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In-vitro antibacterial activity of various extracts from *Terminalia ivorensis* A. Chev. (Combretaceae) stem bark against some Beta-Lactamase-producing bacteria strains

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Abstract
This study was undertaken to investigate the antibacterial properties of various extracts from the stem bark of *T. ivorensis* on three extended-spectrum β-lactamases-producing bacteria strains growth (*Acinetobacter sp.* IPMQ/C_3GR, *Escherichia coli* KGTNE/RCFQ, and *Klebsiella pneumoniae* ATCC/1833). Antibacterial activity of hydro-alcoholic (EBeth), aqueous (Faq), and hexane (Fhex) extracts was assessed using agar disc-diffusion method and liquid medium micro dilution method. Aqueous fraction (Faq) and hydro-alcoholic (EBeth) extracts showed significant activity ($p \leq 0.05$) against all bacteria strains except hexane fraction (Fhex). Faq and EBeth exhibited the lowest Minimum Inhibition Concentration (12.50 mg/ml), while Minimum Bactericidal Concentration ranged between 12.50 to 25 mg/mL respectively. Therefore, Faq and EBeth extracts of *T. ivorensis* showed a bactericidal power against all bacterial strains tested.

Keywords: Beta-lactamases, *Acinetobacter sp*, *Escherichia coli*, *Klebsiella pneumoniae*, Extracts of *Terminalia ivorensis*, antibacterial parameters

1. Introduction
Plant products play an important role in healthcare delivery as therapeutic remedies in the world, especially in developing countries. This has caused phytomedicine to become an integral part of the healthcare system of many nations. Medicinal plants are rich in bio-resources of drugs, and these chemical compounds play a definite physiological action in the human body system [1, 10]. Investigation of medicinal plants by ethno-botanists as an alternative to the existing synthetic medicines has been on the increase; this is because most man-made medications are now progressively losing their potency to pathogens [23]. Thus, the production of β-lactamases (βLs), particularly carbapenemases and extended-spectrum β-lactamases (ESβLs), by some pathogens, is an important mechanism by which bacteria develop resistance to available antibiotics [14]. Carbapenemases are mainly found in lactose-free fermenters such as Pseudomonas and Acinetobacter species; and to a lesser extent lactose-fermenting Enterobacteriaceae, and they have spread worldwide [9]. ESβLs and carbapenemases are mainly produced by Enterobacteriaceae and Pseudomonas, respectively, and give these bacterial pathogens the exceptional ability to have a broad substrate profile in terms of numbers and classes of antibiotics to which they are resistant to. Pathogenic microorganisms producing ESBLs and carbapenemases have serious clinical consequences as they are generally associated with high rates of morbidity and mortality, increased hospitalization duration, and high treatment costs [13, 14].

Plants still play a major role in the lives of people in different regions of sub-Saharan Africa, both in food and therapeutic [19, 29]. Among the many families used for their therapeutic properties, we can mention the Combretaceae family, whose several taxa are widely used for the treatment of various diseases [25]. The genus *Terminalia* is the second largest genus of the Combretaceae after *Combretum*, with about 200 species. These plants are distributed in tropical regions of the world with the greatest genetic diversity in Southeast Asia [12]. *Terminalia* species range from shrubs to large deciduous forest trees. Mostly they are very large trees reaching in height up to 75 m tall [25]. Members of the genus *Terminalia* are widely used in traditional medicine in several continents in the world for the treatment of numerous diseases including, abdominal disorders, bacterial infections, colds, sore throats, conjunctivitis, diarrhea, dysentery, fever, gastric ulcers, headaches, heart diseases, hookworm, hypertension, jaundice, leprosy, nosebleed, edema, pneumonia and skin diseases [2, 17, 23]. In Ivory Coast, *Terminalia ivorensis*, is found in thick rainforest zone. It is used in traditional medicine to treat many bacterial and fungal infections [11, 24]. Showed that the bark of this plant had anti-fungal activity against *Candida albicans* and *Aspergillus fumigatus*. Then, [11] have subsequently shown that the bark of this plant could be used to eradicate methicillin/oxacillin-resistant staphylococci infections mainly caused by *Staphylococcus aureus* and by coagulase-negative staphylococci, as *Staphylococcus epidermidis*. However, no study has been reported to our knowledge on

the antibacterial activity of this plant on the growth of ESβLs-producing bacteria.

