IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
November 2015 Vol.:4, Issue:4

Antioxidant and Antibacterial Activity of the Extracts of Stem Bark of *Terminalia glaucescens* Planch Ex Benth (Combretaceae) on Enterobacteriaceae Extended-Spectrum Beta-Lactamase Producing (ESBL)

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Submission: 1 November 2015
Accepted: 5 November 2015
Published: 25 November 2015

Keywords: *T. glaucescens*, antioxidant activity, antibacterial activity, ESBL

ABSTRACT

Terminalia glaucescens Planch ex Benth, plant of the Combretaceae family, is rich in secondary metabolites, and is used as traditional medications. The objective of this work is to evaluate the antioxidant and antibacterial activity of the aqueous and ethanol 96% extracts of the stem bark of *T. glaucescens* on enterobacterial extended spectrum beta-lactamases (ESBL) producing. Firstly, the methods of maceration, titration, DPPH tests and diffusion a solid medium in Muller-Hinton®, using different concentrations of extract were carried out to determine the amount of respectively the crude extract, phenols and flavonoids and secondly, to evaluate the antioxidant and antibacterial activity of aqueous and ethanol 96% extracts of the stem bark of *T. glaucescens*. The yields in aqueous and ethanolic 96% extracts were found to be14.8 and 12.75 percent respectively. The quantitative determination of total phenols and flavonoids by the method of Folin Ciocalteu and trichloride aluminum showed that extracts were rich in these compounds. Aqueous and ethanol 96% extracts showed an IC_{50} of 12.8 and 5.27µg/ml respectively. The ethanol 96% extract of *T. glaucescens* showed a greater activity on the ESBL as compared to the aqueous extract. The values for diameter of zone of inhibition for the aqueous and ethanolic 96% extract at 200 mg/ml were found to be 14 ± 0.6 at 23 ± 4.3mm and 15.3 ± 0.6 at 25 ± 2.0 mm respectively for against the ESBL activity. This work aims to explore a scientific basis involved in the traditional use of extracts from the stem bark of *T. glaucescens* against ESBL infections. The results suggest that, extracts of *T. glaucescens* may act as a chemopreventive agent offering effective protection against free radicals.

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INTRODUCTION

The use of synthetic antioxidant molecules is currently of concern because they convey toxicological risk. As a matter of fact new natural antioxidants originating from plant sources are being investigated [1, 2]. Indeed, polyphenols are natural compounds widely spread in the flora and receive growing importance particularly through their beneficial effects on health [3]. Plants that contain secondary metabolites like phenolic compounds in high amount, are sought after by cosmetic, pharmaceutical and herbal medicine industries [4]. *Terminalia glaucescens* Planch ex Benth (Combretaceae) is a tree of 8m high with a gray-black bark deeply fissured. The leaves have a roughly elliptical shape and have ribs at their hairy underside, measuring 8.5 to 15cm long and 2.5 to 7.5cm wide. The flowers are greenish-white, very small and highly fragrant with brown hair at the base of the style [5]. It is a early stage species present in the old Mid Savannas of Côte d'Ivoire. It is found in the region of Toumodi and endemic to the region of Tiébissou and Katiola [6]. Study by Adebayo and Ishola in 2009 [7], has revealed the presence of flavonoids, steroids, tannins, alkaloids and saponins in the extracts of *T. glaucescens*. The studies conducted on this plant reported that the extracts have antimicrobial properties [5]. Among others the fraction in ethyl acetate of the leaves of *T. glaucescens* has demonstrated inhibitory action on *S. typhimurium* ATCC 14028 [8]. Okpekon et al. [9] also showed that the stem bark extracts of *T. glaucescens* is highly active against *Plasmodium falciparum*.

Therefore, it is appropriate to test these extracts of *T. glaucescens* on ESBL, which are responsible for therapeutic failure of human medicines on account of the production of ESBL which limit the spectrum of action of third generation cephalosporins. Further, the study of antioxidant activity of crude extracts of *T. glaucescens* has never been performed.

