Research Article

# Triphytochimique Study and Inhibitory Activity of the Ethanol Extract of the Stem Bark of *Terminalia glaucescens* Planch Ex Benth on Enterobacteriaceae Producing Extended-Spectrum Beta-Lactamase (ESBL)

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**ABSTRACT**

The production of the beta-lactamases extended spectrum plays an important role in antibacterial therapy. The purpose of this work was to study the phytochemistry and the inhibitory activity of the ethanol extract of the stem bark of *T. glaucescens* Planch ex Benth on Enterobacteriaceae producing extended-spectrum beta-lactamase (ESBLE). Characterization methods for coloring, in solid medium diffusion and broth dilution Muller-Hinton® using different concentrations of the plant extract have identified various chemical groups and to evaluate the antibacterial activity of the extract. The characterization of the chemical constituents of the ethanol extract of the stem bark of *T. glaucescens* Planch ex Benth showed the presence of tannins gallic and cathechic, polyphenols and flavonoids. The in vitro antibacterial activity of the ethanol extract from the bark of *T. glaucescens* Planch ex Benth was compared to that of third-generation cephalosporins towards the strains studied. The 96% ethanol extract gave zones of inhibition ranging from 13 ± 0.0 to 23 ± 4.3mm at 200mg/ml from 11.6 ± 0.3 to 21.3 ± 4.2mm to 100mg/ml and 10 ± 0.0 to 20.3 ± 4.2mm at 50mg/ml. The MIC of ethanol extract ranged between 6.25 and 50mg/ml. The strongest activity was observed with *K. pneumoniae* 137C12 (MIC = MBC = 6.25mg/ml). The extract was studied showed bactericidal to 100% (11/11) of the strains studied. This work could provide a scientific basis for the traditional use of *T. glaucescens* Planch ex Benth in infectious diseases.

**Keywords:** *T. glaucescens*, ESBLE, Phytochemistry, 96% ethanol extract, antibacterial activity.

## INTRODUCTION

The therapeutic use of the plants (phytotherapy) is very old and is currently experiencing a resurgence of interest among the public. It is possible to use the whole plants or the products that they provide extraction[1]. Today, they have an important role in the treatment of tropical diseases such as malaria, jaundice, and schistosomiasis[2,3]. Surveys showed that between 3 and 5% of patients in Western countries, 80% of rural populations in the developing countries and 85% of the population south of the Sahara use medicinal plants in primary health care[4]. African flora in general and Ivorian particular, offers a significant reserve of aromatic plants and medicinal character occupying an important place in the Ivorian pharmacopoeia.

However, the chemical composition of medicinal plantsfor health care is not always known by many people. In order to improve this medicine, several phytochemical investigations have been made to provide a scientific justification for the traditional use of medicinal plants in the treatment of bacterial infections.

Enterobacteriaceae producing beta-lactamase extended spectrums are a real concern in human medicine because they can limit the treatment of certain infections. Also, it was observed that the presence of ESBLE is associated with increased morbidity and increased costs of care of the infection.

The evolution of this resistance and the presence of more frequent ESBLE lead clinicians to prescribe more and more carbapenems, which induce selective pressure favorable to the emergence of carbapenemases.

In Côte d'Ivoire ESBLE rates increased from 5.3% in 2005 to 16.8% in 2009. [5]

It should therefore consider orient research toward new ways and above all plants to fight against these plagues. And more into the world, nearly 25% of prescriptions are herbal and 60 to 70% of antibacterial and anticancer drugs are substances of natural origin. [6]

*T. glaucescens* Planch ex Benth (Combretaceae) is used in the treatment of diarrhea, dysentery, tooth decay, burns, ulcerations and sores in the gums and malaria[7,8]. The antibacterial activity of the extracts of leaves was studied in *Salmonella* Typhi and *Salmonella typhimurium*[9]. The same is true for the antiparasitic activity of *Plasmodium falciparum*[10].

