Antibacterial properties of trunk barks of Terminalia ivorensis , a commercial and medicinal species on methicillin-resistant Staphylococci species strains.

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Abstract:

Background: Methicillin-resistant Staphylococcus aureus, Staphylococcus epidermidis and coagulase-negative Staphylococcus infections are a worldwide concern. Terminalia ivorensis, of Combretaceae family plant, is a widely used traditional medicine in Côte d'Ivoire to treat skin diseases (affection in which Staphylococci are implied) including local inflammation and also to treat voice-loss.

Objectives: To investigate the effect in vitro of the extracts of trunk barks of Terminalia ivorensis on methicillin/oxacillin-resistant strains of Staphylococcus aureus, S. epidermidis, coagulase-negative S. and reference strain of S. aureus ATCC 25923.

Methods: Antibacterial activity of aqueous, 70% ethanolic 70% and aqueous residue extracts was assessed using agar disc-diffusion method and liquid medium microdilution method in 96 multi-well micro-titer plates. This method led us to determine minimum inhibition concentration (M.I.C.) and minimum bactericidal concentration (M.B.C.). The presence of major chemical groups was detected qualitatively.

Results: Aqueous and 70% ethanolic 70% extracts showed significant activity against all the bacteria except aqueous residue when compared with the standard antibiotic oxacillin (5µg/ml). M.I.C. for aqueous and 70% ethanolic 70% extracts ranged from 0.83-16.67 mg/ml and 0.156-13.33 mg/ml respectively. Viable cell determination revealed the bactericidal nature of the two barks extracts. The 70% ethanolic 70% extract exhibited the highest activity according to the M.B.C. values. The phytochemical analysis indicates the presence of tannins, saponins, flavonoids, terpen/sterols, coumarins, polyphenols and traces of alkaloid.

Conclusion: The in-vitro antibacterial efficacy shown by the barks of this plant and lushness of chemical compounds, would justify its use in the traditional treatment of some diseases of microbial origin. These compounds might be an alternative source of new therapeutic agents.

Keys words: Terminalia ivorensis, Dermal diseases, Methicillin-resistant, Côte d'Ivoire.

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Introduction

The treatment of bacterial infections is in general based on the use of antibiotics. Rampant inappropriate use of antibiotics has led to selection of strains of multi-drug resistant bacteria, for example penicillinase producing bacteria like the groups of A, G and M. Methicillin/oxacillin-resistant staphylococci infections, mainly caused by Staphylococcus aureus and coagulase-negative staphylococci, such as S. epidermidis are considered one of the major pathogens, causing infections of the skin.

In Côte d'Ivoire like in the other developing countries, infectious caused by methicillin/oxacillin-resistant Staphylococcus spp. continues to be a growing public health concern. Numerous cases of multi-resistant bacteria were reported[1, 2, 3]. The Staphylococci are involved in various illnesses and often responsible for infections most frequently contracted in hospitals (nosocomial infections)[4], starting by simple whitlow to the most serious infections like septicaemia, endocarditis, pneumonia, cellulitis and abscesses[5,6]. These bacteria produce an important number of toxins and extracellular enzymes, and fight against the action of the methicillin/oxacillin and its by-products. So, the effectiveness of antibiotics, considered as the quasi-universal solution to infections, decreases. It is thus important to direct research towards new ways and especially towards the plants which always have been used as a basis for new drugs.

Terminalia ivorensis A. Chev. (Combretaceae) is a woody species belonging to the category I of the commercial sawlog of Côte d'Ivoire. The trunk barks presents a rhytidom peeling of the tree in sheets (fig. 1). Its common name is Framiré. In Côte d'Ivoire, the root of this plant is used against voice-loss, and as an antipyretic[7,8]. The trunk barks are also used against wounds[9]

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and some cutaneous infections.

