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Original article

Methanolic Extract of *Plumbago Zeylanica* - A Remarkable Antibacterial Agent Against Many Human and Agricultural Pathogens

Mukesh Kumar Singh[1]*, Ajit Pandey[1], Hemant Sawarkar[1], Anshita Gupta[1], Bina Gidwani[1], Hemant Dhongade[1], Dulal Krishna Tripathi[2]

[1]Department of Pharmacy, Shri Rawatpura Sarkar Institute of Pharmacy, Kumhari, Durg, Chhattisgarh, India
[2]Department of Pharmacy, Rungta College of Pharmaceutical Sciences and Research, Kohka-Kurud Road, Chhattisgarh, India

Key Words
anti microbial activity, cytotoxic activity, MTT assay, *Plumbago zeylanica*

Abstract

Objectives: The current investigation was carried out to determine the cytotoxic and the antimicrobial activities of methanolic extracts of *Plumbago zeylanica*.

Methods: The stems, leaves, and whole plants were air dried and extracted with methanol by using a Soxhlet extractor for 72 hours at 55 - 60°C. The antimicrobial activities were determined from the zones of inhibition, which were measured by using the agar well diffusion method, and the cytotoxicity assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method.

Results: The methanolic extracts of the stem and the leaves of *Plumbago zeylanica* were tested against six bacterial species and nine fungal species, and both extracts showed antimicrobial activity in a dose-dependent manner. The leaf extract of *Plumbago zeylanica* showed maximum antimicrobial activity against both *Staphylococcus aureus sub sp aureus* and *Fusarium oxysporum*. The stem extract was found to be more antimicrobial against the *Pseudomonas aeruginosa* and the *Penicillium expansum* species. MTT assays were used to test the cytotoxicity of the whole plant extract in the HCT-116 and the K-562 cell lines, and that extract was shown to have weak cytotoxicity in both cell lines.

Conclusion: In the present study, the methanolic stem extracts of *Plumbago zeylanica* were found to possess remarkable antibacterial activities against many human and agricultural pathogens. The extracts were also found to possess significant antifungal activities, but the antifungal activities were less than the antibacterial activities. Finally, the extracts were found to have weak cytotoxicities in the HCT-116 and the K-562 cell lines.

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1. Introduction

The increasing prevalence of multi-drug-resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics are major threats to hu-

*Corresponding Author
Mukesh Kumar Singh. Shri Rawatpura Sarkar Institute of Pharmacy, Kumhari, Durg (C.G.), India.
Tel: +91-96-9169-9320 Fax: +07-71-225-9510
E-mail: mukeshbiotech09@gmail.com

man life. For those reasons, efforts have been made to discover new antimicrobial compounds from various sources, such as microorganisms, animals, and plants. One such resource is folk medicines, a systematic screening of which may result in the discovery of novel effective compounds [1]. Plants are a rich source of bioactive molecules. Plants, as sources of medicinal compounds, have played a dominant role in the maintenance of human health since ancient times [2]. Over 50% of all modern clinical drugs have their origins in natural products, and natural products play an important role in drug-development programs in the pharmaceutical industry.

The methanolic extracts of *Plumbago zeylanica* have been screened against different microorganisms responsible for various infections. *Plumbago zeylanica* belongs to *Plumbaginaceae* family and has been used especially as a laxative, an expectorant, an astringent and an abortifacient. The roots of *Plumbago zeylanica* are used in traditional systems of medicine to cure various ailments such as body pain, headache, fever and inflammation [3]. The present research was done to investigate the antimicrobial and cytotoxic activities of *Plumbago zeylanica*.

2. Materials and Methods

Fresh plant materials were collected from the Indira Gandhi Agricultural University, Sarkanda, Bilaspur, Chhattisgarh, India, in August 2015. The plants were identified and authenticated by the Department of Botany, V.Y.T.P.G. Autonomous College, Durg, Chhattisgarh, India. The plant materials, stems and leaves, were separated and air dried. The dried stems and leaves were powdered and extracted with methanol by using a Soxhlet apparatus for 72 hours (h) at 55 - 60°C. The extract was filtered and concentrated under vacuum in a rotary evaporator at a temperature of less than 50°C. Methanol was then added to increase the volume of the concentrated extract to 100 mL. For the cytotoxic evaluation, the whole plant was extracted with methanol by using the procedure described above, after which the concentrated extracts were lyophilized and kept at 4°C until use.