Given the many excellent anti-infective results obtained with this plant, this study was to evaluate the antibacterial activity of various extracts from *T. ivorensis* stem bark on *in vitro* growth of three βLs-producing bacteria strains.

2. Materials and methods

2.1 Collection and preparation of plant material

The stem bark of *T. ivorensis* was collected at the University Hospital Center (Cocody, Ivory Coast) in February 2017 and authenticated at the National Floristic Center, Félix Houphouet Boigny University, where an herbarium specimen is deposited under the number 8855 since May 17, 1966. Harvested stem bark was air-dried for 14 days and milled with a laboratory grinder (RETSCH AS 200 type). The resulting powders were stored in sealed bottles for subsequent extractions.

2.2 Beta-lactamase-producing bacteria strains

Three βLs-producing bacteria strains comprising of *Acinetobacter sp.* IPMQ/C_3GR, *Escherichia coli* KGTNE/RCFQ, and *Klebsiella pneumoniae* ATCC/1833 were obtained from IPCI (Institut Pasteur of Côte d'Ivoire) biobank. Thus, bacterial inoculum was prepared from young cultures of 18 hours of each tested strain. This preparation was made by homogenizing two well-isolated bacterial colonies using a platinum handle in 10 ml Mueller-Hinton Broth (MHB), and this gives suspension was incubated at 37°C during 3 hours in order to obtain a pre-culture. Then, 1 ml of pre-culture broth was taken and added to 9 ml of MHB concentrated twice, which referred as suspension 10^0 having a charge of 10^6 CFU/ml [21].

2.3 Preparation of the crude hydro-ethanolic extract of *T. ivorensis* and its fractionation

The crude hydro-ethanolic extract was prepared from *T. ivorensis* stem bark powder. Thus, hundred grams (100 g) of dry powder was added in one liter of a mixture of ethanol (70 ml) and water (30 ml), and then the suspension was homogenized in a mixer. The homogenates obtained were first dewatered in a square of white fabric, then filtered successively two times with absorbent cotton and once with Whatman filter paper of 3 mm. The resulting filtrate was concentrated in vacuum using a Buchi rotary evaporator at 60°C. The brown powder obtained, constituting our crude hydroethanolic extract, was codified EBeth [22]. After this step, ten grams (10 g) of EBeth were submitted to liquid/ liquid partition in 400 ml of an n-hexane/water mixture (v/v, 50/50). After decantation, these two phases were separated and concentrated under vacuum using a Büchi rotary evaporator at 60°C which gave two extracts (fractions), that are Fhex (hexane fraction) and Faq (aqueous fraction) [17].

2.4 Extraction yield of different extracts

The yield is the amount of extract obtained from the plant material. In practice, it is determined by the ratio of the mass of the dry extract after evaporation to the mass of the powder of dry plant material used for extraction multiplied by 100 (Eq. 1).

$$\text{Yield}(\%) = \frac{m_E}{m_{DP}} \times 100 \quad (1)$$

Where $\text{Yield}(\%)$ is extraction yield, m_E is the mass in grams of the dry extract, and m_{DP} is the mass in grams of the drug powder.