Accordingly, the study aims to evaluate the *in vitro* antioxidant activity by DPPH free radical method and antibacterial activity of the aqueous and ethanol extracts of *T. glaucescens* against ESBL.

2. MATERIAL AND METHODS

2.1. Material

2.1.1. Bacterial material

Bacterial material is made up of bacterial strains producing extended-spectrum beta-lactamase (ESBL) responsible for various infections and phenotypes (Table 1), isolated at the Institut Pasteur of Côte d'Ivoire and two reference strains *Escherichia coli* ATCC 25922 and *Escherichia coli* ATCC 35212.

Table 1: Biological and clinical phenotypes Product strains

Code	Name of germ	Biological Product	Phenotypes
075C12	***Enterobacter cloacae***	Pus	- ESBL - Cross-resistance to FQ - TG
234C12	***Escherichia coli***	Urines	- ESBL - Cross-resistance to FQ - KG
244C12	***Enterobacter aerogenes***	Trach Tube	ESBL
252C12	***Klebsiella pneumoniae***	Urines	- ESBL - Cross-resistance to FQ - KTGNt
265C12	***Citrobacter koseri***	Pus	- C_3G-R : likely cephalosporinase (hyperproduite chromosomal or plasmid) - Cross-resistance to FQ - KTG

FQ : fluoroquinolones ; ESBL : extended-spectrum beta-lactamase; K : kanamycin ; T : tobramycin ; G : gentamycin ; Nt : Nétilmicin

2.1.2. Plant material

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

2.2. Methods

2.2.1. Sputtering plant material

The barks of *T. glaucescens* were cut into small pieces and dried in the air and kept at ambient temperature (25°C to 30°C) away from direct sunlight for 14 days. The dried barks were ground to fine powder using a mortar. The powdered extracts were stored in glass vials and tightly sealed. Finally they were used to prepare of aqueous and ethanol extracts.

2.2.2. Preparation of aqueous extract

100g powder of the bark of *T. glaucescens* were macerated for 72 hours in 1L of distilled water [10]. The macerate has been wrung into a square of sterile tissue, filtered successively on cotton wool and one fold on filter paper (Whatman paper® 2mm). The filtrate was dried slowly in the stove at 50°C. The powder obtained was stored in a hermetically sealed jar and refrigerated at 4 ° C [11].

2.2.3. Preparation of ethanolic 96% extract

It was carried out using modified Olakunle et al**.** method [10]. A mass of 20g of plant powder was added in 100ml of ethanol 96% and subjected to maceration for 72 hours . The macerate was treated according to the same procedure like the aqueous extract.

2.2.4. Dosing of the polyphénols

Total polyphenols content of the extracts was determined by reported method using Folin Ciocalteu reagent [12]. The gallic acid was used as standard. Briefly, 5ml of Folin reagent (diluted 10 times) was added to 500µl of sample or reference standard (prepared from methanol) with suitable dilutions. After 5 minutes, 4ml of sodium carbonate solution (75mg/ml) were added to the reaction medium. After 15 minutes of incubation at ambient temperature, the absorbance was

measured at 765nm. The concentration of total polyphenols was calculated from the linear regression equation of the calibration range established with gallic acid (0-250mg/l). The total polyphenols content of the extract was expressed in milligrams of gallic acid per equivalent gram of extract (mg/g GAE of extract).

2.2.5. Dosing of the flavonoids

Flavonoids content of the extracts was determined by reported method using aluminum trichloride [13] and the standard chosen was quercetin. At a volume of 0.5ml of sample or reference standard (prepared in methanol) was added successively, a volume of 2.8ml of distilled water, 0.1ml of potassium acetate (98.15mg/ml), 0.1ml of 10% aluminum trichloride solution in methanol and 1.5ml of methanol. After 30 minutes of reaction, the absorbance was read at 415nm. The concentration of flavonoids derived from a calibration established with quercetin (0-100µg/ml) and is expressed in milligram equivalents of quercetin per gram of extract (EQ mg/g extract).

