



Research Article

# Stem Bark's Extracts of *Tieghemella heckelii* (Sapotaceae) Against Imipenem-Resistant *Pseudomonas aeruginosa*: Identification of a Prospective Antibacterial Agent

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## Abstract

**Background:** Stem bark of *Tieghemella heckelii* (Sapotaceae) used as traditional medicine for its therapeutic effect on infectious diseases in Ivory Coast, was tested against imipenem-resistant bacteria for efficacy assessment.

**Methods:** Six extracts (hexane, chloroform, ethyl acetate, ethanol, methanol and sterile distilled water) were prepared and tested on Imipenem-Resistant *Pseudomonas aeruginosa* (IRPA), using broth microdilution for activity evaluation. From this experiment, the Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of the plant extracts were determined in 96-well micro-plates in order to search for both bacteriostatic and bactericidal effects. Thereafter, data analysis was performed using GraphPad Prism 5 software (One-way ANOVA, Bartlett test for equal variance and Turkey Multiple Comparison test). The results were then presented as Mean ± SD for experiment repeated three times.

**Results:** Four extracts (ethyl acetate, methanol, ethanol and sterile distilled water) showed credible potency, with strong, significant and

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moderate growth inhibition of the IRPA tested. The MIC values varied depending on microbial phenotype, and were within the range of 0.048 mg/mL to 12.5 mg/mL. As for the MBC values, also associated to bacteria strain type, they demonstrated both bacteriostatic and bactericidal effects of the active extracts towards Imipenem-resistant *P. aeruginosa*.

**Conclusion:** Stem bark extracts of *Tieghemella heckelii* showed an antibacterial effect towards imipenem-resistant *P. aeruginosa*. They could therefore be used to deplete the prevalence rate of the named resistant strains.

**Keywords:** Antibacterial; Imipenem-resistant *Pseudomonas aeruginosa*; Ivory Coast; *Tieghemella heckelii*

## Abbreviations

ATCC: American Type Collection Culture

DMSO: Dimethyl Sulfoxide

MBC: Minimum Bactericidal Concentration

MIC: Minimum Inhibitory Concentration

IRPA: Imipenem-Resistant *Pseudomonas aeruginosa*

IAI: Intra-Abdominal Infection

SMART: Study for Monitoring Antimicrobial Resistance Trends

## Background

The prevalence of multi-drug resistant gram negative bacteria has increased over time since 2002, in Asia, Europe, Latin America, the Middle East, North America and South Pacific according to the Study for Monitoring Antimicrobial Resistance Trends (SMART) [1]. Among others was *Pseudomonas aeruginosa*, identified as the third most common pathogen in Intra-Abdominal Infection (IAI) at a rate of 5%, and also responsible for nosocomial infections and re-infections [1,2]. *P. aeruginosa* is a non-fermenting gram negative bacterium, intrinsically resistant to many drugs and resistance acquiring towards any antimicrobial agent [3]. It opposes resistance by use of its impermeable outer membrane, expression of efflux pumps, target alteration and production of antibiotic-hydrolyzing enzymes [4]. This multi-resistance mostly to beta-lactam antibiotics could be due either to individual mechanism aforementioned or simultaneous processes. In order to alleviate this phenomenon, public health scientists have addressed since decades, medicinal plants for novel antimicrobial compound discovery. In this context, in Africa and especially in Ivory Coast, where 80% of the populations use traditional medicines, research has been promoting plant-based compounds to find a way out [5]. This because antibiotic resistance modulating effects of plant extracts have been reported [6]. The public health threat could be described by the prevalence rate of imipenem-resistant *P. aeruginosa*, in South Africa, which reached 25% in the year 2004-2009, while in Ivory Coast, it increased from 2.4% to 5.1% between 2014 and 2015 [1,7]. As a result of seeking for solutions to this concern, stem bark's extracts of *Tieghemella heckelii* used as traditional medication was investigated in the present study to assess its *in vitro* antibacterial activity towards imipenem-resistant *P. aeruginosa*.

