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In-Vitro Antibacterial Activity of *Harungana madagascariensis* Lam. Ex. Poir. (Hypericaceae) stem bark extracts on some enterobacteria producing extended spectrum β-lactamases (ESBLS)

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A B S T R A C T

H. madagascariensis is well known for its antibacterial properties against various bacterial genera. The objective of this study was to evaluate the antibacterial activity of bark extracts against enterobacteria producing extended spectrum β-lactamase (ESBLs). Methods agar diffusion and dilution in liquid medium were used. Only the hydro-ethanol extract was more effective inhibition with diameters ranging from 11 mm to 15 mm. The phytochemical analysis of this extract showed the presence of polyphenols, flavonoids, tannins and saponins. Antibacterial parameters showed that the extract was bactericidal on 66.67% of strains and bacteriostatic on 33.33% strains. The minimum inhibitory concentrations (MIC) were ranging from 1.56 mg/ml to 100 mg/ml while minimum bactericidal concentrations (MBC) were ranging from 50 mg/ml to 100 mg/ml. This study shows that *H. madagascariensis* could be a good candidate in the search for new active compounds based on herbal against enterobacteria producting β-lactamase extended spectrum.

Keywords: *H. madagascariensis,* enterobacteria, β-lactamase extended spectrum.

A R T I C L E I N F O

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1. Introduction

Beta-lactams constitute the family of antibiotics most important as well by the number and the diversity of molecules also by their directions in therapeutic and in prophylactic (Gutmann and Williamson, 1987; Cavallo *et al*., 2004; Gangoué-Piéboji *et al*., 2009). However, due to their excessive and often uncontrolled use, we attend the development of resistance into microorganisms, mainly enterobacteria. The production of β-lactamases is the most developed resistance mechanism in bacteria. These enzymes hydrolyze the amide linking of the beta-lactam nucleus which is the main characteristic of this family of antibiotics. The substrate spectrum has expanded over the years and now extends to carbapenems and the latest generation of cephalosporins (Bébrone *et al*., 2010).

Infections caused by multi-resistant bacteria are a real public health problem because they involve high mortality rates, hospital stay extensions and an increase of treatment cost (Holmberg *et al*., 1987). Despite of the strong expansion of bacterial resistance, there is no developing of new antibiotics in view on the horizon (Spellberg *et al*., 2008; Boucher *et al*., 2009). Faced with this impasse, it is more urgent than ever to find new molecules with different mechanisms of action of those previously described or inhibiting bacterial resistance mechanisms of clinical importance (Okusa *et al*., 2009). These new drugs could come from natural sources, especially medicinal plants (Cowan, 1999).

H. madagascariensis Lam. ex Poir. (Hypericaceae) is a shrub native to tropical Africa and Madagascar (Iwalewa *et al*., 2008). It is highly used in traditional medicine for the treatment of anemia, gastroenteritis, nephrosis and malaria (Erah *et al*., 2003; Kamanzi *et al*., 2004). The various parts of this plant are known for their biological, antibacterial, antifungal, antiviral and antiparasitic (Tshibangu *et al*., 2002; Iwalewa *et al*., 2008; Toty *et al*., 2013). With regard to all these previous studies demonstrated the potential of this plant, it seemed necessary to us to investigate its antibacterial activity against enterobacteria producing extended spectrum β-lactamase. The aim of this study was to evaluate the antibacterial activity of aqueous and ethanol extracts of *H. madagascariensis* stem bark on enterobacteria producing extended spectrum β-lactamase.

2. Experimental

Plant material

The plant material included of *H. madagascariensis* stem bark was collected in the period from June 2013 to July 2013 in Lakota (Centre-West of Côte d'Ivoire). It was identified by the Floristic National Center of Felix Houphouët-Boigny University of Abidjan.

Bacterial Material

The bacterial material constituted of clinical strains representing 3 genera of enterobacteria all producing extended spectrum β-lactamase (ESBLs). These strains are obtained from the Bacteriology-Virology Laboratory of the Pasteur Institute of Côte d'Ivoire (Table 1).

Preparation of the plant extract

Grinding

The stem barks were cleared out of their dead parts then cut into small pieces before being dried at sheltered from sunlight for about three weeks. Once dry, they were made into a fine powder using a grinder.

