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DETERMINATION OF ANTIMICROBIAL PROPERTIES OF *Plumbago zeylanica* L .

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ABSTRACT

Background: Numerous studies have been conducted on antimicrobial properties that may be useful to overcome the emerging antimicrobial resistance. *Plumbago zeylanica* (Ela nitul) is especially known as Ceylon leadwort. *Plumbago zeylanica* has been used in traditional medicine to treat various diseases, including skin rashes, scabies, ringworm, hookworms, dermatitis, acne, sores, and ulcers.
Objectives: The objective of this study was to study the antimicrobial activity of *P. zeylanica* against a selected panel of pathogenic microorganisms *in vitro* and to screen the presence of phytochemicals in the aqueous extracts of *P. zeylanica* root, leaf, stem qualitatively. **Material and Methods:** The antibacterial effect of aqueous extracts of roots, leaves and stems of *Plumbago zeylanica* against *Staphylococcus aureus, Acinetobacter baumanniii, Pseudomonas aeruginosa*, *Escherichia coli* and antifungal effect against *Candida albicans* were studied using well diffusion assay and macro-dilution method. Qualitative screening of the phytochemicals was done using standard methods. **Results:** Among the panel of organisms, *Candida albicans* exhibited the highest susceptibility against root extract of *P. zeylanica* followed by other organisms including *Staphylococcus aureus* and *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, respectively. In qualitative analysis, secondary phytochemicals including alkaloids, flavonoids, steroids, saponins, taninns, chalcones, were determined in the aqueous extract root, leaf and stem, respectively. **Conclusion:** *Plumbago zeylanica* root extract has exhibited stronger antifungal and antibacterial activity rather than the leaf extract. Stem extract of *Plumbago zeylanica* didn't show antimicrobial activity against selected organisms. The presence and absence of phytochemicals in different plant materials explain the antimicrobial properties of root, leaf and stem of *P. zeylanica*.

Keywords: Antibacterial, Antifungal, Phytochemicals, *Plumbago zeylanica*

INTRODUCTION

Nature has been the most significant source of medicinal properties for millennia. Many ancient traditions, such as Ayurveda, Siddha, and Unani medicinal systems, have advocated the use of various herbal preparations such as plant juices and extracts for diseases, including infectious ones. 74% of herbal medicines have a modern indication related to their traditional, cultural and sometimes ancient applications **(Pai, 2011)**. Herbal medicines have a time immemorial use and better patient tolerance **(Parveen *et al.*, 2015)**.
Leaves, flowers, berries, bark and/or roots of plants have been used as antibacterial agents, antioxidants, antimalarials, pain relievers and for various other diseases. Therefore, traditional medicine is an essential source for the development of new, less toxic and inexpensive chemotherapy drugs **(Pai, 2011)**. Several plants have antimicrobial properties due to the presence of active ingredients such as essential oils, flavonoids, terpenoids, triterpenoids, glycosides, alkaloids and other natural phenolic compounds. These natural energy compounds are commonly called secondary metabolites, which are not essential for plant survival but act as defence mediators for plants. Therefore, traditional medicine is an important resource for the development of new, less toxic and less expensive chemotherapy agents **(Pai, 2011)**.
Plants are widely used in ethnomedicine around the world. Plant metabolites are proved to be the most important group of compounds that showed a wide range of antimicrobial activity. Phytochemicals are organic chemicals extracted from plants that can be classified as primary and secondary. *Plumbago zeylanica*, a perennial herb or shrub of the Plumbaginceae family. It is an effective medicine for skin rashes, scabies, ringworms, hookworms, dermatitis, acne, sores, and ulcers. Previous studies review the analysis of chemical constituents and pharmaceutical activities. *Plumbago zeylanica* contains carbohydrates, proteins, chlorophyll as primary constituents. Most importantly, it contains trepans, tannin, steroids, saponins, alkaloids and flavonoids as secondary phytochemicals **(Min *et al.*, 2011)** having cytotoxic, antibacterial, wound healing activity, anti-inflammatory activity, antioxidant activity and antiviral activity **(Mandavkar & Jalalpure, 2011)**.
Hospital-acquired infections (HAI) are a major dilemma in the health care sector. Hospital-acquired infections account for 7% in developed countries and 10% in developing countries. *Staphylococcus aureus, Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans* are selected organisms in the following study, which cause HAIs **(Khan *et al.*, 2017)**. Drug resistance strains of *Staphylococcus aureus, Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans* can be found in common in hospital settings such as methicillin-resistant *Staphylococcus aureus*, carbapenem resistance *Acinetobacter baumannii*. The problem of antibiotic resistance, which has limited the use of cheap and old antibiotics, has necessitated the need for a continued search for new antimicrobial compounds **(Khan *et al.*, 2017)**.
The present study was conducted to determine the antimicrobial activity of aqueous extracts of *Plumbago zeylanica* selected organisms including *Staphylococcus aureus, Acinetobacter baumanni, Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans*. Well-diffusion assay, Minimum inhibitory concentration, minimum bactericidal concentration and minimum fungicidal concentration were used to assess *in vitro* activity of aqueous extract of plant materials of *Plumbago zeylanica*.