## Methods

### Plant material

The plant material used was the stem bark of *Tieghemella heckelii* Pierre ex., A. Chev (Sapotaceae). The plant species was authenticated at the herbarium of “Centre Suisse de Recherches Scientifiques en Ivory Coast, Adiopodoumé (Abidjan-Ivory Coast)”, and registered under voucher number 3021. The stem bark was collected within the period of September to October 2014 in the Haut-Sassandra, a mid-west region in Ivory Coast.

### Bacteria strains

Bacteria tested and their antibiogram, were provided respectively by the strains collection bank (Bio-bank) and the National Center for Antibiotics Reference at Institute Pasteur Ivory Coast. These Carbapenemases harboring isolates were made up of six Imipenem-Resistant *Pseudomonas aeruginosa* (IRPA). Additionally, American Type Collection Culture (ATCC 27853; Manassas, VA) strain was used as reference material.

## Methods

### Preparation of extracts

The stem bark was shade dried over 15 days, and powdered in a mortar. Then, the extracts were prepared by macerating successively 200 g of plant powder in 1L of the different solvents of increasing polarity (hexane, chloroform, ethyl acetate, ethanol, methanol and sterile distilled water). Extraction was carried out for 48 hours for each solvent used. After filtration successively on hydrophilic cotton and filter paper, the extracts were dried in an oven at 40°C-50°C to yield a dense residue. Each extract sample was then transferred to a glass vial, and kept into a storage chamber at controlled temperature ( $0 \pm 2^\circ\text{C}$ ) and humidity (< 40%) until use [8].

### Antimicrobial assays

The different plant extracts were serially diluted from 0.048 mg/mL to 25 mg/mL separately in both sterile 96-well microplates, and test tubes using broth microdilution method (Figure 1) [9]. Suspension (50  $\mu\text{L}$ ) was added to each well, and potency (MICs) was evaluated; that is the lowest concentration of plant extract that completely inhibited the growth of the bacterium in the well or test tube [10]. Then, the MBC values were determined by sub-culturing the samples with no visible growth in the MIC assays (Figure 2). For this purpose, the inoculum was diluted from  $10^{-1}$  to  $10^{-4}$  in test tubes, and streak-seeded with a calibrated loop (2  $\mu\text{L}$ ) on Muller-Hinton Agar gel. The set-up included bacterial growth controls containing the test inoculum (50  $\mu\text{L}$ ) and the negative controls without bacterial inoculum. Extracts controls were likewise included into the set-up. The first batch of Petri dishes containing the agar was labelled A, and incubated at  $37 \pm 2^\circ\text{C}$  for 24 hours. Then after reading the MIC values, the tube content which did not show bacteria growth was streak-seeded on Muller-Hinton Agar gel. This second batch was labelled B, and the MBCs were determined by comparing bacteria growth in A and B. The extract is said to be bacteriostatic if the ratio MBC: MIC is equal to 4, whereas it is said to be bactericidal when it is lesser or equal to 2. In addition, classification for growth inhibition was based on the MIC cutoff point. Thus, the extract is said to be strongly active upon bacterium strain, when MIC is lesser or equal to 0.097 mg/mL. From this point, the MIC value increasingly confers moderate and weak activity.

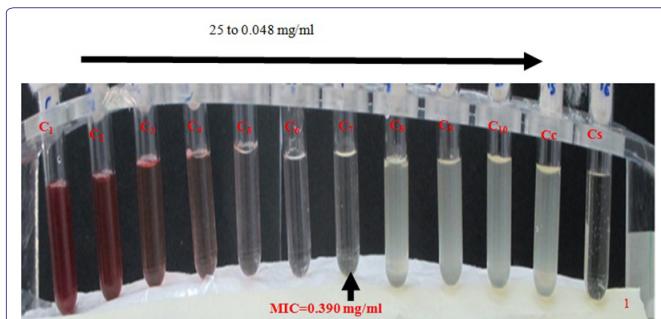


Figure 1: Illustration of the Minimum Inhibitory Concentration.