Extraction

Preparation of aqueous total extract

The aqueous total extracts were prepared according to the method of Zirihi and Kra (2003)**.** We have weighed 100 g of bark powder which were homogenized in one liter of distilled water in a blender. After six cycles of homogenization, each homogenate was spun in a clean white square cloth, filtered three successive times on cotton wool and once on Wattman® filter paper. The filtrate obtained was dried in an oven at 50 °C for 4 days.

Preparation of hydro-ethanol extract

As previously, the method used is those of Zirihi and Kra in 2003 with a slight modification. We have weighed 100 g of bark powder which were dissolved by homogenization in one liter of mix ethanol-water (70/30:v/v) in a blender. After six cycles of homogenization, each homogenate was spun in a clean white square cloth, filtered three successive times on cotton wool and once on Wattman® filter paper. The filtrate was then concentrated in a rotavapor and dried in an oven at 50 °C for 3 days.

Antibacterial activity of plant extracts

This study consisted of the determination of various antibacterial parameters which are the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Efficacy Test extracts

This test allows to prove that the extract has antibacterial activity. The technique of diffusion in Mueller Hinton agar was used. The media were inoculated by flooding with strains 18 h. With the help of a sterile punch, some wells of approximately 6 mm diameter are performed in the agar. Each wells received 50 µL of the substance to test. We have let diffuse on the bench for 30 min and incubated at 37 °C for 18 h. The presence or absence of inhibition zone is noticed (Bssaibis *et al*., 2009). The results are interpreted according to Ponce *et al*., (2003) and Duraffourd *et al*., (1990).

Preparation of inoculum

The bacterial inoculum was prepared with colonies of 18 h and Mueller Hinton Broth (MHB). A single colony of the bacterial culture was taken using a platinum loop and

homogenized in 10 ml of MHB then incubated for 3 to 5 hours at 37 °C for a pre-culture. Then, 0.1 ml of the pre culture broth was taken and introduced into a tube containing 10 ml of MHB. This bacterial suspension performed is evaluated at approximately 10^6 cells/ml and constituted the dilution 10^0 or the pure.

Notation of the inoculum

The notation of the inoculum was performed by a dilution 1/10 from the initial inoculum. Four dilutions at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} were obtained. These various dilutions and the initial inoculum were inoculated with a calibrated loop of 2 µl per streak of 5 cm on Mueller Hinton agar without antimicrobial then incubated at 37 °C for 24 hours. This preparation has constituted box A and was stored at 4 °C.

Preparation of concentration range of plant extracts

The concentration range of the plant extract is prepared in seven numbered test tubes 1 to 7 by the method of double dilution according to a geometrical progression at reason of 1/2.

Inoculation

In a range of 8 hemolysis tubes numbered from C_1 to C_8, 1 ml of the *inoculum* containing the test organism was charged. Then, 1 ml of plant extract according to the concentration range prepared was added into the tubes. This distribution of plant extract was performed as well as obtained concentrations ranged from 100 mg/ml to 1.56 mg/mL. The tube C_8 received in place of the plant extract, 1 ml of sterile MHB that served as growth control. A last tube containing 2 ml of sterile broth was added to the range to serve as a sterility control. The tubes were incubated at 37 ° C for 24 hours.

Determination of minimal inhibitory concentration (MIC)

The MIC is the lowest concentration of the substance for which there is no growth visible to the naked eye after an incubation time of 18 h to 24 h. Its determination was performed by observing the trouble induced by the growth of germs studied in each tube. The MIC will be the lowest concentration for which there is no trouble with the naked eye.

Determination of minimal bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) is the lowest concentration of substance that leaves at the most 0.01% of surviving germs. Using a calibrated loop 2 µl, tube contents without trouble were collected and streaking on Mueller Hinton agar starting with the tube of MIC. The seeding was performed by parallel streaks of 5 cm on the surface of the agar (Box B). After 18 h of incubation at 37 °C, the number of colonies on the streak was compared to those of the *inoculum* numbering box (box A). Thus, the first experimental tube of which the number of germs on its streak is less than or equal to 10^{-4} dilution correspond to the MBC.

