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ANTI-BACTERIAL ACTIVITY OF *PLUMBAGO ZEYLANICA* L. ROOTS ON SOME PNEUMONIA CAUSING PATHOGENS

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ABSTRACT: The anti bacterial activity of polar and non-polar extracts prepared from the roots of *Plumbago zeylanica* L. (Plumbaginaceae), a plant widely used in Ethiopian traditional medicine for various ailments were investigated using hole plate diffusion method against some pneumonia causing pathogens. The aqueous extract did not exhibit any activity while petroleum ether extract was found to have strong anti-bacterial effects as compared to the ethanol extract which showed a significant activity. Activity guided chromatographic purification of the petroleum ether extract led to the isolation of three compounds, of which the compound identified as 5-hydroxy-2-methyl-1, 4-naphthoquinone, plumbagin, found to be the active component on the tested microorganisms. Minimum inhibitory concentration value of this particular compound showed comparative activity resembling the commonly used broad spectrum antibiotic, tetracycline. The strong antibacterial effect of the petroleum ether extract is discussed to show that it was attributable to this compound rather than the other two that were found to have trace of activities.

Key words/phrases: Anti-bacterial activity, Minimum inhibitory concentration, *Plumbago zeylanica*, pneumonia

INTRODUCTION

Pneumonia was one of the top five (in adults) and the top three (in infants) causes of out patient visits to Ethiopian health institutions in 1998 and 1999 (Ministry of Health, 1999/2000). The most predominant pathogen of bacterial infection among patients with pneumonia is *Streptococcus pneumoniae* followed by the other common pathogens like, *Klebsiella pneumoniae*, *Haemophilus influenzae* and *S. aureus* (Cheesbrough, 1984).

Antibiotic resistant bacterial strains are increasingly emerging world wide as a result of abuse of indiscriminate use of anti-microbial drugs that resulted in significant public health problems (Sheers, 1993; Hart and Kariuri, 1998). The reports of different studies conducted in Ethiopia revealed high prevalence rates of antibiotic resistance of bacterial pathogens, which has created

immense clinical problems in the treatment of most infectious diseases like pneumonia (Dodge and Wallace, 1975; Messele Gedebou and Alebachew Tassew, 1980; Lindtjorn *et al*., 1989; Davis, 1994; Abraham Assefa and Girmay Yohannes, 1996; Dawit Wolday and Worku Erge, 1997; Hart and Kariuri, 1998; Ashenafi Belhu and Lindtjorn, 1999).

One way to control this problem is through the development of alternative antibiotics by screening medicinal plants for their possible anti-bacterial effects. With this regard, the present investigation has been undertaken on one of the traditionally used medicinal plant (Dawit Abebe and Ahadu Ayehu, 1983).

Plumbago zeylanica L. (Plumbaginaceae) local name '*Amera* (Amharic), *Mertes* (Oromifa)' is a shrub widely distributed in the West and Northwest regions of Ethiopia between 1500–2200 m above sea level. It has been reported that plumbagin, the major constituent of the roots of this plant possessed anti-bacterial activity against *Bacillus cerus*, *B. pumilis*, *Staphylococcus aureus* and *Klebsiella aerogenes* (Vandervijver and Lotter, 1971), *B. subtilis*, *E. coli*, *Proteus vulgaris*, *Salmonella typhimurium, Staphylococcus aureus* and *Pseudomonas aeruginosa* (Ahmed *et al*., 1998; Satyavati *et al*., 1987), *Neisseria gonorrhoea* (Gundidza and Manwa, 1990), anti fertility and abortive activity (Bhargava, 1984), anti-feedent and insecticidal effects on insect pests (Gujar, 1990).

The purpose of this study was therefore to evaluate the anti-bacterial property of this plant against clinical isolates and reference strains of the major pneumonia causing pathogens.

MATERIALS AND METHODS

Plant material

The roots of *P. zeylanica* L. (Plumbaiginaceae) were collected near Delomena a town 534 km from Addis Ababa, Balie zone, Oromiya region at an altitude of 1200 m above sea level. A herbarium specimen was deposited (Herbarium No. GH. 10) at the herbarium of Department of Drug Research, Ethiopian Health and Nutrition Research Institute (EHNRI).

General

One dimensional ^{1}H and ^{13}C NMR spectra and two dimensional ^{1}H, ^{1}H-Cosy and ^{1}H, ^{13}C-HSQC spectra were recorded with a Varian Unity Inova spectrometer (400 MHz) in Deuterated chloroform ($CDCl_3$)at 298 K using Tetra methyl silane (TMS) as internal standard. Column chromatography (CC) was carried out over silica gel (Merck, mesh 230–240 μm) using gradient elution with Petroleum ether (Pet. ether) and Ethyl acetate (EtOAc).