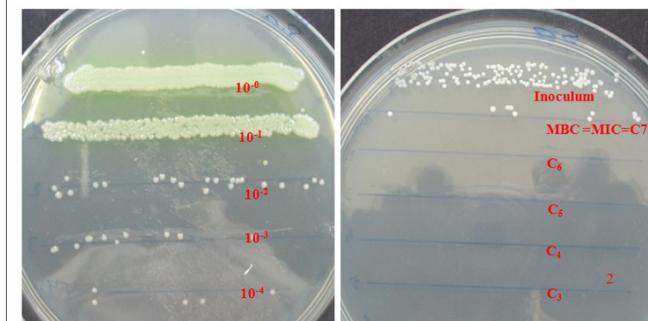


Figure 2: Illustration of the Minimum Bactericidal Concentration.

### Statistical analysis

Data analysis was performed using GraphPad Prism 5.01 software, Microsoft, San Diego, California, USA (ANOVA, Bartlett test and Turkey test).

### Results

The Ethanol extract, which showed moderate growth inhibition for bacterium 1076 C/11, exhibited weak activity towards bacteria 489C/11 and 891C/11. The potency of the extract was demonstrated at a minimum inhibition concentration value in the range 3.125 mg/mL < MIC < 12.5 mg/mL. This was displayed by a bacteriostatic effect against 489C/11, and a bactericidal activity over the remaining tested bacteria (Table 1). Additionally, the one-way analysis of variance did not show significant activity difference ( $P = 0.9865$ ,  $R^2 = 0.001506$ ) of the extract on the whole bacteria tested. This trend was confirmed by the Bartlett's test for equal variances of the efficacy within the range of 0.390 mg/ml to 12.5 mg/mL.

Evaluation of the ethyl acetate extract's activity showed growth inhibition of all IRPA tested, with a MIC figure of 6.25 mg/mL for strains 489C/11, 1076C/11, 1780C/14, which constitute the first set of bacteria tested and 12.5 mg/mL for 891C/11, 1060C/11 and 1810C/10 another bacteria set (Table 2). Using the one-way analysis of variance, the MIC means difference over each group of strains was not significant ( $P = 0.1134$ ,  $R^2 = 0.2149$ ), which trend was also ascertained by the Bartlett's test ( $P = 0.1913$ ) for both strains groups.

Methanol extract mixed with different IRPA inoculum strongly prevented the bacteria growth with MIC highest value of 0.048 mg/ml for bacterium 891C/11 and 0.097 mg/ml for strains 489C/11, 1060C/11 and 1810C/10. For 1076C/11, the extract displayed moderate anti-IRPA potency at MIC value of 3.125 mg/ml and weak activity at 6.25 mg/ml for 1780C/14. Additionally, apart from bacterium 1060C/11, which the extract showed bacteriostatic effect for, a

Strain	Code	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC	Effect
<i>P. aeruginosa</i>	1810C/10	12.5 ± 0.001	12.5 ± 0.001	1	Bactericidal
	1060C/11	12.5 ± 0.001	25 ± 0.001	2	Bactericidal
	1780C/14	12.5 ± 0.001	12.5 ± 0.001	1	Bactericidal
	891C/11	6.25± 0.001	12.5 ± 0.001	2	Bactericidal
	489C/11	6.25 ± 0.001	25 ± 0.001	4	Bacteriostatic
	1076C/11	3.125 ± 0.005	6.25 ± 0.001	2	Bactericidal
	ATCC 27853	0.39 ± 0.001	0.78 ± 0.001	2	Bactericidal

**Table 1:** Antibacterial activity of ethanol extracts of *Tieghemella heckelii* on imipenem-resistant and reference strains of *Pseudomonas aeruginosa*.

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration, values are expressed as mean ± SD of 3 experiments (ANOVA, Bartlett test and Turkey test).

