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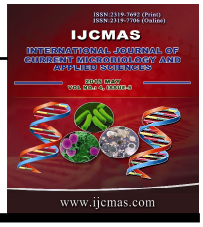

Original Research Article

Antibacterial activities of three latex plants of Asclepiadaceae family used in traditional medicine in South Togo

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Keywords

Pergularia daemia, Secamone afzelii, Leptadenia hastata, antibacterial potentials, time-dependence

A B S T R A C T

This study was undertaken to evaluate the *in vitro* antibacterial potentials of the extracts of *Pergularia daemia*, *Secamone afzelii*, and *Leptadenia hastata* against six human pathogenic bacteria, and the influence of plant material harvest times on the antibacterial activities. The agar well diffusion method was used for the susceptibility of bacteria to the extracts, and peptone water microdilution in 96 well-plates was used to determine the MICs and MBCs. The results indicated that all tested plants extracts exhibited various antibacterial activities. Among the tested species, *P. daemia* was the most active. Analysis of MICs and MBCs showed that *P. daemia* had a bactericide effect against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and *S. aureus*, and a bacteriostatic effect against *S. typhi* and *K. pneumoniae*. The diameters of inhibition zones of the leaves extract collected in the morning were significantly better than those collected in the afternoon or evening, showing that the leaves of *P. daemia* should be collected early in the morning to optimize the activity of the plant. This study supports the medicinal use of the three species for bacterial infections.

Introduction

Infectious diseases remain a major public health problem throughout the world. They still represent the main cause of the high mortality rates recorded in developing countries, whereas, in industrialized countries, an alarming incidence of antibiotic resistance is observed.

The emergence of multi-drug resistant phenotypes is a major public health problem in the treatment of bacterial infections (Okusa, 2012). The real challenge for scientists worldwide today, is to find continuously new drugs to combat resistant microorganisms, or compounds which are

able to inhibit the resistance's mechanisms of pathogenic microorganism therefore restoring the activity of antibiotics (Oseni et *al*., 2014). In modern medical practice, the alarming worldwide incidence of antibiotic resistance causes an increasing need for new compounds. Medicinal plants represent a valuable source for this kind of compounds (Hatano et *al.*, 2005). Indigenous herbal remedies are widely used against many infectious diseases, but only few of them have been studied chemically and biologically in order to identify their active constituents (Longanga et *al*., 2000).

In Togo, numerous plants are traditionally used against infectious diseases. Among them are *Pergularia daemia*, *Secamone afzelii*, and *Leptadenia hastata*, three latex plants from Asclepiadaceae family. The family Asclepiadaceae consists of about 130 genera and 2000 species distributed all over the world. Some of them are tropical and subtropical shrubs, often twining, or perennial herbs. The latex cells usually contain a latex rich in triterpenes and other constituents include: cyanogenetic glycosides, saponins, tannins and cyclitols (Evans, 2002). Plant latex is a good source of various secondary metabolites, which shows growth inhibition effect against bacteria, fungi, viruses, tumours and cancer cell lines (Ujwala and Karpagam, 2013). *P. daemia* is used as anthelmintic, laxative, antipyretic and expectorant, and is also used to treat infantile diarrhoea and malarial intermittent fevers (Sutar and Pal, 2014). *Secamone afzelii* is used in traditional medicine for stomach problems, diarrhoea, gonorrhoea, malaria, cough, catarrhal conditions, diabetes, and as galactogogue (Gill, 1992; N'Guessan et *al.*, 2009). *Leptadenia hastata* is used in the management of onchocercosis, scabies, hypertension, catarrh, skin diseases, sexual potency, and wound-healing (Thomas, 2012). Some pharmacological investigations were conducted to prove their therapeutic potentials (Abere and Onwukaeme, 2012; Raghavamma et *al.*, 2013; Anywar et *al*., 2014). But in Togo, a little is known about these species.

The aim of the present study is to evaluate *in vitro* antibacterial potentials of the aqueous and ethanolic extracts of *P. daemia*, *S. afzelii*, and *L. hastata* against six selected human pathogenic bacteria, and then, the influence of plant material harvest times on the antibacterial activities of *P. daemia*.

Material and Methods

Plants material

The leaves of *Pergularia daemia* and *Secamone afzelii* were collected on "Université de Lomé" campus, whereas the ones of *Leptadenia hastata* were collected from Tsévié located at 35 km north of Lomé (Togo). All the plants material were obtained in October 2014 and identified at the Herbarium of Botanical Department of "Université de Lomé" with the following voucher numbers: *Pergularia daemia* (TG12743), *Secamone afzelii* (TG12744), and *Leptadenia hastata* (TG12741).