MATERIALS AND METHODS

Test Organisms

Staphylococcus aureus (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 10662), *Candida albicans* (ATCC 10231) Clinically isolated *Acinetobacter baumannii* strains were obtained from the University of Sri Jayewardenepura, Sri Lanka. *S. aureus* and *C. albicans* cultures were maintained in Chocolate agar (LAB M, LONDON), SDA (HIMEDIA, INDIA), respectively and all the other cultures were maintained in Nutrient Agar (HIMEDIA. INDIA). Stock cultures were prepared in nutrient broth or SDA broth with 15% of glycerol and stored at -20ºC. Subcultures were maintained using the same culture media.

Plant Materials

Plumbago zeylanica (Ela- nitul) plants were obtained from Maharagama, Western Province, Sri Lanka. The *Plumbago zeylanica* plant was authenticated by the Bandaranayeka Memorial Ayurvedha research institute, Nawinna, Maharagama (Accession No: 3001). The plants were thoroughly washed with distilled water to remove dust and muddy particles and air-dried to eliminate moisture content. These plant materials, including roots, leaves, and stems were cut into small pieces and later ground into fine powder form. Further, 30 g of these powders were mixed separately with 100 ml of water in a 250 ml round bottom flask and kept for heating at a constant temperature of 60 ºC for 20 minutes. Final volumes were adjusted to 100ml again to achieve the concentration of 0.3 g/ml. The extracts were filtered through double-layered muslin cloths followed by Whatman filter paper no. 1 and preparations were stored at 4 ºC for further use with sealed lids. Plant extractions

after filtration through muslin cloths (Before filtration of Whatman No.1 filter papers) are shown in Figure 1.