This is in view of contribute to the search for new molecules inhibitory beta-lactamases and to enable populations of low-income countries to take real advantage of the use of medicinal plants in their

pharmacopoeia that we studied phytochemistry and the inhibitory effect of the powder of the stem bark of *T. glaucescens* Planch ex Benth (Combretaceae), a characteristic plant of the savanna regions on the bacteria producing extended spectrum beta-lactamases.

## MATERIALS AND METHODS

### Plant material

The stem bark of *T. glaucescens* Planch ex Benth (Combretaceae) was collected in 2012 April in the region of Belier (Ahougnansou-Allahou S/P Tiébissou) in the center of Côte d'Ivoire and identified by the National Centre of Floristic University Felix Houphouet Boigny (Côte d'Ivoire).

### Bacterial material

The bacterial material consisted of bacterial strains producing beta-lactamase extended spectrum responsible for various infections isolated at the Pasteur Institute of Côte d'Ivoire and two references strains *Escherichia coli* ATCC 25922 and *Escherichia coli* ATCC 35212.

### Spraying of plant material

The bark of *T. glaucescens* Planch ex Benth were cut into small pieces and dried in the open air, away from sunlight and the temperature (25°C to 30°C) for 14 days. The dried barks were ground to a fine powder using a mortar. The powders obtained were stored in hermetic glass flasks. These powders were used in the preparation of the ethanol extract.

### Preparation of the ethanol extract

It was performed according to the method Olakunle *et al*.[11]. Twenty grams of plant powder were dissolved in 100ml of ethanol by maceration for 72 hours. The macerate was wrung within a square of clean cloth, filtered successively on hydrophilic cotton and once on filter paper (Whatman ® 2mm). The filtrate was slowly dried in a stove at 50°C. The powder obtained is ethanol extract and was stored in a sealable jar and preserved at refrigerator at 4°C.

### Testing phytochemicals

Order to highlight the main chemical groups present in the extracts tested, testing different characterizations were performed.

#### *Research sterols and polyterpenes (Salkowski reaction)*

A one milliliter of the extract was added to two drops of sulfuric acid ($H_2SO_4$) concentration. The presence of sterols and polyterpenes resulted in a yellow or red coloration of the extract.

#### *Research of the alkaloids (reaction DRAGENDORFF and Bouchardat)*

Six milliliters of plant extract were evaporated. The residue was taken by six milliliters of alcohol 60 ° and the alcoholic solution thereby obtained was divided into two test tubes.

In the first tube was added two drops of Dragendorff reagent. The appearance of a precipitate or orange color indicated the presence of alkaloids.

In the second tube was added two drops of reactive Bouchardat. The appearance of a reddish brown color indicated a positive reaction to the presence of alkaloids.

#### *Polyphenols*

Two milliliter of extract was added a drop of alcoholic solution of ferric chloride at 2%. The appearance of a dark green or lighter or darker blue color indicated the presence of polyphenolic derivatives.

#### *Research flavonoids*

For this research, two milliliter of the extract was evaporated to dryness in a porcelain dish on a sand bath. The residue was taken after cooling in five milliliter alcohol hydrochloric half. The successive addition of three magnesium shavings and three drops of isoamyl alcohol showed an intense pink or violet in the presence of flavonoids[12].

#### *Research saponosides*

A volume of two milliliters of the extract were evaporated and taken up in five milliliters of water. After vigorous stirring, the foaming of more than one centimeter, stable and persistent high for 30 minutes indicated the presence of saponins.

#### *Research tannins (reaction Stiasny)*

*Research catechol or condensed tannins*

A volume of five milliliters of each extract was evaporated and a quantity of 10ml of a reagent solution Stiasny was added to the residue. This mixture was placed in a water bath at 80°C for 30 minutes and cooled to room temperature.

Positive feedback had resulted in the formation of large flakes brown clear or dirty precipitates.

*Research gallic tannins*

The above solution was saturated and one to two drops of the alcoholic solution of iron chloride to 2% has been added. The positive reaction resulted in the appearance of a blue-black coloration characteristic intense tannins Gallic.