Microorganisms

The microorganisms used for the antibacterial tests were Gram-positive (*Staphylococcus aureus* ATCC 29213 and clinical strain of *Staphylococcus aureus*), and Gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and clinical strains *Salmonella typhi*, *Klebsiella pneumoniae*) bacteria. The ATCC strains were obtained from the American Type Culture Collection, via National Institute of Public Health of Togo; whereas the clinical strains were from the laboratory of Navrongo Health Research Centre (Ghana).

Extractions

The leaves of the three plants were washed thoroughly under running tap water, and each plant material was reduced to small fragments. The plant samples were dried in air condition room for two weeks. After drying, the plant parts were powdered using a grinding machine. Aqueous extraction was made by boiling 50 g of powder in 500 mL distilled water for 20 minutes. After cooling at room temperature, the extract was filtered with Whatman n°1 paper and evaporated dried. Ethanolic extraction was performed by maceration of 50 g powder in 500 mL of 70% (v/v) ethanol, while being shaken for 48 hours with a magnetic stirrer. The preparations was filtered with Whatman n°1 paper and evaporated dried. The extracts were preserved at 4°C in refrigerator till used.

Antibacterial sensitivity assay

The agar well diffusion method was used to investigate the antimicrobial properties of the extracts as described in the National Committee for Clinical Laboratory Standards (NCCLS, 2003; Rupapara et *al*., 2015). The bacterial strains grown on nutrient agar at 37°C for 18 to 24 h were suspended in a saline solution (0.9%, w/v) to a turbidity of 0.5 Mac Farland standards (10^8 cfu/ml). The suspension was used to inoculate Mueller Hinton agar 90 mm diameter Petri dishes with a sterile cotton swab on a wooden applicator.

A sterilized steel borer of an internal diameter of about 6 mm was used to bore holes in the Mueller-Hinton media plates. The dried ethanolic extract was dissolved in dimethyl-sulfoxide 1% (DMSO), and the aqueous extract in distilled sterilized water. The extracts, positive and negative controls were dispensed into these holes. A duplicate of each plate was made. The plates were kept at room temperature for 1 hour for the extract to diffuse into the media before it was incubated at 37°C for 24 hours. Antibacterial activities were evaluated by measuring inhibition zone diameters around the wells containing the extract (CASFM, 2014). Ciprofloxacin (5µg/ml) and DMSO 1% in sterilized distilled water were used as positive and negative controls respectively.

Determination of Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

MICs and MBCs were determined using the peptone water microdilution in 96 well-plates according to National Committee for Clinical Laboratory (NCCLS, 2003). The same 0.5 Mac Farland suspensions were diluted with peptone water to inoculate 96 well-plates containing 2-fold serial dilutions of extracts. The extracts concentrations were ranged from 20 to 0.039 mg/ml.

The final volume in wells was 200 µl. Plates were incubated at 37°C for 24 h. MIC was recorded as the lowest extract concentration demonstrating no visible growth in the broth. MBC was recorded as the lowest extract concentration killing 99.9% of bacterial inocula. MBC values were determined by removing 100 µl of bacterial suspension from subculture demonstrating no visible growth and inoculating nutrient agar plates that were incubated at 37°C for 24 h.

The influence of harvest time on the activity of extracts

To evaluate the influence of the harvest time in the biological activities of the plant extracts, the leaves of *Pergularia daemia* were collected at three different times (GMT) of day (Morning before 8:00 am, Afternoon between 12:00 am to 1:00 pm, and Evening after 5:30 pm). The collected

plant materials were treated in the same conditions as the previous, and the extracts were tested on the same bacterial strains using the agar well diffusion method.

Data analysis

Data were keyed into SPSS 20.0 (Chicago, USA). The results of each inhibition zone are presented as mean ± standard deviation (SD) of the mean of duplicates. Data were analyzed using the one-way analysis of variance (ANOVA). P-values of less than 0.05 were considered statistically significant.

Results and discussion

Antibacterial sensitivity assay

The results of antibacterial sensitivity assay of the three tested plants extracts are presented in table 1. The antibacterial activities were observed in various ways with zones of inhibition diameters ranging from 6.5 ± 0.7 mm to 22.5 ± 2.1 mm. Among the three plant species tested, *P. daemia* was the most active. The extracts of *P. daemia* inhibited the growth of all tested bacteria except the aqueous extract which was inactive against *S. aureus* and *K. pneumoniae*.