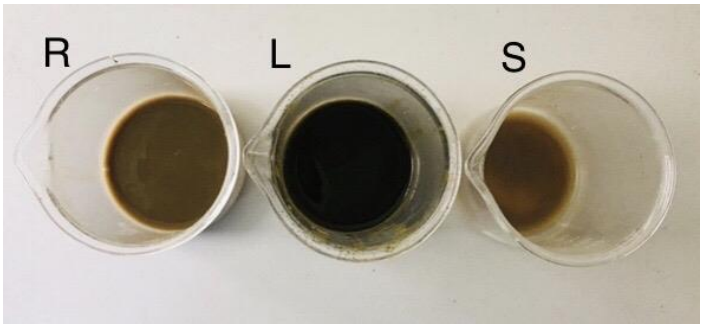

Figure 1 Extraction of plant materials
Legend: R: Roots, L: Leaves, S: Stems

Antimicrobial Activity

Antibacterial activity

Well diffusion method was conducted to assess the antimicrobial activity of aqueous extract of *Plumbago zeylanica in vitro* on Muller Hilton agar medium followed by the determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Wells were made in Muller Hilton agar plates. Then 100 µl of 0.3g/ml of aqueous extract was added into the wells. Same volumes of standard controls were added as required for each plate. Gentamycin (8 µg/ml) was used as a standard positive control for all the antibacterial tests **(Liu *et al.*, 2016)**. The plates were sealed and left for diffusion process for 2 hours and incubated for 24 hours at 37 ºC. The zone of inhibition was measured in millimetres. The macro-dilution method was conducted to investigate the MICs of selected organisms. A set of Khan tubes was prepared by dispensing 1 ml of nutrient broth and 1 ml of 0.3 g/ml of plant extract. Then two-fold dilution series was prepared up to a 1:512 ratio. Each well was inoculated with 10 µl of microbial suspensions. Tubes were sealed and incubated for 24 hours at 37 ºC bacteria. Minimum bactericidal concentration was measured by inoculating the broths used for MIC tests on MHA agar plates. The plates were prepared in triplicates.

Antifungal Activity

The same procedures were followed to determine the antifungal activity of *Plumbago zeylanica* against *Candida albicans*. Sabouraud dextrose agar plates were used to determine antifungal susceptibility tests and minimum fungicidal concentrations against *Candida albicans.* Sabouraud dextrose broth was used to determine the minimum inhibitory concentrations. Nystatin (25 µg/ml) was used as a standard positive control for all the antifungal tests **(Nenoff *et al.*, 2016)**. As usual, these procedures were triplicated to obtain more accurate results.

Qualitative Phytochemical Screening

Fifteen grams of fine powdered root, leaf, stem extracts were prepared using sterile distilled water. The qualitative phytochemical tests were carried out using aqueous extract of all of these samples of *Plumbago zeyalnica* according to standard procedures **(John De Britto *et al.*, 2013; Rufai *et al*., 2016)**.

RESULTS

Antimicrobial properties of *Plumbago zeylanica*

The aqueous extracts *of Plumbago zeylanica*, a traditionally used medicinal plant, were tested against frequently encountered hospital-acquired microorganisms using the routine *in vitro* tests. The well diffusion method showed the higher antimicrobial effect for roots and leaf aqueous extract. In addition, the study showed that both root and leaf extracts except stem extract used in this study exhibited a varying degree of antimicrobial activity against all microorganisms at 0.3g/ml and the concentration below that.
Aqueous extract of *Plumbago zeylanica* roots revealed a much higher antifungal effect than the antibacterial effect for the tested microorganisms. *Plumbago zeylanica* root extract showed antifungal activity against *Candida albicans* with a 35.7 ± 0.6 mm zone of inhibition. Among the tested microorganisms, zones of inhibition were observed *Pseudomonas aeruginosa* (18.3 ± 1.2 mm), *Acinetobacter baumannii* (17.3 ± 0.6 mm), *Staphylococcus aureus* (14.3 ± 0.6 mm). In contrast, there was no activity against gram-negative *Escherichia coli* at a 0.3 g/ml concentration. Aqueous extract of *Plumbago zeylanica* leaf extract was shown antimicrobial effect against *Candida albicans* (17.3± 0.6 mm), *Acinetobacter baumannii* (14.3 ± 0.6 mm) and *Staphylococcus aureus* (12.0 ± 1.0 mm), respectively. On the other hand, *Plumbago zeylanica* leaf extract did not give an inhibition zone against *Pseudomonas aeruginosa*, *Escherichia coli* at a 0.3 g/ml concentration. Eight micrograms per millilitre of gentamicin were used as a positive control for all the bacterial cultures. Gentamicin was given zone of inhibitions against *Staphylococcus aureus* (28.7 ± 0.6 mm*), Acinetobacter baumannii* (27.3 ± 1.2 mm), *Pseudomonas aeruginosa* (28.3 ± 1.5 mm), *Escherichia coli* (27.7 ± 0.6 mm), respectively. 25 µg/ml Nystatin was the positive control used against *C. albicans.* Nystatin was shown 21.0 ± 2.0 mm of inhibition zone against *Candida albicans* (Fig 2).