The bacterial strains were obtained from the Microbial-type Culture Collection, Chandigarh, India. The bacterial strains studied for antibacterial activity were *Escherichia coli (E-coli)* (MTCC 739), *Staphylococcus aureus (S. aureus*, MTCC 2940), *Micrococcus luteus (M. luteus)* (MTCC 2470), *Bacillus subtilis* (MTCC 121), *Staphylococcus aureus sub sp aureus* (*S.aureus Sub.aureus*, MTCC 96), and *Pseudomonas aeruginosa* (MTCC 2453). The fungal strains were obtained from the GD Rungta College of Pharmaceutical Science & Research, Bhilai, Chhattisgarh, India. The fungal strains studied for antifungal activity were *Botrytis cinerea* (KACC 43524), *Fusarium oxysporum (F. oxysporum)* (KACC 42109), *Penicillium expansum (P. expansum)* (KACC 40815), *Penicillium chrysogenum* (KACC 40399), *Rhizoctonia solani* (KACC40136), *Fusarium moniliforme* (KACC 41031), *Geotrichum candidum* (OKI 605/8402), *Candida albicans* (*C. albicans*) and *Yarrowia lipolytica* (*Y. lipolytica*) (KACC 41237).

Agar well diffusion was employed for the antibacterial assay of the plant extracts [4, 5]. A loop full of the bacterial strain was inoculated in 5 mL of each nutrient broth in a test tube and incubated on a rotary shaker at 140 rpm for 24 h. Mueller Hinton Agar no. 2 was prepared for the study. The test bacterial strain (1 mL) was inoculated into the media (inoculums size: 108 cells/mL) when the temperature had reached 40 - 42°C, and care was taken to ensure proper homogenization. The experiment was performed under strict aseptic conditions. After the medium had solidified, a well was made in the plates with the help of a cup-borer (0.85 cm). The test compound was introduced into the well, and the plates were incubated for 24 h at 37°C. Microbial growth was determined by measuring the diameter of the zone of inhibition. Methanol was used as the control. The control activity was deducted from the experimental activity, and the result obtained was plotted. Experiments were performed in triplicate.

For the antifungal activity, the agar well bioassay using PDA medium (Hi- Media, 39 g) was employed [6, 7]. Fungi were inoculated, and the procedures were similar to those mentioned above. The treated fingi and the controls were kept in an incubator at 37°C for 24 to 72 h, and the zones of inhibition were measured. Three to four replicates were maintained for each treatment.

The cytotoxicity assays were performed using the MTT method [8]. Human colon cancer cells (HCT-116) and leukemia cells (K-562) were used for the MTT assay. The cells were harvested (1×10^5 cells/well) and inoculated into 96-well microtiter plates. The cells were washed with phosphate buffered saline (PBS), and the cultured cells were then incubated for 72 h with the test sample at a final concentration of 25 - 800 μg/mL. One hundred μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg Ml-1 in PBS, pH 7.2) were added to each well, and plates were incubated for 4 h at 37°C. After incubation, 200 μL of dimethyl sulfoxide (DMSO) were added to each well, the absorbance of each well was measured at 570 nm by using a microplate reader, and the surviving cell fraction was calculated. The inhibition of cell viability was calculated using % cytotoxicity = (1-[absorbance of treated cells/absorbance of untreated cells]) × 100.