2.5 Antibacterial activities of different extracts

2.5.1 Sensitivity tests

The agar well diffusion method known as the Kirby-Bauer method [6] as adopted earlier [22] was used. The inoculum (0.1 ml) of each bacteria strains (10^6 CFU/ml) was spread on Muller-Hinton Agar (MHA) plates. Wells of 6 mm diameter were punched into the agar medium and filled with 75 µl of each plant extracts (200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 mg/ml concentration) and oxacillin as positive control separately (125, 62;5, 31.25, 15.62, 7.81, 3.9, 1.95 and 0.97 mg/ml concentration). The plates were incubated at 37°C for 18 - 24 hours. After incubation, the results were recorded by measuring the diameter (mm) of the zones of growth inhibition. Clear inhibition zones around discs indicated the presence of antibacterial activity.

2.5.2 Determination of minimum inhibitory concentrations (MIC)

The MIC of the various extracts on the beta-lactamases-producing bacteria strains growth, was determine using method described by [22]. So, MIC is defined as the lowest concentration of the antimicrobial agent that inhibits the microbial growth after 18 - 24 hours of incubation.

2.5.3 Determination of minimum bactericidal concentrations (MBC)

For the determination of the MBC of the various extracts, the dilution macro method in a liquid medium described by [22] was used. Thus, in hemolysis tubes, 1 ml of each plant extracts (400, 200, 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml concentration) and oxacillin as positive control (250, 125, 62.5, 31.25, 15.62, 7.81, 3.9 and 1.95 mg/ml concentration), were inoculated with 1 ml of an inoculum (10^6 CFU/ml) for 18 hours, leading to a halving of each concentration. Two other tubes, one of which, without antibacterial agent, serves as growth control and the other without germ, for sterility control, were also prepared. Then, MHA plates cast in antimicrobial-free petri dishes were seeded in 5 cm streaks with 0.1 ml of the all tubes content and incubated at 37 °C for 24 hours. The MHA plates were examined for bacterial growth and the MBC was taken as the concentration of plant extract that did not exhibiting any bacterial growth on the freshly inoculated agar plates.

2.5.4 Determination of the antibacterial power (AP)

The antibiotic power (AP) of each extract tested was determined according to the value of the MBC/MIC ratio. Thus, an extract is bactericidal if this ratio is less than or equal to 4 and bacteriostatic when it is greater than 4 [7].

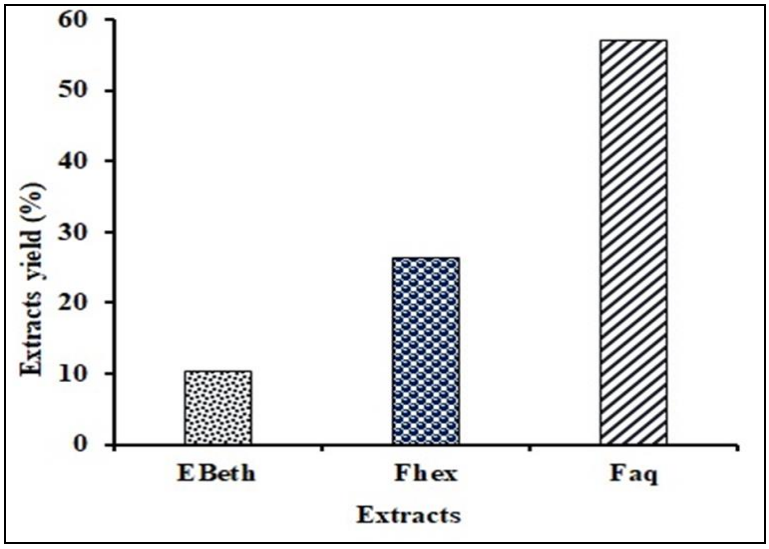
2.6 Statistical Analysis

All experiments were performed in triplicates according to a Completely Randomised Design [18]. Results were expressed as mean ± Standard Error (SE). Statistical significance was established using Analysis of Variance (ANOVA) models to estimate the effect of plant extracts on the growth of three Beta-lactamases-producing bacteria strains. Means were separated according to Duncan's multiple range analysis ($p \leq 0.05$), with the help of the software STATISTICA version 10 [26].