Our study consisteds of the research of the antimicrobial activity biological activity of the aqueous, 70% ethanolic and aqueous residue extracts of the trunk barks of Terminalia ivorensis A. Chev. (Combretaceae) against opposite some bacterial methicillin/oxacillin-resistant bacteria strains of Staphylococcus spp., which implied in some dermal diseases,. in other to verify its claimed ethno-medicinal use in the treatment of skin infections. To do with, the different extracts underwent a screening phytochemical.

Materials and methods:

Vegetable materials

The trunk barks part of T. ivorensis were collected in Tiassalé, Côte d'Ivoire, in December 2008, and identified by Pr Aké-Assi of the Laboratory of Vegetable Biology University Félix Houphouët Boigny of Cocody-Abidjan. A voucher specimen (voucher n° 8855) is deposited in the Herbarium of National Floristic Center of Abidjan.

Bacterial strains

Microorganisms were obtained from the Laboratory of Bacteriology-Virology of the Institute Pasteur of Côte d'Ivoire. They consist of:

- 01 strain reference of Staphylococcus aureus ATCC 25923
- 14 strains of S. aureus resistant to oxacillin, cefoxitine, rifampicin, ciprofloxacin, tetracycline, gentamycin and some to vancomycin.
- 01 strain of S. epidermidis resistant to cefoxitine, cotrimoxazole, erythromycin, ciprofloxacin, oxacillin and gentamicin.
- 04 strains of coagulase-negative Staphylococcus resistant to fusidic acid, cefoxitin, erythromycin, fosfomycine, cotrimoxazol and oxacillin.

Preparation of extracts

1. Aqueous extract

The barks were cleaned, cut out in small dises, then dried at the temperature of the laboratory 25-27^{0}c for three weeks and powdered. Briefly, 100g of bark powder was extracted in 100ml of distilled water, by grinding, in mixer blinder of the type Moulinex, according to the Zirihi and Kra method[10]. After three cycles of extraction , the filtrate was concentrated in Rotary evaporator® at 60^{0}c, then the paste obtained was freeze dried to obtain the total aqueous extract (codified ITV_{aq}).

2. 70% Ethanolic extract

Thirty grams of ITV_{aq} were soaked in 300ml of 70% ethanol for 1 hour, with constant stirring, After a total exhaustion of the substance with solvent, we obtained a hydro-alcoholic upper phase and a deposit. Thanks to a funnel separating , the 70% hydro-alcoholic phase and the deposit were separate[11]. The 70% hydro-alcoholic phase was filtered through Wattman® 3mm and dried with the drying oven at 40^{0}c, to obtain the 70% ethanolic extract noted ITV_0.

Antimicrobial activity

Test of the substance sterility

This test was carried out soaking 100mg substance to be tested in 10 ml of Thioglycholate broth. After incubation for 24 hours at 37^{0}c, the broth was sown on a Mueller Hinton (M.H) agar and a sabouraud dextrose agar in petri dishes, and then incubated in the same conditions as previously. The dishes are kept 3 to 5 days in a drying oven at 37^{0}c. If there is growth of microorganisms, the extract is submitted to a sterlization by passing on a Millipore® filter (ha: 0,45**u**m) and the test of sterility is taken. The extracts that do not bear any germ were submitted to a test of effextiveness.

Antibacterial activity test

The effectiveness test is a test of detection of the existance of a antibacterial activity of the extract. It was determined by M.H agar diffusion method using the application of blotting *inoculums* of 2.10^6 UFC/ml[12,13]. The discs were permeated with 50*u*l at six concentration of each ectract (12,5-400 mg/ml) employing a 2 fold serial dilution of the crude extract . They were dried under an oarweed-flux extractor hood for 15 min and deposited with sterilized pliers, on the surface of the Petri dishes containing the agar sown beforehand with a suspension of the *inoculums*. The Petri dishes were put to incubate at 37^{0}c for 24 hours, after 30 min for pre-diffusion. We observed, the presence or not of inhibition zone.

