Asian Journal of Research in Biochemistry

7(4): 157-162, 2020; Article no.AJRB.64072
ISSN: 2582-0516

Inhibitory Activity of β-lactamases by Hydro-Ethanolic Extracts of *Harungana madagascariensis*, a Plant of the Ivorian Pharmacopoeia

Toty Abalé Anatole[1*], Aka Ayébé Edwige[1], Guédé Kipré Bertin[1], Konan K. Fernique[1], Adrien Jehaes[2], N. Guessennd[1], Otokoré D. Albert[3], M. Dosso[1] and M. Galleni[2]

[1]Laboratoire de Bactériologie-Virologie, Unité des Antibiotiques, des Substances Naturelles et de la Surveillance des Résistances des Micro-Organismes aux Anti-Infectieux (ASSURMI), Institut Pasteur de Côte d'Ivoire, 01 BP 490 Abidjan 01, Côte d'Ivoire.
[2]Centre d'Ingénierie des Protéines, Université de Liège, Institut de Chimie B6a, Allée du 6 Août, 11 Start Tilman-B4000, Liège, Belgium.
[3]Laboratoire de Pharmacodynamie Biochimique, UFR Biosciences, Université Félix Houphouët Boigny d'Abidjan, 22 BP 582 Abidjan 22, Côte d'Ivoire.

Authors' contributions

This work was carried out in collaboration among all authors. Author TAA performed the analysis, produced and interpreted the manuscript. Authors AAE and GKB performed the statistical analysis, read and corrected the manuscript. Authors KKF and AJ managed the literature searches and the analyses. Authors NG, ODA, MD and MG defined the theme of the work, supervised and gave scientific support to the work. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJRB/2020/v7i430168
Editor(s):
(1) Dr. Mohamed Fawzy Ramadan Hassanien, Zagazig University, Egypt.
(2) Dr. Khadiga Mohamed Abu-Zied, National Research Centre, Egypt.
Reviewers:
(1) Arleto Tenorio dos Santos, São Paulo State University, Brazil.
(2) Abdul Samad Aziz, Maharashtra University of Health Sciences, India.
Complete Peer review History: http://www.sdiarticle4.com/review-history/64072

Original Research Article

Received 20 October 2020
Accepted 25 December 2020
Published 29 December 2020

ABSTRACT

Infection due to multi-resistant bacteria is a public health concern. Unfortunately, the prospect of developing new antibiotics seem not to be on the horizon. Faced with this impasse, medicinal plants could be an alternative for the development of new molecules. The aim of this study was to determine the inhibitory effect of *H. madagascariensis* extracts on β-lactamases. Extraction of bioactive compounds from trunk barks and leaves of the plant was done in an ethanol-water mixture

**Corresponding author: E-mail: totyabale@yahoo.fr;*

(70:30). Anti-β-lactamase activity was evaluated by spectrophotometry after the removal of tannins and a phytochemical screening was used to identify the groups of compounds. Concentrations inhibiting 50% of enzyme activity were 0.005±0.001 mg/mL (CTX-M-15), 0.01±0.001 mg/mL (P99) and 0.027±0.009 mg/mL (NDM-1) for bark extracts and 0.704 mg/mL for leaves extracts. Phytochemical screening revealed the presence of flavonoids in bark extracts. The ethanolic extracts of the trunk bark exert a good inhibitory activity on CTX-M-15, P99 and NDM-1 β-lactamases and this activity could be attributed to the presence of the flavonoids. Further studies by bio-guided fractionation of the ethanolic extracts of the bark could yield fractions with high inhibitory potential of β-lactamases.

Keywords: β-lactamases; hydro-ethanolic extracts; H. madagacariensis; Ivorian pharmacopoeia.

1. INTRODUCTION

The production of β-lactamases by Enterobacteriaceae is a global problem that deserves special attention because it is responsible for therapeutic failures of infections caused by these organisms. Infections due to these multi-resistant bacteria leads to an increase in mortality rate, prolonged hospital stay and an increase in the cost of treatments [1]. Despite the strong expansion of bacterial resistance, the prospect of developing new antibiotics seem not to be in the horizon [2,3]. In the face of this impasse, it is imperative to look for new molecules with mechanisms of action different from those hitherto described or inhibiting the resistance mechanisms of bacteria of clinical importance [4]. These new molecules could come from natural sources, especially medicinal plants [5].