3. Results

3.1 Extraction yields of different extracts

The extract yields from stem bark of *T. ivorensis* by different extraction solvents are shown in Fig.1. The extract yields varied from 10.25–57 g/100 g of dry weight (DW), respectively, showing significant differences among the solvent used. Within the extraction solvents, aqueous fraction (Faq) showed the maximum yield followed by hexanic fraction (Fhex). The extracting ability of different solvents used followed the order: aqueous > hexane > aqueous-ethanol.



EBeth: Crude hydroethanolic extract; Fhex: hexan fraction; Faq: aqueous fraction

Fig 1: Effects of extracting solvent on the extract yield (%) from *T. ivorensis* stem bark

3.2 Antibacterial activities of different extracts

At the end of the incubation time, a progressive decrease in the diameter of the growth inhibition zone for each bacterial strain tested was observed when a reduction in the concentration from 200 to 0.78 mg/ml of each plant extracts (Table 1) and from 125 to 0.97 mg/ml of oxacillin (Table 2) were performed. Thus, for Acinetobacter sp. IPMQ/C3GR, the diameter of inhibition zones exhibited by EBeth fraction ranged between 10.6 and 20.4 mm, the one obtained with Fhex was between 8.66 and 17 mm, while the diameter of inhibition zones shown by Faq ranged between 10.67 and 21 mm. Concerning E. coli KGTNE/RCFQ, the diameter of inhibition zones ranged between 11 and 20.4 mm, 9.66 and 20 mm, and 10.67 and 20 mm exhibited by EBeth, Fhex and Faq, respectively. As for the diameters of K. pneumoniae ATCC/13883 growth inhibition zones, they ranged between 13 and 20.75 mm, 10 and 20.33 mm, and 10.33 and 20.67 mm exhibited by EBeth, Fhex and Faq, respectively (Table 1). Otherwise, the zones of inhibition exhibited by oxacillin used as a positive control ranged between 11 and 25 mm whatever the strain tested (Table 2).

Table 1: Diameter of zones of inhibition (mm) on the tested bacteria strains growth by various extracts from *T. ivorensis* stem bark

Extracts	Extracts concentrations (mg/ml)								
	200	**100**	**50**	**25**	**12.5**	**6.25**	**3.12**	**1.56**	**0.78**
Acinetobacter sp. IPMQ/C$_3$GR									
EBeth	20.40 ± 1.00^{h}	16.40 ± 1.67^{efg}	13.20 ± 0.80^{bcd}	10.60 ± 1.34^{a}	-	-	-	-	-
Fhex	17 ± 2.00^{fg}	15 ± 0.57^{cde}	12.67 ± 0.57^{b}	10.33 ± 0.57^{a}	8.66 ± 0.57^{a}	-	-	-	-
Faq	21 ± 2.07^{h}	18.33 ± 0.57^{g}	15.00 ± 1.00^{cef}	13.00 ± 1.00^{bd}	10.67 ± 0.57^{a}	-	-	-	-
E. coli KGTNE/RCFQ									
EBeth	20.40 ± 1.67^{e}	16.60 ± 2.60^{d}	13.40 ± 1.67^{bc}	11.00 ± 1.87^{a}	-	-	-	-	-
Fhex	20 ± 1.00^{e}	17 ± 1.00^{d}	13.66 ± 2.00^{bc}	11.67 ± 1.15^{ab}	9.66 ± 0.57^{a}	-	-	-	-
Faq	20 ± 1.00^{e}	16.67 ± 0.57^{d}	14.00 ± 1.00^{c}	11.00 ± 0.00^{a}	10.67 ± 0.57^{a}	-	-	-	-
K. pneumoniae ATCC/13883									
EBeth	20.75 ± 0.95^{g}	18.50 ± 0.57^{fg}	16.00 ± 2.16^{de}	13.00 ± 1.41^{bc}	-	-	-	-	-
Fhex	20.33 ± 1.52^{g}	17.33 ± 1.52^{ef}	14.33 ± 0.57^{cd}	10.33 ± 0.57^{a}	10.00 ± 0.00^{a}	-	-	-	-
Faq	20.67 ± 0.57^{g}	18.67 ± 0.57^{fg}	14.00 ± 1.00^{cd}	11.67 ± 1.55^{ab}	10.33 ± 1.52^{a}	-	-	-	-

EBeth: Crude hydroethanolic extract; Fhex: hexan fraction; Faq: aqueous fraction; Values with the same superscript character in each column are not significantly different ($p < 0.05$).