Phytochemical screening

The different groups of compounds (sterols, polyterpenes, alkaloids, tannins, polyphenols, flavonoids, saponins and quinones) contained in the extracts were demonstrated using the methods described by Békro *et al*. (2007), Wagner (1983), Hegnauer (1973), Ronchetti and Russo (1971) reported by Bidié *et al*., 2011. Thus, Liebermann reagent, ferric chloride reaction, cyanidin reaction, Stiasny reagent, Borntraegen reagent, Dragendorffet de Bouchardât reagents and the persistence of the foam were used to put in a preminent position respectively sterols and polyterpenes, polyphenols, flavonoids, tannins, quinones, alkaloids and saponins.

3. Results and Discussion

Yields of extraction

The ethanol-water mixture (70/30) was the best extraction solvent with 17.4 mg of extracts obtained, representing a yield of 17.4. As for water, it allowed to obtaine 4.6 mg of extract, representing a yield of 4.6%.

Phytochemical screening

The phytochemical screening of the ethanol extract 70% of *H. madagascariensis* stem bark allowed to detect the presence of secondary metabolites such as polyphenols, flavonoids, tannins and saponins. The others chemical groups researched, namely sterols, polyterpenes, quinones and alkaloids were not present (table 2).

Extract efficacy

The effectiveness test showed that the aqueous extract was not effective on all bacterial strains tested. This lack of effectiveness has resulted in the absence of inhibition zone whatever the bacterial genus and the extract concentration (table 3). The ethanol extract 70% was the most effective with inhibition diameters ranging according to the concentration of extracts. At a concentration of 100 mg/ml, all strains were revealed sensitive with diameters from 11 mm to 15 mm. *K. pneumoniae* 46L/14 strain was the most sensitive for a diameter of 15 mm followed by 32L/14, 77L/14 strains and *E. cloacae* 21L/14 with a inhibition diameter of 14 mm. At concentrations of 50 mg/ml and 25 mg/ml, the inhibitory activity of the extract was low with diameters ranging from 0 mm to 13 mm and 0 mm to 11 mm respectively. For the same bacterial strain, it was allowed to observe a variation in the activity depending on the concentration extracts.

Antibacterial parameters

The minimum inhibitory concentration (MIC) of ethanol extract 70% on *Escherichia coli* strains was 25 mg/ml while the minimal bactericidal concentration (MBC) of the extract on these strains was 50 mg/ml or 100 mg/ml. The highest MIC and MBC having been obtained respectively with 61L/14 strains on the one hand and 45L/56L and 56L/14 strains on the other.

With regard to *K. pneumoniae* strains, the lowest value of MIC (1.56 mg/ml) was obtained on 31L/14 strain and the highest value (100 mg/ml) was observed on 19L/14 strain. On all other *K. pneumoniae* strains, the observed MICs were 6.25 mg/mL, 25 mg/mL and 50 mg/mL. MBC was equal either to 50 mg/mL, or 100 mg/mL. However, the maximum concentration of 100 mg/mL, no value of CMB could not be determined for the 32L/14 strain.

For *E. cloacae* strains, MIC values were the lowest. These ranged from 3.12 mg/mL to 12.5 mg/mL. MBC was similar to that of other bacterial genera tested namely 50 mg/mL

and 100 mg/mL. The report MBC/MIC for bacterial genera included in this study gave values ranging from 1 to 64. However, the report could not be determined for the strain 32L/14 because the minimum bactericidal concentration could be found (Table 4).

Discussion

The aim of this study was to evaluate the antibacterial activity of two extracts of *H. madagascariensis* stem bark on enterobacteria species producing extended spectrum β-lactamase. It is a plant used in traditional medicine for the treatment of bacterial infections such as diarrhea, dysentery and typhoid fever. All *Enterobacteriaceae* species taken into account in this study are class A β-lactamases producing such as TEM-1, SHV-11, SHV-12, CTX-M-15 and class D β-lactamases like OXA-1. These enzymes confer increased resistance to β-lactam thus resulting major therapeutic failures.

The bacteria studied showed variable sensitivity with regard to ethanolic extract 70% only with inhibition zones ranging from 11 mm to 15 mm. In view of the extraction yields, we can say that ethanol 70% (ethanol-water 70/30: v / v) is a good solvent. Indeed, it has been shown that the solvent used for extraction can greatly differentiate the compounds present in extracts from the same plant part. Moreover, Eloff (1998) showed in a study that the antimicrobial compounds are not soluble in water. This could explain the difference of activity observed in efficacy tests which showed that the ethanol extract was more active than the aqueous extract.