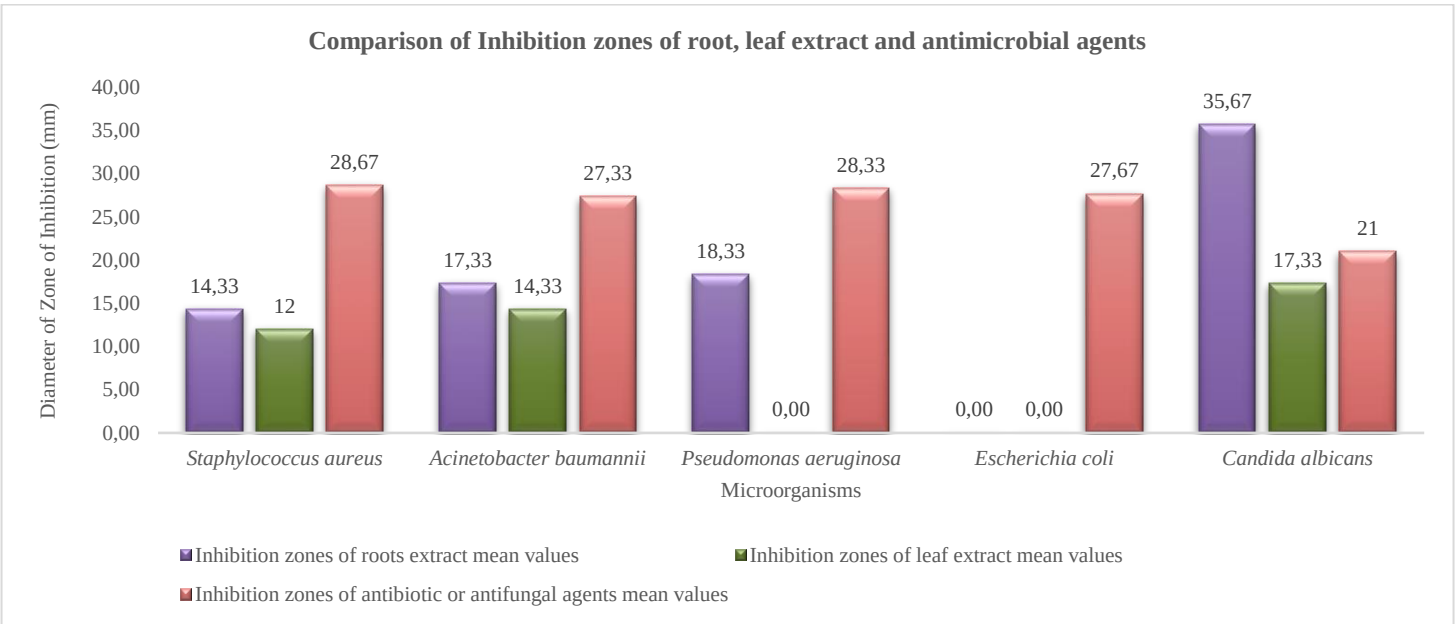

Figure 2 Well diffusion assay result of *Plumbago zeylanica* aqueous extracts

The effectiveness of the extracts against tested organisms was determined by evaluating minimum inhibitory concentration followed by minimum bactericidal and fungicidal concentrations. MIC, MBC and MFC were performed for only those microorganisms which showed inhibition zones for well diffusion assay. *Plumbago zeylanica* root extract exhibited the lowest MIC value against *Candida albicans* (0.8 ± 0.3 mg/ml) than other tested organisms. When compared to the MIC values of root extract, leaf extract also showed similar results (2.3 mg/ml) for *Staphylococcus aureus* and *Acinetobacter baumannii*. High MIC values were observed among other organisms for the leaf extract than the root extract (Tab 1). Minimum bactericidal and fungicidal concentrations were contributed to further confirmation of MIC values. MBC of *Acinetobacter baumannii* was 2.4 mg/ml and MFC of *Candida albicans* was 1.6 ± 0.7 mg/ml against root extract of *Plumbago zeylanica.* Among all three plant extracts, *Plumbago zeylanica* root extract was found to show strong antimicrobial activity (Tab 2).