Harungana madagascariensis Lam. Ex Poir. (Hypericaceae) is a shrub native to tropical Africa and Madagascar [6]. It is widely used in traditional medicine for the treatment of anemia, gastroenteritis, nephroses and malaria [7,8]. Recently, our team showed that this plant had antibacterial effect on Enterobacteriaceae producing β-lactamases with broad spectrum [9]. The aim of this study was to determine the inhibitory effect of the hydro-ethanolic extract of *H. madagascariensis* on pure β-lactamases.

2. MATERIAL AND METHODS

2.1 Plant Materials

Plant materials consisted trunk barks and leaves of *H. madagascariensis* Lam. Ex. Poir. (Hypericaceae). They were harvested from Lakota, in West-Central of Cote d'Ivoire, from June to July 2013. The identification was carried out at the National Floristic Center of the Félix Houphouët Boigny University of Cocody.

2.2 Enzymes and Proteins

β-lactamases were selected from each of the four classes, namely active-serum β-lactamases KPC-2 and CTX-M (class A), P99 (class C), OXA-48 (class D) and metallo-β-lactamases, NDM-1 (class B). They were extracted and purified by the Center of Protein Engineering (CIP) of the University of Liège.

2.3 Preparation of the Ethanolic Extract 70%

The trunk bark and leaves of *H. madagascariensis* were dried and grinded into a fine powder using an IKA Labortechnik MFC® grinder. The hydro-ethanolic extracts were prepared according to the method described by Zihiri et al. [10] with modifications. The extracts obtained were stored in jars in a refrigerator.

2.4 Elimination of Tannins

The extract (1 g) and 100 mg skin powder (EDQM, Strasbourg, France) were introduced into a flask containing 150 mL of milliQ water. The flask was heated in a water bath at 100 °C for 30 min. After heating, the contents of the flask were transferred to a 250 mL volumetric flask and then made up to 250 mL with milliQ water. This was followed by decantation, filtration and lyophilization. The lyophilisates were dissolved in the various buffers (PBS, HEPES (MP Biomedicals, LLC) and HEPES + $ZnCl_2$ (Acros Organic)) and filtered on microfilters of 42 µm.

2.5 Inhibition of β-lactamases by Extracts of *Harungana madagascariensis*

The activity of the different β-lactamases was determined in the presence and absence of extracts of *H. madagascariensis* [11]. The reaction volume was 500 µl, consisting of the various concentrations of plant extract, 5 µL of

enzyme, 5 µL of 100 µM nitrocefin and adjusted with different volumes of buffer. After homogenization, the enzyme was added and then the activity was measured with a spectrophotometer at the wavelength of 482 nm for 300 s. The residual activity of the enzyme was determined and the percentage inhibition of the extracts was calculated thus:

$$(1)\ \text{Residual activity} = \frac{\text{Initial velocity (Samples)}}{\text{Initial velocity (Control)}} \times 100$$

(2) Inhibition (%) = 100% — Residual activity

2.6 Phytochemical Sorting

The different groups of compounds (sterols, polyterpenes, alkaloids, tannins, polyphenols, flavonoids, quinones and saponins) in the ethanolic crude extract and the ethanolic extract treated with the skin powder were investigated according to the methods [12,13,14,15] and reported by [16].

2.7 Statistical Processing of Data

Graphical representations of data was done using Graph Pad Prism 5.0 and Excel 2007 (Microsoft, USA). Analysis of one-factor variance (ANOVA) was used for statistical tests at significance level of 5% using the STATISTICA 7.1 software.

3. RESULTS AND DISCUSSION

The ethanolic extract of the trunk bark had a strong inhibitory activity on β-lactamases CTX-M-15, NDM-1 and P99 with concentrations inhibiting 50% of their activity (IC50) of 5 µg/mL, 27 µg/mL and 10 µg/mL (Table 1). The residual activity was 25.8%, 33.2% and 32.2% for CTX-M-15, P99 and NDM-1 respectively (Fig. 1). No significant difference was observed between the activity of this extract on the enzymes tested at $P < 0.001$.

Table 1. Concentration of the bark extract inhibiting 50% of the activity of the enzymes

	CI_{50} (mg/mL)	
Enzymes	**Leaf extracts**	**Bark extracts**
P99	0.704^{b}	0.010 ± 0.001^{a}
CTX-M-15	$> 2.24^{c}$	0.005 ± 0.001^{a}
NDM-1	$> 2.24^{c}$	0.027 ± 0.009^{a}

**IC50 values with the same letter are statistically identical*

Considering the ethanolic extract of the leaves, only P99 was inhibited by this extract with an IC50 of 704 µg / mL. For other enzymes, the IC50 of the extract was greater than the maximum tested concentration of 2.24 mg / mL.