3. Results

The antibacterial activities of the stem and the leaf extracts of *Plumbago zeylanica* are given in Table 1. Compared to the antibacterial activities of the methanolic leaf extract, those of the methanolic stem extracts against all six tested bacterial strains were much higher. The highest antibacterial activities for the methanolic stem extracts against all bacterial strains tested were found at the highest concentration (1,200 μg/μL), and the zones of inhibition ranged from 12 mm to 17 mm. The highest activities of the methanolic stem extracts were against *Pseudomonas aeruginosa*, *M. luteus* and *E-coli*, with zones of inhibition of 17, 15 and 14 mm, respectively. The methanolic leaf extracts at concentrations from 450 to 1,500 μg per 100 μL showed low antibacterial activity, but significant activity was found at a concentration of 1,800 μg per 100 μL. The test organisms that were very sensitive at a concentration of 1,800 μg per 100 μL were *S.aureus Sub.aureus* (inhibition zone: 20 mm), *S. aureus* (inhibition zone: 16 mm), and *M. luteus* (inhibition zone: 13 mm).

Different concentrations of *Plumbago zeylanica* extract

were also tested to determine their activities against different fungal strains (Table 2). All the concentrations of the test solution inhibited the fungal species, but with varying degrees of sensitivity. The antifungal activities were very low at concentrations of 300 - 500 μg per 100 μL for methanolic stem extracts and 450 - 750 μg per 100 μL for methanolic leaf extracts. The diameters of inhibition zones ranged from 5 - 13 mm among the different fungal species for both the methanolic stem and leaf extracts. An increased activity was observed with increasing concentration of the test solution. The maximum zones of inhibition were found at concentrations of 1,200 and 1,800 μg per 100 μL for the methanolic stem and leaf extracts, respectively. Among the test organisms, high zones of inhibition were observed in *P. expansum* (12 mm) and *F. oxysporum* (13 mm) for the methanolic stem and leaf extracts, respectively. The highest zones of inhibition against *C. albicans* and *Y. lipolytica* were 11 mm for both the methanolic stem and leaf extracts. This result compared to the result in the previous paragraph shows that the antibacterial potential of the methanolic extracts of this plant is greater than its antifungal potential.

The crude methanolic extract of the whole plant *Plumbago zeylanica* was evaluated for cytotoxic activity on two cancer cell lines (HCT-116 and K-562). The extracts were tested at concentrations of 25 - 800 μg/mL after 72-h treatment. The results show that the methanolic extracts of this plant had no significant cytotoxicity against either cell line. In the HCT-116 cell line, the percentage of cytotoxicity was 42.1% at a concentration of 800 μg/mL while that in the K-562 cell lines was 28.4% (Fig. 1).

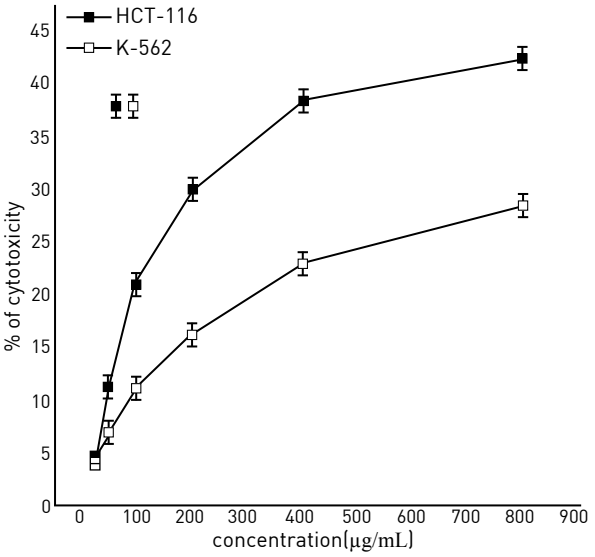

Figure 1 *In-vitro* cytotoxic activity of a methanol extract of *Plumbago zeylanica on* human colon cancer cells (HCT-116) and leukemia cells (K-562). The values are shown as the means of triplicate measurements, and the error bars represent the standard errors of the mean.

Table 1 Antimicrobial activity of *Plumbago zeylanica*

Extract	Conc (μg/100 μL)	Zone of Inhibition (mm)					
		E-coli	*S. aureus*	*M. luteus*	*S.aureus Sub.aureus*	*P. areuginosa*	*B. subtilis*
Stem	300	9	8	9	8	10	9
	500	11	9	11	9	11	11
	1,000	12	10	11	9	11	11
	1,200	14	12	15	12	17	13
	Control	9	8	9	9	8	9
Leaf	450	8	5	11	11	7	7
	750	8	6	11	11	7	7
	1,500	11	8	12	12	9	9
	1,800	11	16	13	20	12	12
	Control	8	10	10	11	9	9

*Control was methanol (100 μL).