2.2.6. Evaluation of antioxidant activity

The activity of antioxidant extracts of *T. glaucescens* was evaluated by the DPPH test.

The measure of the ability of an antioxidant to fix free radicals is done by measuring the decrease in the purple coloration, induced by the reduction of DPPH by the radicals, method of Pajero et al. [14]. A coloration ranging from purple to yellow was obtained. The solution of DPPH was prepared by dissolving 5mg of DPPH in 250ml of methanol. This solution of DPPH (0.02mg/ml) was stored at 4°C in the absence of light. In a batch of eight tubes containing 2.5ml of each plant sample, different concentrations (0-100µg/ml) were added to 2.5ml of the solution of DPPH. The control tube was prepared by adding 2.5ml of DPPH and 2.5ml of ethanol. After 15 minutes of reaction in the darkness, the DO reading was performed at 517nm using a spectrophotometer (BioMerieux).

The antioxidant activity is determined by the following expression:

$$\text{Activity (\%)} = 1 - \frac{\text{DO sample x 100}}{\text{DO dpph}}$$

2.2.7. Preparation of the concentration range

The concentration range was prepared by dissolving 2g of the aqueous or ethanolic extract of the bark of *T. glaucescens* in 10ml of distilled water or ethanol 48°. The solution obtained was used to perform the range of double dilution.

2.2.8. Evaluation of antibacterial activity

The antibacterial activity of the extracts of *T. glaucescens* was evaluated by the method of diffusion in solid medium.

The wells were made using a Pasteur pipette in agar gel previously seeded and 50µl of the substance to be tested is deposited. The agar was incubated at 37°C for 18 to 24 hours. The reading was made by measuring the diameter of inhibition around each well using a sliding caliper. The diameters of inhibition zone were expressed in mm according to the criteria expressed in 2003 by Ponce et al. [15]. The strain is then said resistant, sensitive, very sensitive and extremely sensitive respectively for a diameter less than 8mm, between 9 and 14mm, between 15 and 19 mm and equal to 20 mm respectively.

3. RESULTS AND DISCUSSION

3.1. Yield of the extracts (aqueous and ethanolic) obtained by maceration process

The aqueous extract demonstrated best extractive yield when compared with that of the ethanolic extract.

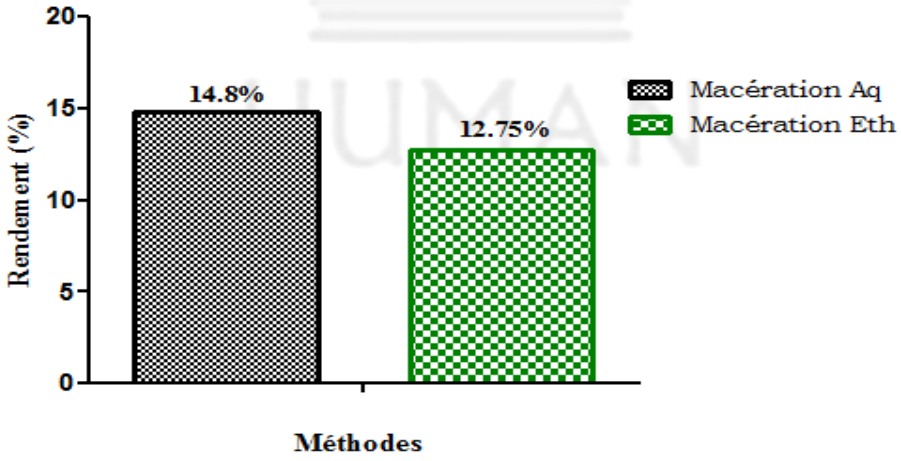

Figure 1: Percentage yield of extract for *T. glaucescens* by maceration

3.2. Total polyphenols content

The determination total polyphenols content in extracts of *T. glaucescens* was made by using the colorimetric method of Folin-Ciocalteux. The results showed that the ethanolic extract has a high content of total phenols (552 ± 4mg GAE/g) as compared to that of the aqueous extract (390.7 ± 1.8mg GAE/g) (Figure 3).