Table 2: Diameter of zones of inhibition (mm) on the tested bacteria strains growth by Oxacillin

Bacteria	Oxacillin concentrations (mg/ml)							
	125	**62.50**	**31.25**	**15.62**	**7.81**	**3.90**	**1.95**	**0.97**
Acinetobacter sp. IPMQ/C$_3$GR	20 ± 0.01^{h}	17 ± 0.05^{f}	14 ± 0.00^{d}	11 ± 0.00^{a}	-	-	-	-
E. coli KGTNE/RCFQ	23 ± 0.00^{j}	$18 \pm 0,96^{g}$	15 ± 0.00^{e}	12 ± 0.00^{b}	-	-	-	-
K. pneumoniae ATCC/13883	25 ± 0.01^{k}	21 ± 0.00^{i}	17 ± 0.00^{f}	13 ± 0.00^{c}	-	-	-	-

Values with the same super script character in each column are not significantly different ($p < 0.05$)

The antibacterial parameters, namely MIC, MBC, and the antibacterial power of the different extracts are shown in Table 3. MICs ranged between 12.50 and 25 mg/mL, whatever plants extracts used against all bacteria strains tested (Table 3). Furthermore, Fhex and Faq exhibited the greatest inhibition against all tested bacteria strains with lowest MIC (12.50 mg/mL). Concerning the MBCs of plants extracts, which ranged from 12.50 to 125 mg/mL, the lowest MBCs (12.50 and 25 mg/mL) were obtained with Faq against the growth of *K. pneumoniae* ATCC/13883, *E. coli* KGTNE/RCFQ and *Acinetobacter sp*. IPMQ/C_3GR respectively. Therefore, only Faq and EBeth showed a bactericidal power against all bacterial strains tested, because their MBC/MIC ratio is less than or equal to 4, depending on the strain (Table 3).

Table 3: Antibacterial parameters of various extracts from *T. ivorensis* stem bark on the tested bacteria strains growth

Bacteria	Antibacterial agents	MIC (mg/ml)	MBC (mg/ml)	AP	Antibacterial effects
Acinetobacter sp. (IPMQ/C_3GR)	EBeth	25±0.00	100±0.00	2	Bactericidal
	Fhex	12.50±0.00	100±0.00	8	Bacteriostatic
	Faq	12.50±0.00	25±0.00	4	Bactericidal
	Oxa.	15.62±0.00	125±0.00	8	Bacteriostatic
E. coli (KGTNE/RCFQ)	EBeth	25±0.00	100±0.00	2	Bactericidal
	Fhex	12.50±0.00	-	-	-
	Faq	12.50±0.00	25±0.00	4	Bactericidal
	Oxa.	15.62±0.00	125±0.00	8	Bacteriostatic
K. pneumoniae (ATCC/13883)	EBeth	25±0.00	100±0.00	1	Bactericidal
	Fhex	12.50±0.00	100±0.00	8	Bacteriostatic
	Faq	12.50±0.00	12.50±0.00	4	Bactericidal
	Oxa	15.62±0.00	-	-	-

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; AP: Antibacterial Power; EBeth: Crude hydroethanolic extract; Fhex: hexan fraction; Faq: aqueous fraction, Oxa: Oxacillin