Table 1 MIC results of *Plumbago zeylanica*

Minimum Inhibitory Concentrations against bacterial strains			
Organisms	MIC values (mg/ml) (Mean ± Standard Deviation)		
	Root extract	Leaf extract	Gentamicin (8 µg/ml)
Staphylococcus aureus (ATCC 25923)	2.4	2.4	0.4 ± 0.1
Acinetobacter baumannii (Clinically isolated)	2.4	2.4	0.3 ± 0.1
Pseudomonas aeruginosa (ATCC 10662)	3.9 ± 1.4	6.3 ± 2.7	1.2 ± 0.8
Escherichia coli (ATCC 25922)	6.3 ± 2.7	15.6 ± 2.7	0.7 ± 0.3
Minimum Inhibitory Concentrations against fungal strains			
Organism	MIC values (mg/ml) Mean ± Standard Deviation		
	Root extract	Leaf extract	Nystatin (25 µg/ml)
Candida albicans (ATCC 10231)	0.8 ± 0.3	3.1 ± 1.4	1.1 ± 0.6

Table 2 MBC and MFC results of *Plumbago zeylanica*

Minimum Bactericidal Concentrations			
Organisms	MBC values (mg/ml) (Mean ± Standard Deviation)		
	Root extract	Leaf extract	Gentamicin (8 µg/ml)
Staphylococcus aureus (ATCC 25923)	3.1 ± 1.4	3.9 ± 1.4	0.7 ± 0.3
Acinetobacter baumannii (Clinically isolated)	2.4	4.7	0.4 ± 0.1
Pseudomonas aeruginosa (ATCC 10662)	7.8 ± 2.7	7.8 ± 2.7	1.3 ± 0.6
Escherichia coli (ATCC 25922)	9.4	18.8	1.2 ± 0.8
Minimum Fungicidal Concentrations			
Organism	MFC values (mg/ml) (Mean ± Standard Deviation)		
	Root extract	Leaf extract	Nystatin (25 µg/ml)
Candida albicans (ATCC 10231)	1.6 ± 0.7	3.9 ± 1.4	2.3 ± 1.5

Screening of preliminary phytochemicals

The preliminary phytochemical screening of the aqueous extract of the root of *Plumbago zeylanica* showed the presence of primary phytochemicals including carbohydrates, reducing sugars, proteins and amino acids as similarly leaf extract and secondary phytochemicals including alkaloids, flavonoids, steroids, saponins, tannins, terpenoids, glycosides and chalcones. Aqueous extract of *P. zeylanica* leaf exhibited the presence of the same phytochemicals and three other chemicals, including phenolic flavonoids, anthraquinones and cardiac glycosides except the steroids. Compared to the root and leaf aqueous extracts of *Plumbago zeylanica*, aqueous stem extract showed a few types of phytochemicals, as shown in Tab 3.

Table 3 Qualitative screening results of *Plumbago zeylanica*

Test	**Aqueous**		
	Root	Leaf	Stem
Carbohydrates	+	+	+
Reducing sugars	+	+	+
Protein	+	+	-
Amino acid	+	+	+
Alkaloids	+	+	-
flavonoids	+	+	+
Phenolic flavonoids	-	+	-
Steroids	+	-	-
Saponins	+	+	-
Tannins	+	+	+
Phlobatannins	-	-	-
Terpenoids	+	+	-
Anthraquinones	-	+	-
Anthocyanosides	-	-	-
Glycosides	+	+	-
Cardiac glycosides	-	+	-
Chalcones	+	+	+

Legend: (+): Presence; (-): Absence

DISCUSSION

Over the past few years, there has been a growing concern over natural materials as novel sources of antimicrobial agents. *Plumbago zeylanica* is a medicinal herb used in traditional medicine to manage various health conditions such as digestive weakness, constipation, piles, rheumatism, muscular pains, etc (**Min *et al*., 2011; Mandavkar & Jalalpure, 2011**). It has anti-inflammatory, antibacterial, anti-tumor, antifungal properties **(Dang *et al*., 2011; Yang *et al*., 2010**). The plant has medicinal, pharmaceutical and therapeutic significance in some parts of the world (**Hao & Xiao, 2020)**.

This study was carried out to investigate the antimicrobial activities of *Plumbago zeylanica* components (Root, leaf and stem) on four bacterial species *Staphylococcus aureus, Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli.* and fungus *Candida albicans*. This study aimed to identify the antimicrobial potential of different parts of *P. zeylanica* against selected multidrug-resistant bacteria which are causing hospital-acquired infections.