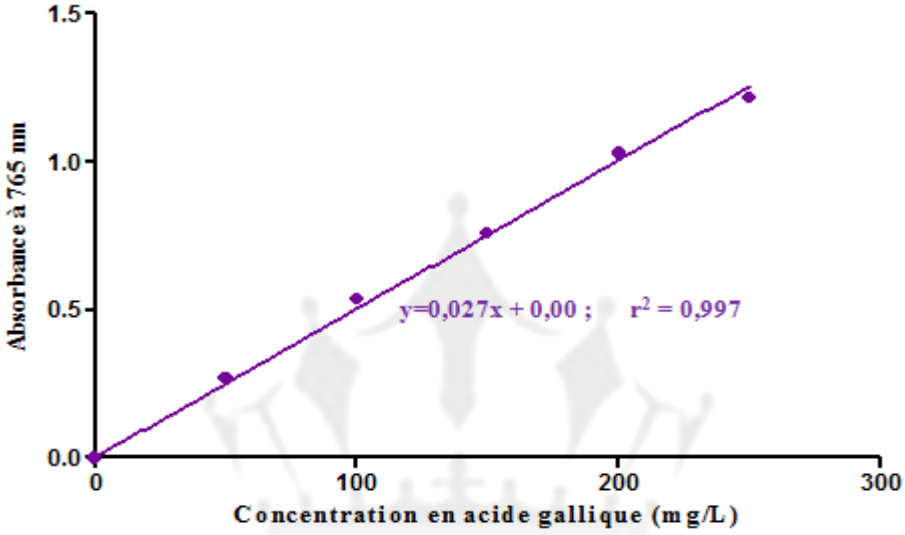

Figure 2: Calibration curve gallic acid (mean ± SD of three trials).

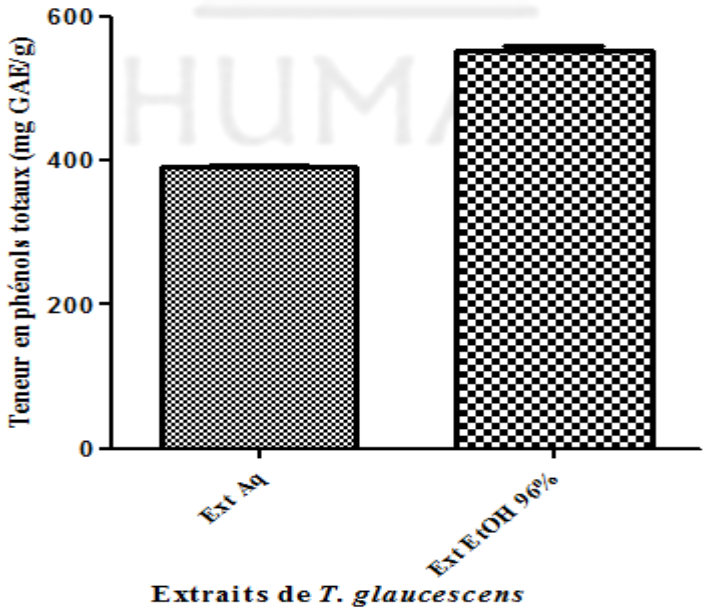

Figure 3: Total phenolics content of extracts (mean ± SD of three trials)

3.3. Flavonoid content

The results revealed that the aqueous extract is rich in flavonoids (42.87 ± 6mgQE/g) as compared to the ethanol extract (35.84 ± 2.7mgQE/g).

