plumbagin	Standard
	Antibacterial activity (Mean±SE)				
<i>Escherichia coli</i> (MTCC 1195)	16.7±0.14**	11±0.98*	4.3±0.90*	25.6±0.56**	12.5±0.76
<i>Salmonella typhi</i> (MTCC 733)	14.3±0.04**	10±0.87**	3.7±0.87*	24.3±0.23**	5.5±0.27
<i>Klebsiella pneumoniae</i> (MTCC 2405)	9.2±0.73*	8.5±0.56*	-	19.8±0.76**	10.5±0.87
<i>Serratia marcescens</i> (MTCC 2645)	8.6±0.07*	7.3±0.67*	-	17.6±0.65**	13±0.53
<i>Proteus vulgaris</i> (MTCC 1771)	5.9±0.55*	4.3±0.9*	-	10.1±0.14**	12.0±0.98
<i>Pseudomonas aeruginosa</i> (MTCC 2642)	4.8±0.87*	3.2±0.78*	-	9.6±0.67**	19.0±0.68
<i>Staphylococcus aureus</i> (MTCC 1430)	12±0.54**	9.8±0.67**	2.7±0.94*	21.6±0.55**	4.5±0.87
<i>Bacillus subtilis</i> (MTCC 1272)	8.0±0.61*	6.5±0.8*	-	14.5±0.76**	12.0±0.53

Each value represented mean±SE; * Activity significantly lower than standard antibiotic (p<0.05); ** Activity significantly higher than standard antibiotic (p<0.05)

TABLE 2. Minimum inhibitory concentration of crude extracts and plumbagin of *Plumbago zeylanica* L. root. (µg/disc). Values are means of three replicates

Bacterial Strain	Minimal Inhibitory Concentration (µg/Disc)			
	Chloroform	Methanol	Aqueous	Plumbagin
<i>Escherichia coli</i> (MTCC 1195)	< 6	< 13	> 16	< 1
<i>Salmonella typhi</i> (MTCC 733)	< 6	< 13	> 16	< 1
<i>Klebsiella pneumoniae</i> (MTCC 2405)	> 13	> 14	-	< 1
<i>Serratia marcescens</i> (MTCC 2645)	> 13	> 15	-	> 1
<i>Proteus vulgaris</i> (MTCC 1771)	> 16	> 18	-	< 2
<i>Pseudomonas aeruginosa</i> (MTCC 2642)	> 15	> 18	-	>3
<i>Staphylococcus aureus</i> (MTCC 1430)	< 8	< 11	> 20	< 1
<i>Bacillus subtilis</i> (MTCC 1272)	> 13	> 16	-	> 2

Serratia marcescens (7.3±0.67 mm). The methanolic root extract significantly inhibited *Staphylococcus aureus*. Inhibition of the remaining bacterial strains was lower. The aqueous extract showed significantly less activity.

MINIMUM INHIBITORY CONCENTRATION

The results for the minimum inhibitory concentrations of plumbagin and extracts on the test organisms

are shown in Table 2. Plumbagin and chloroform extract had the lowest MICs for *Escherichia coli*, *Salmonella typhi* (<1 µg/disc and <6 µg/disc, respectively), *Staphylococcus aureus* (<1 µg/disc and <8 µg/disc), *Klebsiella pneumoniae* (<1 µg/disc and <13 µg/disc), *Serratia marcescens*, *Bacillus subtilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa* (>1–3 µg/disc and >13–16 µg/disc). The methanolic extract showed significant activity against the bacteria more at concentration of >11–18 µg/disc.

DISCUSSION

Recently, much attention has been directed toward extracts and biologically active compounds isolated from popular plant species. The use of medicinal plants plays a large role in covering the basic health needs in developing countries; these plants may offer new sources of antibacterial, antifungal and antiviral agents with significant activity against infective microorganisms (Munoz-Mingarro et al., 2003; Coelho de Souza et al., 2004). The antimicrobial activity of medicinal plants against various pathogenic bacteria has been demonstrated (Tadhani and Subhash, 2006; Lino and Deogracious, 2006; Doughari, 2006; Aberé et al., 2007). Alcohol extract of *Plumbago scandens* and *Plumbago zeylanica* root showed antibacterial activity against *Salmonella paratyphi*, *Staphylococcus aureus*, *Escherichia coli* and *Shigella dysenteriae* (Beg and Ahmad, 2000; De Paiva et al., 2003).

Plumbagin occurs as a group of secondary metabolites in members of Plumbaginaceae. Plumbagin compounds of natural origin are deposited in cell vacuoles, where they are dissolved (in glycoside form). Plumbagin exerts antimicrobial, antifungal, antiviral and antiparasitic effects (Babulaa et al., 2005). Plumbagin exhibits relatively specific antimicrobial activity. The growth of *S. aureus* and *C. albicans* was completely inhibited (De Paiva et al., 2003). Here we investigated plumbagin isolated from the root of *Plumbago zeylanica*. The *in vitro* antimicrobial activity and the minimum inhibitory concentration of plumbagin and crude extract of *Plumbago zeylanica* were assessed by the agar disc diffusion method. The results were compared with the standard antibiotic streptomycin. The results showed that plumbagin exhibits good activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Serratia marcescens* (MIC <1 µg/disc) and moderate activity against *Proteus vulgaris* and *Pseudomonas aeruginosa* (MIC >2 µg/disc). These results revealed significantly higher activity against both gram-positive and gram-negative bacteria than that of crude extracts and standard antibiotic (Tab. 1). Methanol, chloroform and aqueous extracts of *Plumbago zeylanica* root were tested against *Staphylococcus aureus* and *Bacillus cereus* (gram-positive), *Escherichia coli*, *Enterobacter aerogens*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus vulgaris* and *Pseudomonas aeruginosa* (gram-negative). The chloroform root extract of *Plumbago zeylanica* showed significantly increased activity against *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus* (MIC, <6 µg/disc), and lower activity against the remaining strains (*Klebsiella pneumoniae*, *Serratia marcescens* *Bacillus subtilis*,

Proteus vulgaris and *Pseudomonas aeruginosa*) (MIC, 13–16 µg/disc). The methanol and aqueous extracts showed moderate and lower activity against gram-positive and gram-negative bacterial strains.

These results demonstrate that plumbagin and crude extract from the root of *Plumbago zeylanica* exhibit antimicrobial activity against different pathogenic bacteria.

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