Homogeneity of fractions was tested on Thin layer chromatography (TLC), (Silica gel, Merck). The spots were visualized by spraying with 5% methanolic KOH. Solvent systems used for TLC and CC were the following: A) Pet. ether/EtOAc (30:1); B) Pet. ether/EtOAc (18:1); C) Pet. ether/EtOAc (9:1); and D) Pet. ether/EtOAc (4:1).

Extraction and isolation

The powdered roots (301g) were extracted with Pet. ether (40–60° C) and ethanol successively using soxhlet apparatus, which were concentrated in vacuo to give 5.6 g and 26.18 g gummy residues, respectively. Ten point one grams of the powdered roots of the plant were also separately macerated with cold water and lyophilized in super MODULYO freeze dryer. All these crude extracts were tested for their anti-bacterial effects. The petroleum ether extract which showed stronger anti-bacterial activity was chromatographed on silica gel, four fractions each 50 ml were collected and analyzed by TLC using solvent systems B and C. Fractions 1 and 2 yielded two compounds which were found to have slight activity against the tested organisms. Fractions 3 and 4 that contained the major compound and minor impurities were further purified using CC (solvent systems A and B) to yield the highly active orange colored crystal, plumbagin (420 mg).

Test organisms and culture media

The test organisms included in the study were *Staphylococcus aureus* (ATCC 25923), *Streptococcus pneumoniae* (ATCC 7465) and *Klebsiella pneumoniae* (ATCC 13883) obtained from reference strains and the clinical isolates from Clinical Bacteriology Laboratory of the Ethiopian Health and Nutrition Research Institute (EHNRI). All these organisms were maintained on Soybean Casein Digest Agar (SCDA) except for *Streptococcus pneumoniae* (ATCC 7465) which was maintained on blood agar. The organisms were then kept at 4° C prior to use.

Standard antibiotics

Tetracycline and chloroamphenicol being the most commonly prescribed broad spectrum antibiotics and quite cheap in their price were selected as a positive control. Tetracycline hydrochloride (Sigma, lot No. 114F-0163) stock solution was prepared in a concentration of 2 mg per ml in distilled water. Chloramphenicol (Park Davis, lot No. 15256) stock solution was prepared in a concentration of 4 mg per ml in ethanol.

Inoculum

Culture of the test organisms were prepared following the method adopted from Matsen (1980) and Leven *et al*. (1979). In brief, four to five morphologically identical colonies were inoculated in 5 ml of Soybean Casein Digest Broth (SCDB) and incubated for 24 hours at 37° C. The turbidity of the broth culture was then equilibrated with similar broth to match that of a half

of McFarland standard to obtain approximately the organisms number in the range of $1x10^6$ to $5x10^6$ CFU/ml. Then the suspension was diluted to 1:100 and used as a starting inoculum for the test.

Preliminary screening test for anti-bacterial activity

Preliminary anti-bacterial activity was carried out for the crude plant extracts by using hole plate diffusion method (Vanden Berghe and Vlietinck, 1991). The agar plate was prepared for each organism separately as follows. Two hundred fifty microliters of the standardized inoculum was mixed with 20 ml SCDA at a temperature of 45–50° C in sterile condition and allowed to solidfy at room temperature. Different concentrations of petroleum, ethanol and aqueous extracts of the root were prepared with solvents used for extraction and added in the hole (10 mm diameter) which was made with sterile borer on the solidified agar. The plate were then left at room temperature for 2 hours in order to favour diffusion and incubated at 37° C for 24 hours. Similarly, a hole was prepared for each solvent used in the extraction process as a negative control. After 24 hours of incubation, the inhibition zones were determined for petroleum ether, ethanol, aqueous extracts and pure solvents, respectively.

Anti-bacterial activity and minimum inhibitory concentration (MIC) determination of plumbagin

The MIC of Plumbagin and the standard drugs, tetracycline and chloramphenicol against the tested organisms were determined using tube dilution method (Reeves, 1978) by inoculating the standardized test organisms into 2 ml of SCDB tubes containing different concentrations of the compound, the standard drugs and petroleum ether as controls. The concentrations that were included in the study were 0.05, 0.1, 0.2, 0.3, and 0.4 mg/ml in liquid media and one more tube was used only with broth media without the test compound and standard drugs in order to provide the appropriate growth control. All the tubes were incubated at 37° C for 24 hours in aerobic environment except for standard organism of *Streptococal pneumoniae* which was incubated in anaerobic jar with candle.