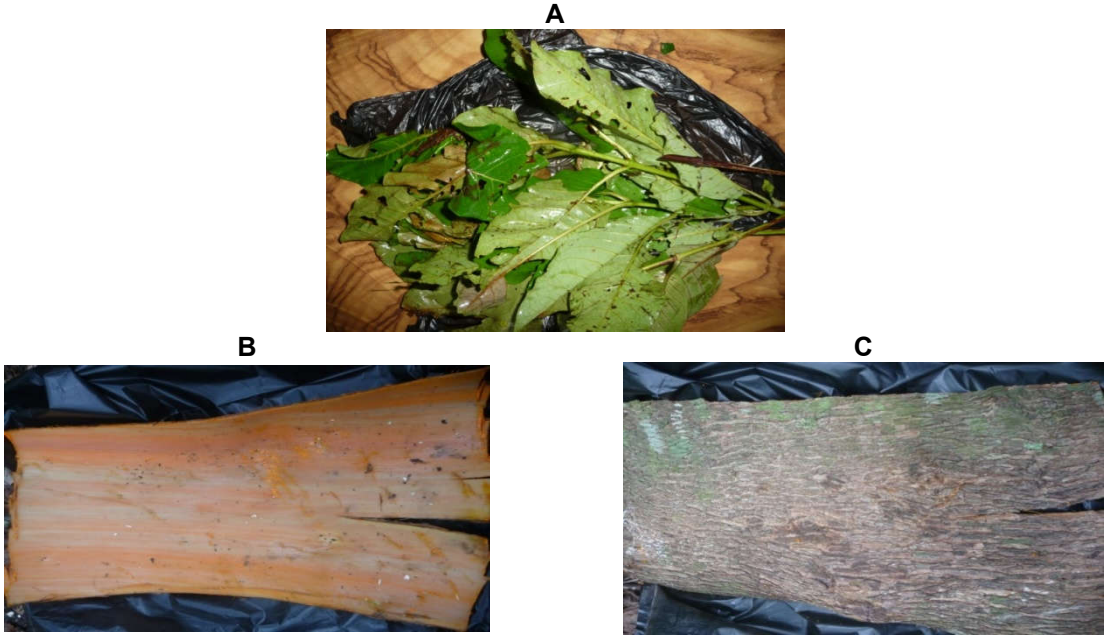

Fig. 1. Different parts of *Harungana madagascariensis* studied
A: Leaves
B: Inner side of the trunk bark
C: External face of the trunk bark

At this maximum concentration, the residual activity of the P99 enzyme was 35.4%. The inhibitory activity of the *H. madagascariensis* leaf extract on CTX-M-15, NDM-1 and P99 was significantly different at P <0.001. Our findings corroborate those of Gangoué-Piéboji et al. [11] on 16 medicinal plants in Cameroon and showed good inhibitory activity of P99 by *Garcinia lucida* extracts and pure products at 0.012 mg / mL.

Several studies for the detection of new effective inhibitors of β-lactamases capable of replacing commercial inhibitors have been undertaken on medicinal plants with convincing results. Zhao et al., in 2002 tested the direct inhibitory effect of epigallocatechin gallate, a green tea extract compound, on penicillinase extracted from a resistant strain of *Bacillus cereus*. They showed that this compound inhibits the activity of penicillinase at IC50s of 10 µg / mL and 44 µg / mL depending on whether there was a pre-incubation of 18 hours or 30 min respectively and restores the antibacterial activity of Penicillin [17]. Similarly, 1,4-naphthalenedione isolated from the leaves of *Holoptelea integrifolia* and the salicylsalicylic acid isolated from the roots of *Fissistigma cavaleriei* were studied. The results of these studies have shown that both compounds inhibit β-lactamase activity and the synergistic action of 1,4-naphthalenedione with amoxicillin has been demonstrated [18,19]. Other similar studies have shown the inhibitory activity of plant extracts on penicillinases and metallo-β-lactamases and demonstrated their synergistic action with certain β-lactams towards enterobacteria, non-fermenting Gram negative bacilli and *Staphylococcus aureus*-producing β-lactamases [20,21,22].

The inhibition of CTX-M-15 and NDM-1 by the ethanolic extract of the trunk bark of *H. madagascariensis* has a promising prospect, because these two β-lactamases are very problematic in bacterial infections. CTX-M-15, in addition to being the most expressed β-lactamases, leads to resistance to third and fourth generation cephalosporins and NDM-1 is a metallo-β-lactamase responsible for carbapenem resistance which are antibiotics of last resort in case of serious infections.