The ethanolic extract of *S. afzelii* inhibited the growth of tested bacteria with zones of inhibition diameters ranging from 6.5 ± 0.7 mm to 17.5 ± 2.1 mm, whereas the aqueous one was inactive against *P. aeruginosa* ATCC 27853, *S. typhi*, and *K. pneumoniae*. The extracts of *L. hastata* were active against *S. aureus* ATCC 29213, *S. aureus*, *Salmonella typhi*, and *K. pneumonia*e, and inactive against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.

The ethanolic extracts were more active than the aqueous and inhibited the growth of all tested bacteria except ethanolic extract of *L. hastata* against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. Among the tested bacteria, *S. aureus* ATCC 29213 was the most sensitive. Ciprofloxacin, which was used as a positive control, had zones of inhibition diameters ranging from 24 ± 2.8 mm to 32.5 ± 2.1 mm and DMSO 1% in distilled water, which was used as the negative control, showed no activity. Hence, any inhibitions observed in the plant extracts were not due to the solvent.

Some phytochemical components such as tannins, saponins, terpenoids, alkaloids, flavonoids, phenols and steroids, were incriminated in the pharmacological properties of plants species (Alagesaboopathi and Sivakumar, 2011). Previous studies showed in India that the ethanolic extract of *P. daemia* reveals the presence of medicinally valued bioactive components like tannins, saponins, terpenoids, alkaloids, flavonoids, phenols and steroids (Sridevi et *al.*, 2014, Raghavamma et *al.*, 2013). The presence of alkaloids, tannins, cardiac glycosides and saponins in the leaves extract of *S. afzelii* was demonstrated in Nigeria (Abere and Onwukaeme, 2012).

Leptadenia hastata was reported to contain alkaloids, saponins, phenolic glycosides, tannins, flavonoids, proanthocyanidins and triterpenes (Thomas, 2012). So the antibacterial activities observed can be due to the presence of these components. In agreement with our results, Raghavamma et *al.*, (2013), found in India that the methanolic extract of leaves of *P. daemia* was active against *E. coli, Pseudomonas aeruginosa*, and *S. aureus.* Mensah et *al.*, (2006), in Ghana, demonstrated that the methanolic extract of aerial part of *S. afzelii*, inhibited the growth of *S. aureus* and *E. coli* and was inactive against *P. aeruginosa*.

Table.1 Diameters of growth inhibition zones of tested plants extracts

Microorganisms / Extracts		*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 29213	*S. aureus*	*S. typhi*	*K. pneumoniae*
PdF	Aq	12.5±2.1	13 ± 1.4	12.5 ± 2.1	0	13.5 ± 2.1	0
	Eth	21.5±0.7	22.5 ± 2.1	21 ± 2.8	13 ± 2.8	17 ± 1.4	14.5 ± 0.7
SaF	Aq	9.5 ± 2.1	0	14 ± 1.4	11.5 ± 0.7	0	0
	Eth	17.5±2.1	8.5 ± 2.1	13.5± 2.1	13.5 ± 0.7	6.5 ± 0.7	8 ± 1.4
LhF	Aq	0	0	13 ± 1.4	11.5 ± 0.7	13.5 ± 0.7	0
	Eth	0	0	13.5± 0.7	13 ± 2.8	13 ± 2.8	11 ± 1.4
Ciprofloxacine		29.5±0.7	25 ± 0	32.5± 2.1	28 ± 1.4	29 ± 1.4	24 ± 2.8