E-coli, Escherichia coli; *S. aureus, Staphylococcus aureus*; *M. luteus, Micrococcus luteus*; *S.aureus Sub.aureus, Staphylococcus aureus sub sp aureus*; *P. areuginosa, Psrudomonas areuginosa*; *B. subtilis, Bacillus subtilis.*

Table 2 Antifungal activity of *Plumbago zeylanica*

Extract	Conc (μg/100 μL)	Zone of Inhibition (mm)								
		B. cinerea	*F. oxysporum*	*P. expansum*	*P. chrysogenum*	*R. solani*	*F. moniliforme*	*G. candidum*	*C. albicans*	*Y. lipolytica*
Stem	300	6	5	8	6	6	8	7	8	6
	500	7	7	11	7	6	8	7	9	8
	1,000	8	8	12	7	7	7	7	11	9
	1,200	9	9	12	9	7	8	9	11	11
	Control	8	6	9	8	6	7	8	7	9
Leaf	450	7	9	7	7	6	8	7	7	4
	750	7	11	11	8	6	5	7	8	8
	1,500	8	11	9	9	7	4	8	8	9
	1,800	8	13	9	9	7	8	9	9	11
	Control	7	8	8	8	6	7	8	8	11

*Control was methanol (100 μL).

B. cinerea, Botrytis cinerea; F. oxysporum, Fusarium oxysporum; P. expansum, Penicillium expansum; P. chrysogenum, Penicillium chrysogenum; R. solani, Rhizoctonia solani; F. moniliforme, Fusarium moniliforme; G. candidum, Geotrichum candidum; C. albicans, Candida albicans; Y. lipolytica, Yarrowia lipolytica.

4. Discussion

Antimicrobial activity of methanolic extract of stem and leaves of *Plumbago zeylanica* were evaluated by agar diffusion method. The activity was investigated against 6 bacterial and 9 fungal strains. The concentrations were 300 - 1200 μg/100 μL for stem extract and 450 – 1,800 μg/100 μL for leaves extract. Results revealed that the stem extract showed prominent antibacterial and antifungal activity as compared to leaves extract. According to Arunbala, 2014 the active ingredients of parts of plant are better than extracts with methanol than chloroform [9]. Similarly, in our work we also proved that methanolic extract of stem posses' better antimicrobial activity than leaves; this may be due to presence of active constituents in plants. Based on these results and literature reported; the leaves and stem of *Plumbago zeylanica* can exhibit therapeutic potential in treatment of bacterial, malarial and fungal infections including cancer. *Plumbago zeylanica* stem extract has been much known to posses' prominent antimicrobial properties [10].

Plumbago zeylanica is a medicinal plant whose cytotoxicity activity was determined by MTT method. Percentage of cytotoxicity indicated that for the non-cancerous human clone tumor cell line HCT-116 and K-562, the growth was inhibited by detoxified methanolic extract of *Plumbago zeylanica*. Only the live cells have the ability to reduce the tetrazolium dye but dead cells cannot do. When the MTT is reduced, the quaternary amine is converted into a tertiary amine by opening the tetrazolium ring. The absorption spectrum of the molecule changes due to the displacement of H-atom at high pH. Thus, the subsequent cell numbers can be estimated and the absorbance is directly correlated to number of viable cells. The result and data revealed that with the increase in concentration of extract its toxicity increases [11].

5. Conclusion

In the present study, the methanolic stem extracts of *Plumbago zeylanica* were found to possess remarkable antibacterial activities against many human and agricultural pathogens. The extracts were also found to possess significant antifungal activities, but the antifungal activities were less than the antibacterial activities. Thus, this plant shows potential as a source for an antimicrobial agent. Further study is necessary to identify other bioactive compounds that can be used to fight microorganisms.

Conflict of interest

The authors declare that there are no conflicts of interest.

ORCID

Mukesh Kumar Singh. http://orcid.org/0000-0001-5753-2700.

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