### Antibacterial activity of extracts

#### *Detection of bacterial strains producing beta-lactamase*

The characterization of Enterobacteriaceae by producing extended spectrum beta-lactamases (ESBLE) was made by the diffusion technique. On the surface of agar disks of ceftazidime, cefotaxime, cefepime, aztreonam and amoxicillin + clavulanic acid were disposed 3cm on center from each other. After 24 hours of incubation at 37°C, a

synergy image showed the production of extended-spectrum beta-lactamases.

***Preparation of bacterial inoculum***

Two isolated colonies for bacterial culture on 18 hours were homogenized in 10ml of Muller-Hinton broth and incubated for three hours at 37°C for pre-culture. A levy of 0.1ml of broth pre-culture was diluted into a tube containing 10ml of Muller-Hinton broth. This bacterial suspension was made up of bacterial inoculum dilution $10^0$.

***Enumeration of the bacterial inoculum***

The bacterial inoculum was diluted from 10 by 10, to the $10^{-4}$ dilution. The initial bacterial inoculums and four successive dilutions were inoculated with a calibrated bight 2µl on Muller-Hinton agar with striations of five centimeters long constituting the box A.

***Preparation of the range of concentration of the substance***

The concentration range was prepared in a string of seven test tubes numbered $T_1$ to $T_7$, by the method of dual dilution in liquid medium. This concentration range varied from 200 to 3.12mg/ml. In the tube$T_1$, 2g of plant extract were dissolved in 10ml of distilled water, the concentration of 200mg/ml was obtained. A volume of five milliliters of tube $T_1$ was transferred into the tube $T_2$, and then homogenized. This operation was repeated until tube$T_7$, and at the end of the tube $T_7$ five milliliters were rejected. The content of the tubes was filtered through a membrane (MILLEX GV ®) of 0.45µm and stored in the refrigerator at 4°C.

***Testing antibacterial***

*Method of diffusion in a solid medium*

Wells were made using a Pasteur pipette and a quantity of 50µl of the test substance is deposited. The agar was incubated in an incubator at 37°C for 18 to 24 hours.

The reading was done by measuring the diameter of inhibition around each well area by using a caliper. The diameters of the inhibition zone were expressed in mm according to the following criteria expressed in 2003 by Ponce *et al.* [13]

Said resistant strain is sensitive, highly sensitive and highly sensitive respectively to a diameter smaller than 8mm, between 9 and 14mm, between 15 and 19mm and more than or equal to 20mm.

*Methods of broth dilution*

The concentration range of 96% ethanol extract of the stem bark of *T. glaucescens* Planch ex Benth added the seven experimental tubes and tube growth (Tc) containing 1ml of inoculum of bacteria test according Dosso and Faye-Kette[14] and Koné *et al*. [15] A volume of one milliliter extract the highest concentration (200mg/ml) was transferred into the tube $T_1$, the following concentration in the tube $T_2$, and so on until the lowest concentration in the tube $T_7$. The growth control (Tc) was represented by one milliliter of 48% ethanol and two milliliters of Muller-Hinton broth used as sterile sterility control (Ts). All tubes were incubated at 37°C for 24 hours. The MIC is the concentration of the first tube from which we observe any disturbance to the naked eye. This operation was repeated three times.

**Mode of action of the extract**

The MBC/MIC ratio has clarified the mode of action of a substance[16]. If the MBC/MIC ratio is less than or equal to two, the substance is said to be bactericidal, however,if it is more than two, the substance is said to be bacteriostatic.

## RESULTS

**Performance of the extraction method**

From 20g powdered stem bark *T. glaucescens* Planch ex Bench and macerated in 160ml of 96% ethanol (Merck, Darmstadt) for 72 hours, an extraction yield of 12.75% was calculated.

**Tri phytochemical**

Tri phytochemical tests, carried out on the 96% ethanol extract are shown in Table 1.