Finally, the presence or absence of growth at each concentration of plumbagin and the standard antibiotics was examined and the MIC that completely inhibited the growth of bacteria for both test compound and standard drugs were recorded. All the tests were carried out in triplicate and the results were reported as the average of these replications.

RESULTS AND DISCUSSION

Petroleum ether, ethanol and aqueous extracts of the roots of the plant were tested against standard organisms and clinical isolates of *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Streptococcus pneumoniae*. Petroleum ether extract exhibited the highest degree of anti-bacterial activity as compared to the ethanol extract that showed significant effect against tested organisms, but the aqueous extract did not possess any anti-bacterial activity. Table 1 provides the summary of the anti-bacterial activities of four different concentrations of the extracts with reference to the test organisms. In order to locate the active substance in the petroleum ether extract, the extract was analyzed by a thin layer chromatography (TLC).

Table 1. Anti-bacterial activities of crude extracts of *P. zeylanica* root against the standard and clinical isolates tested.

No.	Bacteria	Conc.(mg/ml)	Inhibition Zone (mm) on SCDA		
			H_20 extract	EtOH extract	Pet ether extract
1	*Staphyloccus aureus* (ATCC 25923)	0.1mg/ml	-	-	-
		0.3mg/ml	-	2	5
		0.5mg/ml	-	2	6
		1mg/ml	-	2	8
2	*Streptococcus pneumoniae* (ATCC 7465)	0.1mg/ml	-	-	3
		0.3mg/ml	-	1	4
		0.5mg/ml	-	2	6
		1mg/ml	-	2	7
3	*Klebsiella Pneumoniae* (ATCC 13883)	0.1mg/ml	-	2	2
		0.3mg/ml	-	2	4
		0.5mg/ml	-	2	4
		1mg/ml	-	3	5
4	*Staphyloccus aureus* (clinical isolate)	0.1mg/ml	-	-	-
		0.3mg/ml	-	2	3
		0.5mg/ml	-	2	6
		1mg/ml	-	2	7
5	*Streptococcus pneumoniae.* (clinical isolate)	0.1mg/ml	-	-	-
		0.3mg/ml	-	1	3
		0.5mg/ml	-	1	5
		1mg/ml	-	2	6
6	*Klebsiella pneumoniae* (clinical isolate)	0.1mg/ml	-	-	-
		0.3mg/ml	-	2	2
		0.5mg/ml	-	2	3
		1mg/ml	-	2	4

The numbers indicated diameters of zone of inhibition; '-', indicated the absence of zone of inhibition; solvents used for extraction were tested as a negative control but did not show any growth on the above mentioned organisms.

The TLC showed five components that were visualized by the spraying reagent. Repeated silica gel column chromatography fractionation and purification readily separated three of these components. The anti-bacterial activity of these components were tested individually with tube dilution method against the test organisms using petroleum ether as a control. The chromatographic fraction occurring as orange spot (deep violet with the reagent) on TLC produced a marked zone of inhibition of bacterial growth while the remaining fractions containing the remaining two components showed traces of activity. The active orange colored substance, plumbagin, was found to be sparingly soluble in hot water and moderately in ethanol but more soluble in petroleum ether, chloroform and benzene. This showed that the substance occurred in high concentration in petroleum ether but in lower concentrations in the ethanol extracts.

Plumbagin showed a strong activity against both gram-positive and gram-negative microorganisms tested, with MIC value of 0.1 mg per ml against standard organisms of *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883), *Streptococcus pneumoniae* (ATCC 7465) and clinical isolates of *Staphylococcus aureus*. The activities against clinical isolates of *Streptococcus pneumoniae* (MIC 0.2 mg per ml) and *Klebsiella pneumoniae* (MIC 0.4 mg per ml) were less pronounced as compared to the above mentioned organisms (Table 2). In order to have a better view on the anti-bacterial activity of plumbagin, a comparison was made with standard broad spectrum antibiotics namely tetracycline and chloroamphenicol as a positive control (Table 3).