The agar well diffusion technique is frequently used to assess the antibacterial activity of plant extracts. Furthermore, the dilution procedure is crucial for quantitative screening, to determine the MIC and MBC values. The MIC is considered the gold standard for determining the susceptibility of microorganisms to antimicrobials. A lower MIC value implies that a lower concentration of a drug is required to inhibit the organism's growth **(Gonelimali *et al.*, 2018)**.

In this study, the agar well diffusion assay was done as a screening test for three plant extracts against tested bacteria. All the components of the plant extracts (leaf, stem and root) showed noticeable activity in the agar well diffusion assay against the tested organisms. But the root extract showed many noticeable results compared with the antibiotic used. When compared to the root extract, leaf extract has shown lower antibacterial activity. Aqueous extract of *Plumbago zeylanica* stems did not show antimicrobial activity against all the tested microorganisms.

The activity of *Plumbago zeylanica* against *Candida albicans* strain was significantly higher (36 mm) when compared to the corresponding antifungal (25 µg/ml of Nystatin was given 21 ± 2.0 mm). Aqueous extract of *Plumbago zeylanica* roots revealed a much higher antifungal activity than the antibacterial activity

against the tested microorganisms. When considering bacterial species, *Pseudomonas aeruginosa* (18.3 ± 1.2 mm) and *Acinetobacter baumannii* (17.3 ± 0.6 mm) displayed higher activity than other bacterial species.

Plumbago zeylanica root extract did not give an inhibition zone against *Escherichia coli* at 0.3 g/ml concentration. Aqueous extract of *Plumbago zeylanica* leaf extract was effective for *Staphylococcus aureus, Acinetobacter baumannii* and *Candida albicans.*

The MIC results suggest that *Candida albicans* is more susceptible to the root extract of *Plumbago zeylanica* compared to the selected bacterial species. According to the results of this study, root extract was more effective against *staphylococcus aureus* and *Acinetobacter baumannii*. However, when compared to the MIC values of root extract, leaf extract also showed the same value for *Staphylococcus aureus* and *Acinetobacter baumannii*. Other organisms required high concentration of leaf extract than the root extract. The minimum inhibitory concentrations of the extracts showed that the *Plumbago zeylanica* root extract had the highest antimicrobial activity than the leaf extract.

The results obtained regarding the minimum bactericidal and fungicidal concentration showed the same pattern as observed in MIC results. *Candida albicans* was more susceptible to both root and leaf extract of *Plumbago zeylanica* compared to other tested microorganisms. *Pseudomonas aeruginosa* was given the highest inhibition zones to root extract in well diffusion assay among all the tested bacterial strains. But MIC and MBC results confirmed it has less susceptibility to root extract rather than *Acinetobacter baumanii and Staphylococcus aureus.* However, these results of minimum bactericidal and fungicidal concentrations confirmed that the extracts evaluated against selected pathogens exhibited *Plumbago zeylanica* root extract had the highest potency as an antimicrobial than the leaf extract.

The nature of cell wall structure makes gram-positive bacteria more susceptible to different chemical compounds than gram-negative bacteria **(Manandhar *et al.*, 2019)**. Because gram-negative bacteria contain an outer membrane with a lipopolysaccharide layer which is impermeable to certain antimicrobial agents **(Wintola & Afolayan, 2015)**. In this study, *Staphylococcus aureus* has shown more susceptibility to root and leaf extracts rather than gram-negative bacteria except for the *Acinetobacter baumannii.*

The antimicrobial properties of plant extracts depend on the chemical composition of different parts of the plant. *Plumbago zeylanica* contains a variety of important chemical compounds. According to the qualitative screening of the phytochemicals, carbohydrates, reducing sugar, protein and amino acids and secondary phytochemicals including alkaloids, flavonoids, saponins, tannins, terpenoids, glycosides and chalcones appeared in both root and leaf extracts. Compared with the root and leaf extracts, leaf extract showed fewer types of phytochemicals including carbohydrate, reducing sugars, amino acid, flavonoids, phenolic flavonoids, tannins and chalcones.