3.1 Phytochemical Sorting

The phytochemical analysis of the ethanolic extract (70%) of the trunk bark of *H. madagascariensis* revealed the presence of tannins, flavonoids and saponins in the crude extract. As for the extract treated with skin powder, it contained only flavonoids and saponins (Table 2). Some previous studies have shown that in addition to these families of compounds, alkaloids, steroids, polyterpenes and cardiac glycosides were present in the plant [23,24]. Flavonoids are hydroxylated phenolic substances synthesized by plants in response to microbial infection [25]. Their activity is probably due to their ability to form complexes with extracellular proteins and also with the bacterial membrane [5].

Several studies have demonstrated the inhibitory activity of flavonoids on β-lactamases. Thus, tests for the inhibition of the activity of a semi-purified metallo-β-lactamase extracted from *Stenotrophomonas maltophilia* by flavonoids showed that galangine and quercetin irreversibly inhibit metallo-β-lactamase even after addition of

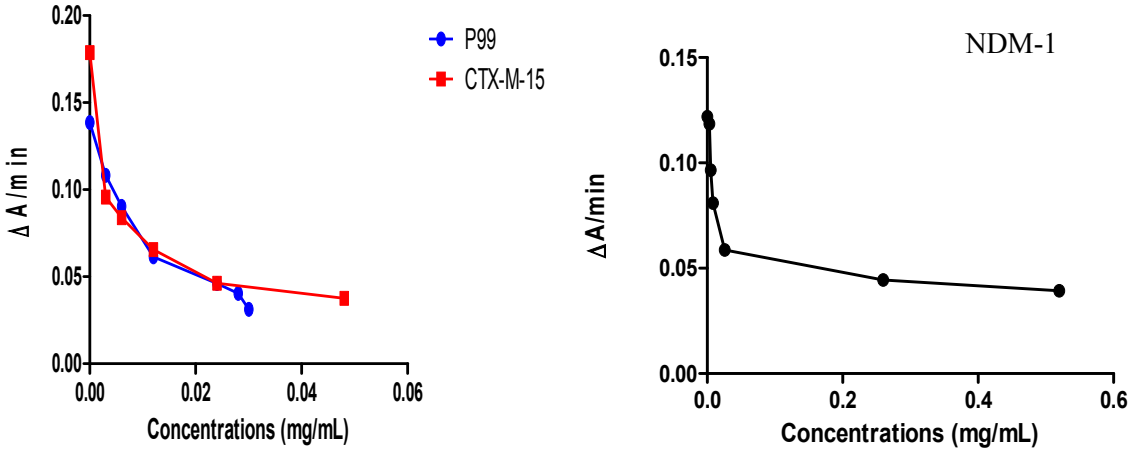

Fig. 2. Variation of the activity of the enzymes P99, CTX-M-15 and NDM-1 as a function of the concentration of the ethanolic extract 70% of the trunk bark of *Harungana madagascariensis*

Table 2. Chemical composition of the crude extract and of the extract treated with skin powder

Compounds		Crude extract	Extract treated vith skin powder
Sterols et Polyterpenes		-	-
Polyphenols		+	+
Flavonoids		+	+
Tannins	Catechecal	+	-
	Gallic	-	-
Quinones		-	-
Alkaloids	Bouchardât	-	-
	Dragendorff	-	-
Saponines		+	+

NB: +: Presence, -: Absence

$ZnCl_2$. It has been deduced that flavonoids are good inhibitors of metallo-β-lactamases because they do not chelate zinc [26]. Similarly, quercetin and its analogue, penta-o-ethylquercetin, are potent inhibitors of NDM-1, a metallo-β-lactamase [27].

The mechanism of action of flavonoids is a non-competitive inhibition as shown by Boussoualim et al. [28] contrary to the conventional inhibitors clavulanic acid, sulbactam and tazobactam which exert a competitive inhibition. It was deduced from this result that the difference in position and the number of hydroxyl groups would be responsible for the difference in the type of inhibition observed [28].

The inhibitory activity of β-lactamases observed in *H. madagascariensis* may therefore be due to the presence of flavonoids in the ethanolic extract of the bark.

4. CONCLUSION

The ethanolic extract of the bark of *H. madagascariensis* exerts a dose-dependent inhibitory activity on β-lactamases CTX-M-15, P99 and NDM-1 and this activity could be due to a flavonoid. It would therefore be interesting to carry out further studies by fractionation, extraction and elucidation of the compounds responsible for the inhibition of the β-lactamases tested and their mode of action.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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