Strain	Code	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC	Effect
<i>P. aeruginosa</i>	1810C/10	12.5 ± 0.001	25 ± 0.001	2	Bactericidal
	1060C/11	12.5 ± 0.001	25 ± 0.001	2	Bactericidal
	1780C/14	6.25 ± 0.001	12.5± 0.001	2	Bactericidal
	891C/11	12.5 ± 0.001	25 ± 0.001	2	Bactericidal
	489C/11	6.25 ± 0.001	12.5 ± 0.001	2	Bactericidal
	1076C/11	6.25 ± 0.001	12.5 ± 0.001	2	Bactericidal
	ATCC 27853	6.25 ± 0.001	12.5 ± 0.001	2	Bactericidal

**Table 2:** Antibacterial activity of ethyl acetate extracts of *Tieghemella heckelii* on imipenem- resistant and reference strains of *Pseudomonas aeruginosa*.

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration, values are expressed as mean ± SD of 3 experiments (ANOVA, Bartlett test and Turkey test).

Strain	Code	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC	Effect
<i>P. aeruginosa</i>	1810C/10	0.097 ± 0.003	0.097 ± 0.003	1	Bactericidal
	1060C/11	0.097 ± 0.003	0.39 ± 0.001	4	Bacteriostatic
	1780C/14	6.25 ± 0.001	6.25 ± 0.001	1	Bactericidal
	891C/11	0.048± 0.001	0.097 ± 0.003	2	Bactericidal
	489C/11	0.097 ± 0.003	0.195 ± 0.005	2	Bactericidal
	1076C/11	3.125 ± 0.005	6.25 ± 0.001	2	Bactericidal
	ATCC 27853	0.097 ± 0.003	0.195 ± 0.005	2	Bactericidal

**Table 3:** Antibacterial activity of methanol extracts of *Tieghemella heckelii* on imipenem-resistant and reference strains of *Pseudomonas aeruginosa*.

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration, values are expressed as mean ± SD of 3 experiments (ANOVA, Bartlett test and Turkey test).

Strain	Code	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC	Effect
<i>P. aeruginosa</i>	1810C/10	6.25 ± 0.001	12.5 ± 0.001	2	Bactericidal
	1060C/11	6.25 ± 0.001	12.5 ± 0.001	2	Bactericidal
	1780C/14	6.25 ± 0.001	12.5 ± 0.001	2	Bactericidal
	891C/11	3.125 ± 0.005	6.25 ± 0.001	2	Bactericidal
	489C/11	3.125 ± 0.005	6.25 ± 0.001	2	Bactericidal
	1076C/11	3.125 ± 0.005	6.25 ± 0.001	2	Bactericidal
	ATCC 27853	3.125 ± 0.005	6.25 ± 0.001	2	Bactericidal

**Table 4:** Antibacterial activity of aqueous extracts of *Tieghemella heckelii* on imipenem- resistant and reference strains of *Pseudomonas aeruginosa*.

MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration, values are expressed as mean ± SEM of 3 experiments (ANOVA, Bartlett test and Turkey test).

bactericidal effect was demonstrated for all strains assayed (Table 3). The statistical analysis also showed significant variation of MIC means difference within the extract concentrations range of 0.048 to 12.5 mg/mL (Turkey Multiple Comparison test), which was confirmed by the one-way analysis of variance ( $P < 0.0003$ ;  $R^2 = 0.7244$ ).

Residual Aqueous extract tested on IRPA in the screening experiment, revealed growth inhibition towards strains 489C/11, 891C/11 and 1076C/11 with a MIC value of 3.125 mg/mL, activity against 1060C/11, 1780C/11 and 1810C/10 with MIC value of 6.25 mg/mL (Table 4). Another important result is the bactericidal effect displayed

by the extract against all IRPA tested. Ultimately, the Turkey Multiple Comparison test showed significant variation of the extract efficacy within the concentrations range. This was confirmed by the one-way analysis of variance ( $P = 0.0253$ ;  $R^2 = 0.3353$ ).