Minimal Inhibitory Concentration Evaluation

The majority Inhibitory Concentration (M.I.C) was determined by the micro-dilution method carried out in liquid medium using 96-well micro-titer plates[14]. Each plate divided up in 8 lines of 12 colums. In the first and the second columns of each micro-plate, were distributed 100 *u*l of the sterilized Müeller-HintonBroth (M.H.B) that served to check barreness of the culture medium. The third column containing 50 *u*l of broth and without extract was used as witness, after inoculation, to control the quality and the groeth of the bacterial strains. The eight following columns received 50 *u*l of broth. The twelth column was filled with 100 *u*l of solution mother of the extracts (400mg/ml). A series of successive dilutions (1,56-400mg/ml) of the crude extract had been prepared in micro-titer plates according to a geometric progression of ratio 2. An aliquot of 50 *u*l of standadized suspension of bacteria were grown to exponential phase in Müeller-Hinton broth (2.10^6 bacteria/ml[15]) were added to each cupule from the third to the twelfth column. The final volume of each well was of 100 *u*l. We obtained final concentrations of 0.156mg/ml at 40mg/ml. The plates were incubated at 37^{0}c for 24 hours. The M.I.C was indicated by the well that contained the lowest concentartion of extract showing no turbidity was visible with the naked eye.

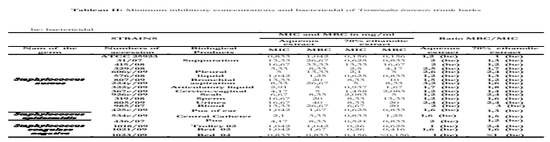
Minimal Bacterial Concentrations Evaluation

To determine M.B.C to a numeration of the standardized suspension of bacteria. For that, we sowed using a gauged handle at 10 *u*l in strias of 5cm, a M.H agar in Petri dishes, the dilutions 10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} of the standardized suspension of bacteria, and which correspond respectively at 100%, 10%, 1%,0.1% and 0.01% of survivors. The dishes were incubated at 37^{o}c for 24 hours. On the following day, after the M.I.C determination, 10 μl of each well without growth (concentration=MIC) were spread in strips of 5cm on Müeller-Hinton agar in Petri dishes and incubated at 37^{0}c for 24 hours. The number of surviving bacteria was counted for each cup. The concentration of the cupule which had a n inferior number or equal to 0.01% of surviving bacteria in relation to the suspension of the beginning in 24 hours is M.B.C. The extracts were judged bactericidal if the relation M.B.C/M.I.C was inferior or equal to 4.

Results

Antibacterial activity

The tests of sterility carried out were negative. No colony was obsereved on the two mediums used. On the three crude extracts tested, only the aqueeous residue did not show a good ctivity on the bacteria tested (fig.6), giving relatively small inhibition zones (of 6~10mm at 400mg/ml). 70% Ethanolic extract showed higher activity (fig.5) with relatively wide inhibition zones (of 12~17mm at 400 mg/ml) than aqueaous extract (of 10~14mm at 400mg) (fig.3). The diameter of reference antibiotic (oxacillin) on the ATCC 25923 strain is higher (26 mm) (fig.2 and 4). However, the 19 remaining strains showed a resistance to the oxacilline (5µg) action, while the aqueous and 70% ethanolic extracts were active(table I).



The aqueous and 70% ethanolic extracts, gave respectively the lowest M.I.C of 0.833mg/ml and 0.156mg/ml. The proportion M.B.C/M.I.C showed that aqueous and 70% ethanolic extracts have a bactericidal activity on the tested strains (table II)

Tableau I: Zone of growth inhibition (mm) of aqueous, 70% ethanolic and aqueous residue extracts, of *Terminalia ivorensis* trunk barks.