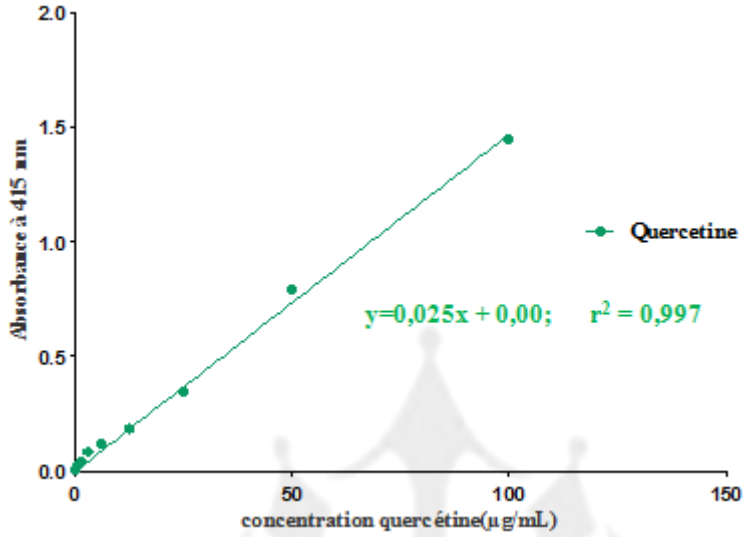

Figure 4: calibration line quercetin (mean ± SD of three trials).

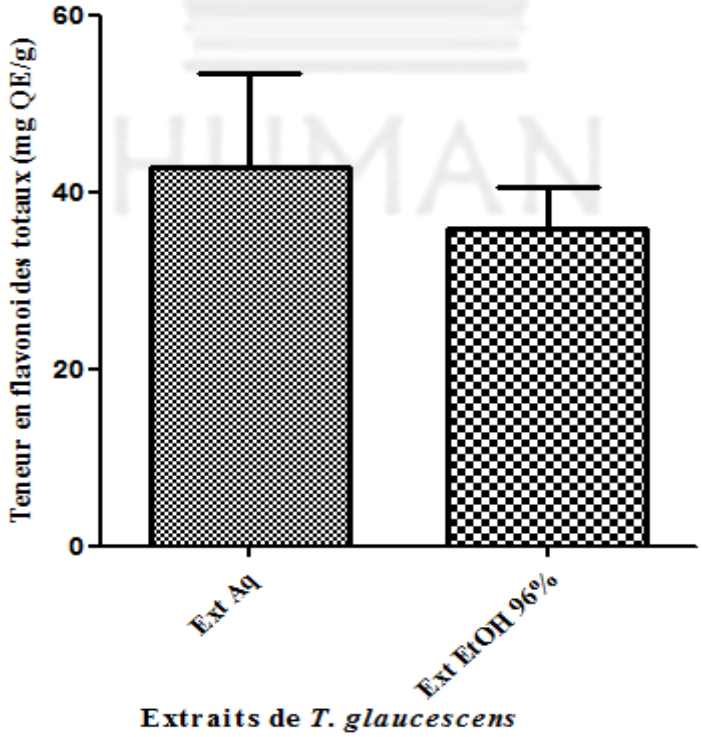

Figure 5: Content of Flavonoids (mean ± SD of three trials)

Table 2: Content of total phenols and flavonoids extracts *T. glaucescens*

	Phenols content (mg GAE/g)	Flavonoids content (mg QE/g)
96% ethanolic extract	552	35.84
aqueous extract	390.7	42.87

On the basis of content of phenols and flavonoids in both extracts of *T. glaucescens* (Table 2), we can conclude that flavonoids represent 6.49% of total phenols in the ethanolic extract. While composition is 10.97 percent in the aqueous extract.