4. Discussion

Since the discovery of ESβLs-producing coliforms in the mid-1980s, over 100 types of different enzymes have been described and have become a worldwide problem. Thus, the EsβLs-producing bacteria are a clinical threat and have been associated with increasing mortality in patients with severe infection. The choice of effective and safe drug to be used against βLs is shrinking day by day [3, 13]. Therefore, due attention is needed to develop alternative agent from medicinal plants including *T. ivoirensis* for effective management of βLs and other problematic multidrug-resistant bacteria. However, extraction is the initial step prior to analysing phytochemical component or biological activities of medicinal plants. Thus, the choice of extraction solvent and method will affect yield and biological activity of plant extracts [15, 29]. In the present investigation, results showed that among all the solvent extracts; the aqueous fraction extract had the highest yield than those from plants extracted using other solvents (Aqueous ethanol and hexane). The differences in the extract yields from *T. ivorensis* stem bark might be ascribed to the different availability of extractable components, resulting from the varied chemical composition of plants [27]. The amount of bioactive components that can be extracted from a plant material is mainly affected by the vigor of the extraction procedure, which may probably vary from sample to sample. Amongst other contributing factors, efficiency of the extracting solvent to dissolve endogenous compounds might also be very important [15, 27]. Our findings agree with previous investigation reported in the literature concerning *Terminalia arjuna* [27], *Clerodendrum splendens* [16], and *Cochlospermum planchonii* [31].

Moreover, the antimicrobial potentials of various extracts from stem bark of *T. ivorensis* were investigated against three βLs-producing bacteria strains. In this study, the stem bark extracts of *T. ivorensis* were found to inhibit all tested bacteria strains, indicating that this plant possesses significant *in vitro* antimicrobial properties. The significant activities ($P<0.05$), exhibited by Faq and EBeth extracts from stem bark of *T. ivorensis*, shows that these solvents (aqueous and aqueous-ethanol) dissolved a greater percentage of the bioactive compounds from this plant than hexane. This justifies the preference of water or local gin "koutoukou", obtained from fermented palm wine distillation that known to contain a high concentration of alcohol, as extraction solvents by herbal physician in the preparation of crude drugs from medicinal plant materials [20, 31]. These observations are consistent with those of [5, 20, 31] who reported respectively that the ethanol extract of *Aspila africana, Cyathula prostrata,* and *Cochlospermum planchonii* exhibited the highest activities, followed by those of aqueous extracts against the growth of *S. aureus, P. aeruginosa, K. pneumonia*, and *E. coli*. While the work of [1], showed that the hot aqueous extracts of the root, stem bark and leaf of *Parkia clappertoniana* were more active than their ethanol and cold water extracts against *E. coli* ATCC 11775, *P. aeruginosa* ATCC10145, and *S. aureus* ATCC 12600. In addition, the inability of certain extracts to exert an antibacterial effect on the tested organisms is not enough to conclude to a lack of antimicrobial property, because the power of the extracts depends on the species and age of the plant at harvest, the solvent and extraction method used, and the amount of active compound, which can vary in quality and quantity from one season to another [8, 29, 30]. As a result, the variability found in this study and those reported previously could be attributed to some of the reasons mentioned above.

5. Conclusion

This work has allowed us to highlight the antibacterial properties of various solvents extracts from *T. ivorensis* stem bark on *Acinetobacter sp*. IPMQ/C_3GR, *E. coli* KGTNE/RCFQ and *K. pneumoniae* ATCC/13883, three βLs-producing bacteria strains involved in large number of infectious diseases. The hydro-alcoholic (EBeth) and aqueous (Faq) extracts showed bactericidal powers against

the growth of all tested bacteria strains compared to hexane extract. As has been proven *in vitro*, stem bark extracts from this plant may be a promising natural alternative against the growth of βL-producing bacteria. Therefore, it is recommended that further studies be carried out regarding the extraction process optimization and the identification of the active components in order to exploit them to evaluate the efficacy and safety *in vivo* against the tested pathogens.

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7. Conflict of interest statement
The authors declare no conflict of interest in this research article.

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