Nam of the germ	STRAINS Numbers of accession	Biological Products	Concentration in mg/ml: Aqueous extract 400	200	100	50	70% ethanolic extract 400	200	100	50	Aqueous residue 400	200	100	50	Antibiotic of reference (OX) S µg
	ATCC 25923		13	12	10	7	14	13	11	8	9	7	6	6	26
	31/08	Suppuration	13	12	9	6	14	12	10	8	6	6	6	6	6
	329/08		13	11	9	7	13	12	9	7	6	6	6	6	6
	606y/09	Pleural	12	10	7	6	13	11	9	7	6	6	6	6	6
	576/08	liquid	13	11	8	6	14	12	10	7	8	6	6	6	6
Staphylococcus aureus	807/09	Bronchial	11	9	7	6	13	12	10	8	6	6	6	6	6
	224y/09	aspiration	12	10	7	6	13	11	9	6	6	6	6	6	6
	367c/09	Cervico-vaginal	13	11	9	6	14	12	10	7	7	6	6	6	6
	322y/09	Articulatory liquid	12	10	7	6	14	12	9	6	7	6	6	6	6
	926c/09	Scab	12	10	8	6	13	11	9	7	8	6	6	6	6
	319/08	Sperm	11	9	7	6	12	10	8	6	6	6	6	6	6
	805/09	Urines	10	9	7	6	12	9	7	6	6	6	6	6	6
	995/07	Blood	11	9	7	6	12	10	8	8	6	6	6	6	6
	425c/09	Suppuration of car	13	11	9	7	14	12	10	8	8	6	6	6	6
Staphylococcus epidermidis	534c/09	Central Catheter	13	11	8	6	15	13	11	8	7	6	6	6	6
	436/07	Pus	12	10	7	6	14	12	10	7	8	6	6	6	6
Staphylococcus coagulase negative	1018/09	Trolley 02	14	12	9	7	17	14	11	7	10	7	6	6	6
	1021/09	Bed 02	13	11	8	6	17	15	12	10	8	6	6	6	6
	1023/09	Bed 04	13	11	9	7	18	16	13	10	9	7	6	6	6

OX: Oxacillin

The screening of the chemical constituents showed the presence of saponins , tannins, terpens/sterols, flavonoids and polyphenols in the three extracts. The presence of coumarins was only obsereved in the aqueous and 70% ethanolic extracts and alkaloids were also observed in traces in the aqueous and 70% ethanolic extracts III .

Tableau III: Phytochemical screening constituents of *Terminalia ivorensis* trunk barks.

Types of extracts / chemical Groups	ITV_{aq}	$ITVx_0$	$ITVx_1$	Technical used
Saponins	+	+	+	Sign moss
Flavonoids	+	+	+	$AlCl_3$
Terpens / Sterols	+	+	+	Vanille sulfurique
Coumarins	+	+	-	Borntrager
Tannins	+	+	+	$FeCl_3$ 10%
Alkaloids	±	±	-	Dragendorf
Polyphenols	+	+	+	Fast blue B/NaOH 10%

+: presence ±: trace -: absent

Discussion

The choice of *staphylococcus spp.* for the study was guided by the fact that these bacteria are responsible for various community affections and nosocomial more or less serious (superficial suppuratives infections of the skin and the deep abscesses), according to the immunizing system of the defence of the infected individual. *Staphylococcus spp.* is a good indicator in the description of the antibacterial properties of the extracts of trunk barks of *T.ivorensis.*

The sterility of these three extracts shows that the extractions have been carried out in the aseptic condition. In this study, the aqueous and 70% ethanolic extracts inhibit the growth of the tested microorganisms according to a relation dose-response. Our results showed a higher sensitivity of *staphylococcus coaggulase* negative followed by *staphylococcus epidermidis* and *staphylococcus aureus* to the 70% ethanolic extract than the aqueous extract. While the strongest activity was demonstarted against the reference ATCC 25923 and one *staphylococcus coaggulase* negative (1023/09) strains with 70% ethanolic. Indeed, the aqueous and 70% ethanolic extracts present a good activity than the standard antibiotic (oxacillin: 5 μg) with the concentration retained for the experiment , although our extracts are still crude. These extracts could contain some substances having target sites other than those used by standarrd antibiotics.