The anti-bacterial activity of plumbagin was more pronounced than that of chloramphenicol against all tested organisms. Equivalent activity in terms of potency was observed between plumbagin and tetracycline against standard organisms of *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883) and clinical isolates of *Staphylococcus aureus*. There was also comparable activity between them against standard organisms of *Streptococcus pneumoniae* (ATCC 7465) and clinical isolates of *Klebsiella pneumoniae* and *Streptococcus pneumonia* (Tables 2 and 3). Clinical isolates are basically obtained from symptomatic patients. Hence, they may have high chance of exposure to anti-bacterial agents that may bring change to the molecular and other factors. Therefore, they are expected to be less sensitive as compared to standard organisms with no chance of exposure to the above agents. The over all result showed that plumbagin has an anti-bacterial activity resembling with the commonly used broad spectrum antibiotic, tetracycline.

Table 2. Anti-bacterial activity of plumbagin against the tested organisms compared to that of antibiotic drugs.

Isolated compound and standard drugs	Conc.	Standard organisms			Isolated organisms		
		Staph.aureus ATCC 25923	***Strep.Pneu*** ATCC 7465	*Kleb pneu.* ATCC 13883	*Staph aureus*	*strep.pneu*	*Klb.Pneu.*
Plumbagin	0.05 mg/ml	-	-	-	-	-	-
	0.1 mg/ml	+	+	+	+	-	-
	0.2 mg/ml	+	+	+	+	+	-
	0.3 mg/ml	+	+	+	+	+	-
	0.4 mg/ml	+	+	+	+	+	+
Tetracycline	0.05 mg/ml	-	+	-	-	-	-
	0.1 mg/ml	+	+	+	+	+	-
	0.2 mg/ml	+	+	+	+	+	-
	0.3 mg/ml	+	+	+	+	+	+
	0.4 mg/ml	+	+	+	+	+	+
Chloramphenicol	0.05 mg/ml	-	-	-	-	-	-
	0.1 mg/ml	-	-	-	-	-	-
	0.2 mg/ml	-	-	-	-	-	-
	0.3 mg/ml	-	-	-	-	-	-
	0.4 mg/ml	-	-	-	-	-	-
	1 mg/ml	+	+	-	-	+	-
	2 mg/ml	+	+	+	+	+	-
	3 mg/ml	+	+	+	+	+	+

'+', indicated anti-bacterial activity; '- ', indicated the absence of anti-bacterial activity.

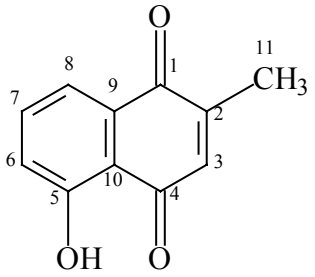
Table 3. Minimum Inhibition Concentration (MIC) values of plumbagin compared to standard antibiotic drugs against tested organisms.

Organisms	Chloramphenicol	Tetracycline	Plumbagin
Staphylococcus aureus (ATCC 25923)	1 mg/ml	0.1 mg/ml	0.1 mg/ml
Streptococcus pneumoniae (ATCC 7465)	1 mg/ml	0.05 mg/ml	0.1 mg/ml
Klebsiella pneumoniae (ATCC 13883)	2 mg/ml	0.1 mg/ml	0.1 mg/ml
Staphylococcus aureus (isolate)	2 mg/ml	0.1 mg/ml	0.1 mg/ml
Steptococcus pneumoinae (isolate)	1 mg/ml	0.1 mg/ml	0.2 mg/ml
Klebsiella Pneumoniae (isolate)	3 mg/ml	0.3 mg/ml	0.4 mg/ml

Identification of the active constituent, plumbagin in the petroleum ether extract was mainly based on 1D (^{1}H and ^{13}C) and 2D (^{1}H, ^{1}H-Cosy and ^{1}H, ^{13}C-HSQC) NMR experiments (Table 4). This compound was reported from a number of *Plumbago* species and *Dorsera rotundifolia* L (Vandervijuer and Lotter, 1971; Vinkenberg *et al.*, 1969). Therefore, this plant has a potential in the development of an anti-bacterial agent. Therefore, it is encouraging to initiate *in vivo* anti-bacterial and toxicological, including cytotoxicity studies followed by clinical evaluation for treating infections caused by susceptible microorganisms.

Table 4. ^{13}C and ^{1}H NMR chemical shifts of Plumbagin in $CDCl_3$ at 298 K vs internal standard (TMS).

Position	δ_C	δ_H (ppm, J= Hz)
1	184.7	-
2	149.6	-
3	135.4	s, 6.75
4	190.2	-
5	161.2	-
6	124.1	dd, 7.18 (8, 2.7)
7	16	br t, 7.55 (8, 7.8)
8	16.5	dd, 7.56 (7.8, 2.8)
9	132.0	-
10	115.1	-
11	16.5	S, 2.17
OH	-	br s, 11.8



Plumbagin

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