**Table 1:** Phytochemical analysis of the 96% ethanol extract of the stem bark of *T. glaucescens* Planch ex Benth.

| Chemical groups | 96% ethanol extract |
|---|---|
| Alkaloids by Dragendorff | - |
| Alkaloids by Bouchardat | - |
| Polyphenols | +++ |
| Flavonoids | ++ |
| Catechic tannins | +++ |
| Gallic tannins | + |
| Saponins | - |
| Steroids and terpenoids | - |

-: Absence; +: Presence, + + means; + + +: abundant.

The phytochemical analysis has shown the presence of polyphenols, catechic and gallic tannins and flavonoids in the 96% ethanol extract of the stem bark of *T. glaucescens* Planch ex Bench. Any alkaloids, saponins, steroidal compounds and terpenoïdic have been identified in this extract as shown in Table 1.

**Testing antibacterial**

***Detection of bacterial strains producing beta-lactamase***

The following antibiotics, ceftriaxone (CRO), aztreonam (ATM), imipenem (IPM), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP) and amoxicillin + clavulanic acid were used to detect the production of beta-lactamase (Figure 1).

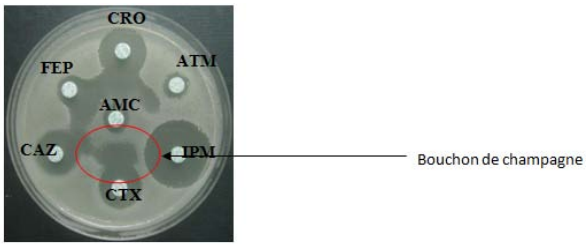



**Figure 1:** ESBLE detection by the method of the double synergy

***Method of diffusion in a solid medium***

No zone of inhibition was observed around the wells filled with 48% ethanol. Table 2 shows the diameters of the inhibition obtained on ESBLE strain and the reference strain according to the concentration areas. Figure 2 shows the activity of the extract on the in vitro growth ESBLE.

**Table 2:** Diameter (mm) of inhibition zones obtained with 96% ethanol extract of *T. glaucescens* Planch ex Benth.

| Code | Strains | Concentration (mg/ml) | | | T=0.0 | Beta-lactam | | |
|---|---|---|---|---|---|---|---|---|
| | | $C_1$=200 | $C_2$=100 | $C_3$=50 | | CRO | CAZ | CTX |
| 033C12 | *K. pneumoniae* | 13±0,0 | 11,6±0,3 | 10±0,0 | 6±0,0 | 06 | 14 | 06 |
| 37C12 | *E. aerogenes* | 16±1,0 | 15±1,0 | 14±1,0 | 6±0,0 | 15 | 17 | 15 |
| 75C12 | *E. cloacae* | 14±0,6 | 11±0,6 | 12±0,6 | 6±0,0 | 12 | 19 | 13 |
| 137C12 | *K. pneumoniae* | 14,6±0,6 | 13,3±0,6 | 11,3±0,6 | 6±0,0 | 15 | 22 | 15 |
| 234C12 | *E. coli* | 15,3±0,6 | 13,6±0,6 | 12,3±0,6 | 6±0,0 | 09 | 16 | 12 |
| 244C12 | *E. aerogenes* | 14±1,0 | 13±1,0 | 11,6±1,1 | 6±0,0 | 20 | 15 | 20 |
| 252C12 | *K. pneumoniae* | 15±1,0 | 14±1,0 | 13±1,0 | 6±0,0 | 13 | 15 | 10 |
| 262C12 | *E. coli* | 17±1,0 | 15±1,0 | 13±1,0 | 6±0,0 | 06 | 09 | 06 |
| 265C12 | *C. koseri* | 23±4,3 | 21,3±4,2 | 20,3±4,2 | 6±0,0 | 06 | 10 | 06 |
| 25922 | *E. coli* ATCC | 16,6±2,0 | 15,6±2,0 | 13,6±1,1 | 6±0,0 | 30 | 28 | 34 |
| 35218 | *E. coli* ATCC | 16,3±0,6 | 15±0,0 | 14±0,0 | 6±0,0 | 42 | 32 | 48 |

CRO: ceftriaxone (30µg) ; CAZ: ceftazidime (30µg) ; CTX: cefotaxime (30µg) ; T: witness ; cup diameter = 6mm