Some authors have already shown that *taphylococcus spp.* are more susceptible towards plant extracts to other

strains (Gram-negative)[19-20]. Several species of *Terminalia* were previously studied and found to have various pharmacological properties[21-22].These properties coincide with the use of *T.ivorensis* in Côte d'Ivoire where its used to treat skin affections according to Coulibaly[23] and N'guessan[9]. The phytochemical screening of the extract of the trunk barks of *T.ivorensis* in has shown the presence of saponins, terpens, tannins, polyphenols. These classes of secondary metabolites are known to possesss antibacterial activities[24]. This would explain the bactericidal action of the trunk barks of *T.ivorensis*. However, negative results obsereved with aqueous residue, donot mean absence of bioactive constituents nor is that the extract inactive. Active compaounds may be present but in insufficient quantities in this crude extract to show activity with the dose levels employed[25] or it could be that its activity is masked by the presence of sugars[26].We could deduce from that, that the antibacterial substances contained in the trunk barks of *T.ivorensis* are more soluble in the 70% ethanol than in water used. The ethanol would then concentrate better the active ingredients[27]. The presence of those active principles would then justify the use of the plant in the treatment of the skin troubles and local infkammation in the Ivorian traditional pharmacopeia.

Conclusion:

The presence of extracts in chemical compounds, would justify their therapeutic effects and overcoat the use of this plant in the traditional treatment of some diseases of microbial origin. Isolation and purification of different compounds is suggested to provide alternative solution to the development of new therapeutic agents.

Thanks

We wish to express our gratitude to the Analytic Chemistry Laboratory of Pharmaceutical Sciences of the University Félix Houphouët Boigny of Cocody and the Institute Pasteur of Côte d'Ivoire for their technical help.

Fig. 1: Part of the trunk bark of *Terminalia ivorensis* A. Chev.

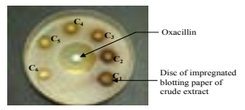


Fig. 2: Action dose-response of aqueous concentrations of *T. ivorensis* on *Staphylococcus aureus* ATCC 25923.

C_1: 400mg/ml C_4: 50mg/ml
C_2: 200mg/ml C_5: 25mg/ml
C_3: 100mg/ml C_6: 12,5mg/ml

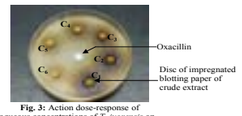


Fig. 3: Action dose-response of aqueous concentrations of *T. ivorensis* on oxacillin-resistant *Staphylococcus aureus*.

C_1: 400mg/ml C_4: 50mg/ml
C_2: 200mg/ml C_5: 25mg/ml
C_3: 100mg/ml C_6: 12,5mg/ml

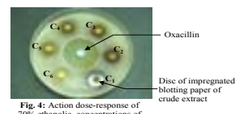


Fig. 4: Action dose-response of 70% ethanolic concentrations of *T. ivorensis* on *Staphylococcus aureus* ATCC 25923.

C_1: 400mg/ml C_4: 50mg/ml
C_2: 200mg/ml C_5: 25mg/ml
C_3: 100mg/ml C_6: 12,5mg/ml

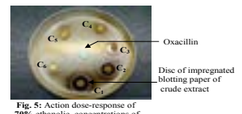


Fig. 5: Action dose-response of **70%** ethanolic concentrations of *T. ivorensis* on oxacillin-resistant *Staphylococcus aureus*.

C_1: 400mg/ml C_4: 50mg/ml
C_2: 200mg/ml C_5: 25mg/ml
C_3: 100mg/ml C_6: 12,5mg/ml

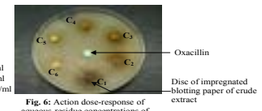


Fig. 6: Action dose-response of aqueous-residue concentrations of *T. ivorensis* on oxacillin-resistant *Staphylococcus aureus*.

C_1: 400mg/ml C_4: 50mg/ml
C_2: 200mg/ml C_5: 25mg/ml
C_3: 100mg/ml C_6: 12,5mg/ml

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