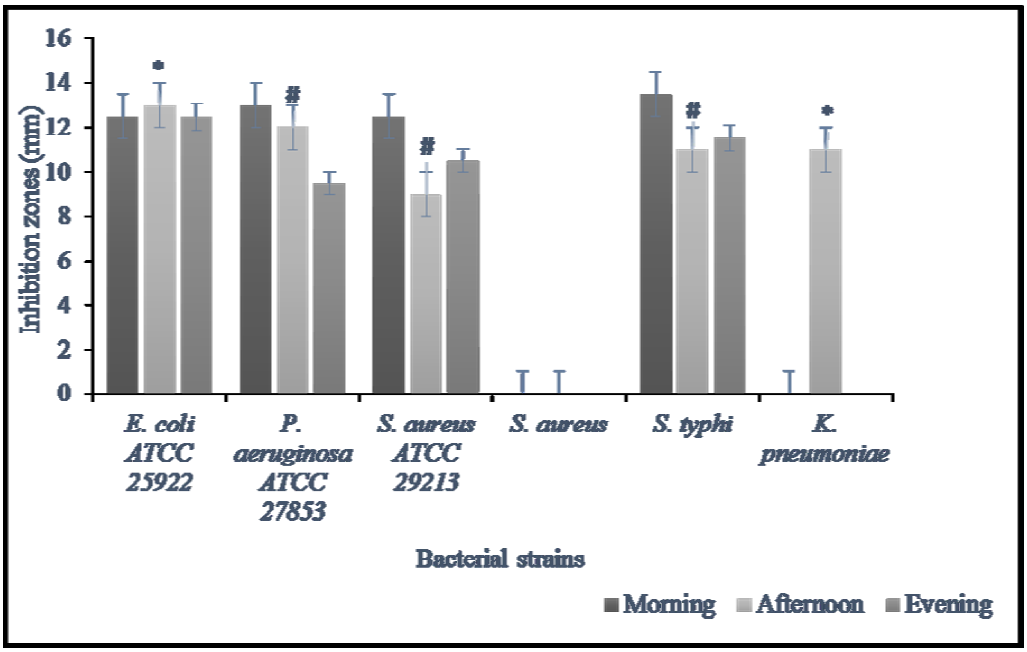
PdF : Leaves of P. daemia, SaF : Leaves of S. afzelii, LhF : Leaves of L. hastata, Aq : aqueous extract, Eth : ethanolic 70% extract,

Table.2 Minimal Inhibitory Concentrations (MIC) and Minimal Bactericidal Concentrations of the active ethanolic extracts (mg/ml)

Bacteria	*Pergularia daemia*				*Secamone afzelii*				*Leptadenia hastata*			
	CMI	CMB	CMB/ CMI	Activity	CMI	CMB	CMB/ CMI	Activity	CMI	CMB	CMB/ CMI	Activity
E. coli ATCC 25922	0.62	0.62	1	Bactericide	1.25	1.25	1	Bactericide	NA	NA	-	-
P. aeruginosa ATCC 27853	1.25	1.25	1		NA	NA	-	-	NA	NA	-	-
S. aureus ATCC 29213	0.62	0.62	1		1.25	2.5	2	Bacteriostatic	1.25	1.25	1	Bactericide
S. aureus	1.25	1.25	1		1.25	2.5	2		1.25	1.25	1	
Salmonella typhi	1.25	2.5	2	Bacteriostatic	NA	NA	-	-	1.25	2.5	2	Bacteriostatic
K. pneumoniae	1.25	2.5	2		NA	NA	-	-	2.5	5	2	