**Table 3:** Antibacterial Settings 96% ethanol extract of *T. glaucescens* Planch ex Benth on clinical ESBLE studied in liquid medium.

| Code | Strains | MIC* (mg/ml) | MBC* (mg/ml) | MBC/MIC | Power |
|---|---|---|---|---|---|
| 033C12 | *K. pneumoniae* | 50 | 100 | 2 | bactericidal |
| 037C12 | *E. aerogenes* | 12.5 | 12.5 | 1 | bactericidal |
| 075C12 | *E. cloacae* | 12.5 | 25 | 2 | bactericidal |
| 137C12 | *K. pneumoniae* | 6.25 | 6.25 | 1 | bactericidal |
| 234C12 | *E. coli* | 12.5 | 12.5 | 1 | bactericidal |
| 244C12 | *E. aerogenes* | 6.25 | 12.5 | 2 | bactericidal |
| 252C12 | *K. pneumoniae* | 6.25 | 12.5 | 2 | bactericidal |
| 262C12 | *E. coli* | 12.5 | 25 | 2 | bactericidal |
| 265C12 | *C. koseri* | 50 | 100 | 2 | bactericidal |
| 25922 | *E. coli* ATCC | 12.5 | 12.5 | 1 | bactericidal |
| 35218 | *E. coli* ATCC | 6.25 | 12.5 | 2 | bactericidal |

MIC*: minimum inhibitory concentration in a liquid medium, MBC * minimum bactericidal concentration in liquid medium

The 96% ethanol extract of the stem bark of *T. glaucescens* Planch ex Benth described zones of inhibition between 13mm and 23mm at 200mg/ml, between 11mm and 21mm at 100mg/ml and between 20mm and 10mm at 50mg/ml. The control (48% ethanol) did not describe any zone of inhibition. Ceftriaxone and cefotaxime have no effect on the three strains tested. While ceftazidime showed an effect on all strains tested.

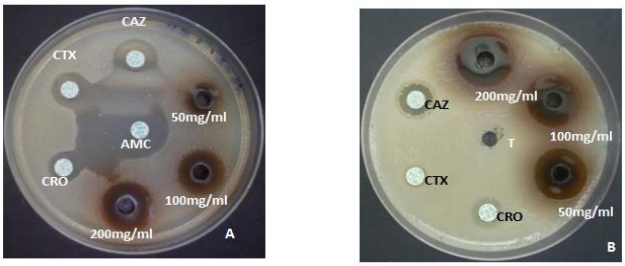
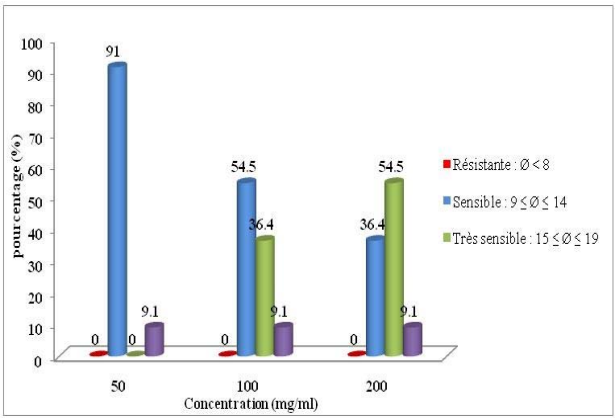



**Figure 2:** Operation of the 96% ethanol extract of the stem bark of *T. glaucescens* Planch ex Benth on *Enterobacter aerogenes* 37C12 ESBLE (A) and *Citrobacter koseri* C3GR 265C12 (B)




Ø = diameter (mm)

**Figure 3:** Different categories of sensitivity of Ponce *et al* [13] obtained with the 96% ethanol extract of the stem bark of *T. glaucescens* Planch ex Benth.