Good inhibitory and bactericidal activities were also observed on the different bacteria tested with MICs ranging from 1.56 to 100 mg/mL and MBC between 50 and 100 mg/mL. This result is of significant importance as it has been reported in the literature that this plant is used in various gastroenteritis treatment (Okoli *et al*., 2002) in which are precisely involved *E. coli*, *K. pneumoniae* and *E. cloacae* taken account in this study.

The phytochemical analysis of ethanol extract has revealed the presence of tannins, polyphenols, flavonoids and saponins. Some previous studies have shown that, besides these families of compounds, alkaloids, steroids, polyterpenes and cardiac glycosides were present in the plant (Omotayo *et al*., 2012; Moulari *et al*., 2006, 2007; Okoli *et al*., 2002; Tona *et al*., 1998). The antibacterial activity observed could be attributed to secondary metabolites found in this extract (Favel *et al*., 1994). In fact, flavonoids are hydroxylated phenolic substances synthesized by plants in response to microbial infection (Dixon *et al*., 1983). Their activity is probably due to their ability to form complexes with extracellular proteins and also with the bacterial membrane (Cowan, 1999). Similarly, the antibacterial activity of polyphenols and tannins could be assigned to their ability to complex macromolecules such as proteins and polysaccharides. Several studies on tannins have shown their toxicity toward filamentous fungi and bacteria and concluded that the antibacterial power of some medicinal plants can be explained by their composition of tannins (Scalbert, 1991).

Table 1: Profile and biological origins of enterobacteria tested

Clinical strains	Biological Origins	Génétique profiles
Escherichia coli		
34L/14	Urines	OXA-1; CTX-M-15
43L/14	Stools	TEM-1; OXA-1; CTX-M-15
Klebsiella pneumoniae		
31L/14	pleural fluid	TEM-1; OXA-1; CTX-M-15
46L/14	Pus	TEM-1; OXA-1; CTX-M-15
Enterobacter cloacae		
21L/14	Urines	TEM-1; OXA-1; SHV-12; CTX-M-15
29L/14	Blood	TEM-1; OXA-1; CTX-M-15
73L/14	Urines	CTX-M-15

Table 2: Chemical composition of ethanol extracts 70%

Compounds	Sterols et polyterpenes	Polyphenols	Flavonoids	Tannins	Quinones	Alkaloids	Saponins
Extrait	-	+++	+++	+++	-	-	+++

NB: +++ : Strong presence , ++ : medium présence, + : weak présence, - : absence

Tableau 3: Diameters (mm) of extracts inhibition zones

	Aqueous extracts			Ethanol extracts		
Strains	**100 mg/ml**	**50 mg/ml**	**25 mg/ml**	**100 mg/ml**	**50 mg/ml**	**25 mg/ml**
Escherichia coli						
34L/14	0	0	0	11	8	8
43L/14	0	0	0	11	8	0
Klebsiella pneumoniae						
31L/14	0	0	0	12	10	0

46L/14	0	0	0	15	13	11
Enterobacter cloacae						
21L/14	0	0	0	14	11	9
29L/14	0	0	0	12	8	8
73L/14	0	0	0	11	9	9

Table 4: Antibacterial parameters of ethanol extract 70%

Strains	MIC (mg/mL)	MBC (mg/mL)	CMB/CMI	Interpretations
Escherichia coli				
34L/14	25	50	2	Bactericide
43L/14	25	100	4	Bactericide
Klebsiella pneumoniae				
31L/14	1,56	100	64	Bacteriostatic
46L/14	6,25	100	16	Bacteriostatic
Enterobacter cloacae				
21L/14	12,5	50	4	Bactericide
29L/14	6,25	100	16	Bacteriostatic
73L/14	3,12	100	32	Bacteriostatic

4. Conclusion

This study not only confirmed the antibacterial power recognized at *H. madagascariensis* but also to show that the ethanol extract of the stem bark could be used in the treatment of infections to bacteria producing β-lactamases extended spectrum. A bioguided fractionation might be previously performed to isolate and identify the compound responsible for this activity.

5. Conflict of interest

No conflict of interest to declare.

6. Acknowledgement

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