## Discussion

The present study aimed at assessing the antibacterial effect of stem barks' extracts of *Tieghemella heckelii* against imipenem-resistant *P. aeruginosa*. Results obtained from experiments carried out, showed plant extract's efficacy against the tested strains. Thus, with the MIC ranging from 0.048 mg/mL to 12.5 mg/mL, they opposed bactericidal effect to most of the tested bacteria. When considering the cutoff point set up in early studies, methanol extract displayed significant antibacterial activity with MIC lowest values of 97 µg/mL and 48 µg/mL against imipenem-resistant *P. aeruginosa* [11]. In evaluating the activity of all plant extracts towards the reference material ATCC 27853, a bactericidal effect was noticed. Moreover, the MIC value of each extract put into contact with the ATCC isolate showed the significance of this trend. Consequently, the methanol extract not only displayed bactericidal effect, but also revealed the lowest MIC value of 0.097 mg/mL. This, followed by the ethanol extract, which showed MIC value of 0.390 mg/mL, and aqueous extract (3.125 mg/mL) along with ethyl acetate extract (6.25 mg/mL). The MIC difference of these extracts may be due to the lipophilic outer membrane of this negative gram bacterium, which is easily penetrated by alcoholic driven secondary metabolites, as compared to aqueous and ethyl acetate extracts. Some of the metabolites probably acted as chelating organic ligands for bacteria metallic-enzyme proteins. These *in vitro* proven bactericidal effects could explain the use of the plant part in traditional medicine to treat ulcers and reinfections caused by *P. aeruginosa*. This result ascertains the plant to display higher antibacterial activity when compared to work done by Ulloa-Urizar et al., [12]. For this group of researchers, which investigated the potency of five Peruvian medicinal plant extracts towards *P. aeruginosa*, the MIC value for the most active, was achieved at 25 mg/mL. The extracts aforementioned were from *M. macrocarpa*, *D. loretense*, *U. tomentosa* and *E. Camaldulensis*. Another comparison made with the faith plant (*T. impetiginosa*) studied by the same research team, showed the bark's extracts of *Tieghemella heckelii* to be highly active. *Carex pumila* extracts also inhibited *P. aeruginosa* according to Hyun et al., but the MIC value (200 µg/mL) proved the plant stem barks used to conduct the current experiment to be more active [13]. Finally, when compared to the study performed by Datta et al., the stem bark's extracts of *Tieghemella heckelii* were active on *P. aeruginosa*, whereas *Piper betle* leaf extract at different concentrations did not show any decrease in bacteria growth [14]. As far as the MBC is concerned, the methanol extract of the stem barks of *Tieghemella heckelii* showed a lower value 97 µg/mL, which is roughly of the same order of magnitude as that obtained by Jahani et al., [15]. Nevertheless, the stem bark's extracts which showed antibacterial activity against these metallic-enzymes producing micro-organisms (Carbapenemases), did not show inhibition against *Enterobacteriaceae* tested in a complementary experiment.

Additional experiment conducted to screen out the prospective compounds responsible for antibacterial activity of the stem barks' extracts revealed saponins, coumarins and flavonoids [15]. These natural products could therefore encompass the activity evaluated.

## Conclusion

To conclude, the present study has shown the antibacterial potential of the tested plant extracts against imipenem-resistant *P. aeruginosa*.

Provided this characteristic of the stem bark of *Tieghemella heckelii*, it could be used as a good candidate for drug resistance modulation.

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## Availability of Data and Materials

Institute Pasteur Ivory Coast/Université Nangui Abrogoua/Centre Suisse de Recherches Scientifiques Ivory Coast.

## Authors' Contributions

BKG conceived, designed, carried out the extraction experiments, performed the bioassays, conducted the statistical analysis and wrote the draft of the manuscript.

NKG designed the bioassays, validated the results of the experiments and participated in the drafted manuscript.

MWK participated in the experiment's design and results validation, in the drafted manuscript and statistical analysis.

VG participated in bioassays design and results interpretation. In addition, VG received the patients who consented for routine sample collection and diagnosis.

JKC contributed in plant extracts preparation and fractionation.

MD designed the bioassays, validated the results of the experiments and participated in the draft manuscript.

All authors read and approved the final manuscript.

## Competing Interests

The authors declare that they have no competing interests.

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