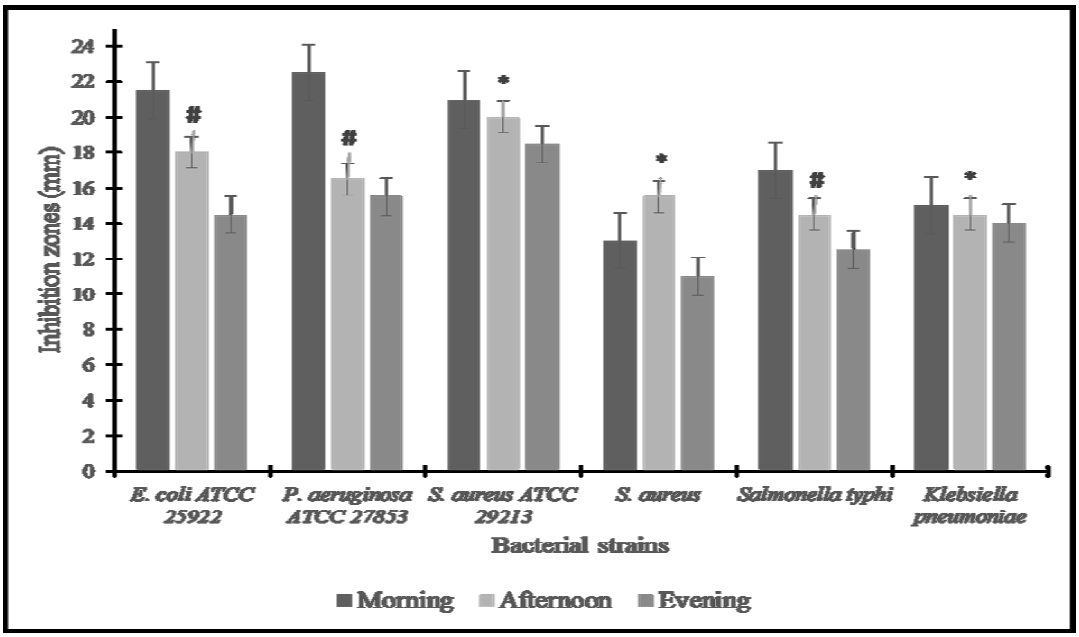
NA: Not active

Figure.1 Variation of inhibition zones of aqueous extracts of *P. daemia* according to plant material harvest time



: significant difference ($p < 0.05$); * : no significant difference or not applicable

Figure.2 Variation of inhibition zones of ethanolic extracts of *P. daemia* according to plant material harvest time



: significant difference ($p < 0.05$); * : no significant difference

Aliero and Wara, (2009), in Nigeria, found that aqueous extract markedly inhibited the growth of *E. coli* at 30 mg/ml and *P. aeruginosa* at 60 mg/ml. Our previous study showed that ethanolic extract of leaves of *L. hastata* inhibited the growth of *E. coli* and *S. typhi* but that study concerned only clinical strains (Hoekou et *al.*, 2012).

MICs and MBCs

The MICs were ranged from 0.62 to 2.5 mg/ml, while the MBCs were ranged from 0.62 to 5 mg/ml. The lowest MICs were obtained for *P. daemia* (Table 2). In order to elucidate whether the observed antibacterial effects were bactericide or bacteriostatic, MBC/MIC ratios were calculated. Extracts with ratios greater than 1 were considered as bacteriostatic, while the extracts with ratios equal to 1 are bactericide (Karou et *al*., 2005; Hoekou et *al*., 2012). Thus, the ethanolic extract of leaves of *P. daemia* had a bactericide effect against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and *S. aureus*, and a bacteriostatic effect against *S. typhi* and *K. pneumoniae*. The extract of leaves of *S. afzelii* had a bactericide activity against *E. coli* ATCC 25922 and a bacteriostatic effect against *S. aureus* ATCC 29213 and *S. aureus.* A bactericidal activity was observed for the extract of *L. hastata* against the two strains of Staphylococcus tested and a bacteriostatic activity against *S. typhi* and *K. pneumoniae*.

The influence of harvest time on the activity of extracts

Biological activities of medicinal plants vary widely depending on the type of plant, plant part, geographic location and solvent used in extraction. It may also depend on the conditions of the plant parts samples. Traditional healers, for the preparation of herbal medicines sometimes follow certain practices such as harvesting plant material early in the morning without greeting anybody along the way (Tchacondo et *al.*, 2012). To check if there is a daily better time to collect the plant material for medicinal uses, the leaves of *P. daemia* (the species most active among those tested in this study) were collected at three times (GMT) of the day: morning before 8:00 am, afternoon between 12:00-1:00 pm, and evening after 5:30 pm. These 3 samples were treated separately, extracted with water and 70% ethanol (v / v). The extracts were tested on the same organisms and the results are represented in figures 1 and 2. These results showed that the diameter of the inhibition zones is time dependent. The diameters of inhibition zones obtained from aqueous or ethanolic extracts were better with the morning collected leaves extracts. Aqueous extract data showed that there was a significant difference in diameters of inhibition zones of *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, and *S. typhi*, between morning collected leaves extract and the afternoon or evening collected leaves extract ($p < 0.05$). The ethanolic extract results, showed also a significant difference in diameters of inhibition zones of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. typhi*, between morning collected leaves extract compared to the evening collected leaves extract ($p < 0.05$). Thus, it appears that the leaves of *P. daemia* should be taken early in the morning to optimize the antibacterial activity.

This study has tested not only the ethanolic extract but also the aqueous decoction of leaves of these three plants. Decoction is the most used form by traditional healers to treat diseases. Therefore the activities obtained for these extracts were consistent with the use of such plants in the treatment of bacterial infections. Although this study has provided useful data concerning the antibacterial activities of leaves extracts of

P. daemia, *S. afzelii*, and *L. hastata*, the toxicological investigations are also necessary to provide the medicinal safety uses of these species.

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