***Method of dilution in liquid medium***

There was a gradual decrease in the intensity of the disorder induced by the growth of bacteria gradually as the concentration of the plant extract increased in the experimental tubes. *K. pneumoniae* 137C12 was more sensitive with MIC and MBC values equal to 6.25mg/ml. The greatest value of MIC was observed with *K. pneumoniae* 33C12 and *C. koseri* 265C12 (50mg/ml) and that of the MBC with these strains (100mg/ml). The MBC/MIC ratios for all strains were less than or equal to two.

The 96% ethanol extract of *T. glaucescens* Planch ex Benth was bactericidal towards the Enterobacteriaceae producing extended spectrum beta-lactamases tested.

## DISCUSSION

This survey aimed to study the phytochemistry and the inhibitory activity of the ethanol extract of the stem bark of *T. glaucescens* Planch ex Benth on Enterobacteriaceae producing extended spectrum beta-lactamases (ESBLE).

The performance of maceration by the alcoholic solvent was 12.7%. This performance may validate the traditional form of use (macerated) *T. glaucescens* Planch ex Benth with regard to traditional usage.

The phytochemical study of the ethanol extract of the stem bark of *T. glaucescens* Planch ex Benth showed that this plant contains catechic and gallic tannins, polyphenols and flavonoids. The richness of this extract of active chemicals may explain the traditional use of *T. glaucescens* Planch ex Benth to treat many diseases such as dysentery, tooth decay, burns, wounds and ulcers in the gums, and malaria[7,8]. Indeed, several authors showed that different types of chemical compounds identified in the extracts of this plant have therapeutic effects[17,18]. These tannins recognized for their antibacterial activity[19,20], polyphenols[21,18] used in their antipyretic and analgesic properties[22] and flavonoids can reduce hypertension[23].

The results of this study showed that the crude ethanol extract of the stem bark of *T. glaucescens* Planch ex Benth has produced larger inhibition zones than antibiotics (cephalosporins) in all cases. The solvent used for dissolving dry residue of the alcoholic extract (48% alcohol) taken as a control gave no zone of inhibition. The crude ethanol extract has been more active on ESBLE strains those common antibiotics. All strains tested resistant to third generation cephalosporins tested (ceftriaxone (30µg), ceftazidime (30µg), cefotaxime (30µg)) according to CA-SFM[24], the zones of inhibition induced by these antibiotics are resistant below 26mm. This opposite was observed among the two reference strains. *E. coli* ATCC 25922 gave diameters of 28mm to 34mm ceftazidime and cefotaxime and *E. coli* ATCC 35218 diameters from 32mm to 48mm ceftazidime and cefotaxime.

These results are in agreement with those of Bolou[9] showed that the extract of the leaves of *T. glaucescens* Planch ex Benth inhibits in vitro growth of various bacterial strains. We can explain these results by the presence of composition in the alcoholic extract as determined by our study and confirmed by the work of Adebayo Ishola[25].

Figure 3 shows that the sensitivity rate of 36.4%, 54.5% and 9.1% of the tested strains ESBLE were respectively sensitive, very sensitive and extremely sensitive to the 96% ethanol extract of the bark the stem of *T. glaucescens* Planch ex Benth to 200mg/ml. Rates of 54.5%, 36.4% and 9.1% of these strains have the same respective categories of sensitivity to 100mg/ml. At 50mg/ml, 91% and 9.1% respectively of the strains were sensitive and highly sensitive to the alcoholic extract[13].

Our results show that ethanol extract has had antibacterial activity by inhibiting the growth of bacterial germs in a dose-response relationship. This allowed us to determine the differents parameters, namely antibacterial MIC and MBC.

With regard to antibacterial powers of the ethanol extract from the bark of *T. glaucescens* Planch ex Benth, the MBC/MIC ratio, shows that the extract has a bactericidal action on all strains studied[16].

## CONCLUSION

The use of plants is a vital necessity for developing countries.

This study demonstrated the presence of tannins gallic and catechic, polyphenols and flavonoids and antibacterial activity of 96% ethanol extract of the stem bark of *T. glaucescens* Planch ex Benth. This extract was bactericidal against all strains studied. This work must continue in order to determine their antioxidant capacity and toxicity or not.

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