Polyphenolic compounds are the major components of plants that are responsible for their antibacterial properties **(Gharpure *et al.*, 2020)**. Their mode of action is assumed to be via enzyme inhibition (phospholipase oxygenase), i.e., via interacting with hydrosulphide groups and bacterial protein inactivation. The structure of flavonoids has been shown to impact their antibacterial activity. Unsubstituted flavones are believed to have the highest antifungal activity, whereas flavanones have the minimum effect. The antifungal activity of these chemicals get reduced when hydroxyl or methyl groups are added to them **(Kumar *et al.*, 2009)**.

Secondary metabolites of the plant extracts show different pharmacological activities including antimicrobial, antioxidant, anti-inflammatory, antiviral, cytotoxic and antimalarial activities **(Mandavkar & Jalalpure, 2011)**. *Plumbago zeylanica* contains secondary phytochemicals including alkaloids, saponins, flavonoids, steroids, coumarins, naphthoquinones and tannins that shows different pharmacological activities **(Subhash *et al.*, 2013)**. Plumbagin is the principal active compound. Plumbagin (5-hydroxy-2- methyl-1,4-naphthoquinone) is primarily present in roots in higher amounts with only about 1% in the whole plant **(Pant *et al.*, 2012)**. Saponins have the ability to produce an inhibitory effect on inflammation. Also, tannins wield antimicrobial properties by iron deprivation or making specific interactions with vital proteins/ enzymes in microorganisms **(Wintola & Afolayan, 2015)**.

Plumbagin induces ROS-mediated apoptosis in human promyelocytic leukaemia cells in vivo plumbagin, a naphthoquinone from the roots of *P. zeylanica*, which possesses anticancer and antibacterial activity also Plumbagin activates EKR 1/2 and Akt via superoxide, Src and pi3- kinase in 3t3-l1 cells. Plumbagin, derived from the plant *P. zeylanica*, has been shown to chronically activate ERK1/2 and inhibit Akt activity in cancer cells **(Yang *et al.*, 2010; Aziz *et al.*, 2008))**.

Plumbago zeylanica exhibits anti-inflammatory properties both *in vitro* and *in vivo* (**Dang *et al.*, 2011)**. The anti-inflammatory and antinociceptive activities of various leaf extracts of *P. zeylanica* (petroleum ether, chloroform, acetone and ethanol) have been studied using *in vivo* experimental models at two dosage levels (200 and 400 mg/kg, orally). The acetone extract significantly ($p < 0.01$) reduced inflammation in the carrageenan-induced rats when compared to the control group **(Sheeja *et al.*, 2010)**.

Plant-based antibiotics offer enormous therapeutic potential and have been successful in the treatment of infectious diseases with fewer or no side effects that are commonly associated with synthetic antibiotics **(Anand *et al.*, 2019)**. Findings of this study and previous studies reveal a potent *in vitro* antimicrobial activity of *Plumbago zeylanica* roots, leaves, and stem **(Sudha Devi & Thenmozhi, 2011; Subhash et al., 2013)**.

CONCLUSION

This study demonstrated the antibacterial potency of aqueous extracts of roots, leaves, and stem of *plumbago zeylanica.* The antibacterial property of these plant extracts could be mainly due to their phenolic and flavonoid content. Furthermore, the plant extracts contain a mixture of compounds that may provide relatively more minor inhibition zones. This could be one of the reasons why plant-based treatments in alternative medicine take time to cure. The secondary phytochemical constituents explain the antimicrobial properties of all three plant materials. But quantitative results are required for more clarification of the antimicrobial activity of roots and leaves as there are few secondary phytochemicals shown by the leaf extract of *P. zeylanica* including phenolic flavonoids, anthraquinones and cardiac glycosides when compared with root extract. However, this may not be compatible with the *in-vivo* effect, as there is still a possibility of the active compounds being destroyed or metabolized within live systems. Further researches are needed to focus on *in vivo* antibacterial assays and interactions with antibiotics, synergetic effects or other medicinal plants or drugs in order to understand *in vivo* antimicrobial effects better.

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