3.4. Test of trapping free radical DPPH

The antioxidant activity of aqueous and ethanol extracts of *T. glaucescens* and antioxidant vitamin C (ascorbic acid) vis-a-vis the DPPH radical was evaluated using a spectrophotometer (bioMérieux). The reduction of this radical was traced by colorimetric method; that is passage from the violet color (DPPH•) to yellow color (DPPH-H) measurable at 517nm.
This reduction capacity is determined by a decrease in the absorbance induced by free radical inhibitors [14].

The results of antioxidant activity of the extracts showed that aqueous and 96% ethanolic extracts of the stem bark of *T. glaucescens* have a strong potential for scavenging the free radicals in concentration-dependent manner with IC_{50} values obtained for both 5.27g/ml and 12.8µg/ml respectively.. The IC_{50} value for vitamin C solution (1.87µg/ml) is taken as control (Figure 6).

These compounds also possess various biological activities such as antibacterial vasodilatory and anti-inflammatory, antiviral, anti-allergic, which may be related to their antioxidant activity [16].

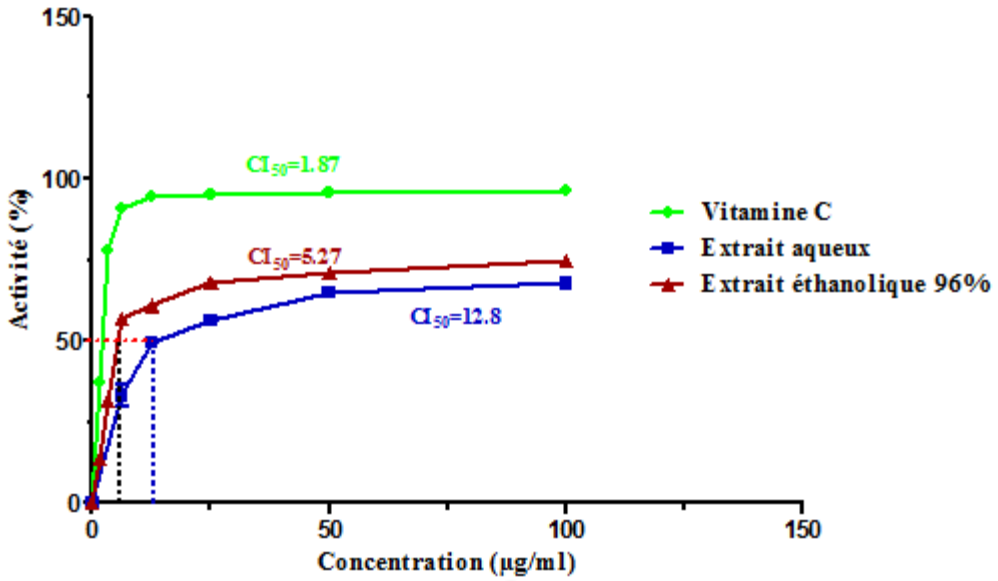

Figure 6: Comparative antiradical activity of extracts of *T. glaucescens* and vitamin C (mean ± SD of three trials).

3.5. Antibacterial activity

Antibacterial activity of the extracts of *T. glaucescens* has been analyzed using the method of diffusion in solid medium vis-a-vis the enterobacteriaceae extended-spectrum beta-lactamases producing and reference strains (Tables 3 and 4).

Table 3: Diameter (mm) of the zone of inhibition obtained with the total aqueous extract of *T. glaucescens* on strains.

		Concentration (mg/ml)			Beta-lactam (C_3G)	
Code	**Germs**	**C_1=200**	**C_2=100**	**Tm=0**	**CRO**	**CTX**
75C12	***E. cloacae***	14±0,6	13±0,6	6±0,0	12±0,0	13±0,0
234C12	***E. coli***	13,6±0,3	11,6±0,3	6±0,0	09±0,0	12±0,0
244C12	***E. aerogenes***	11,6±0,3	10±00	6±0,0	20±0,0	20±0,0
252C12	***K. pneumoniae***	13,6±0,3	12±00	6±0,0	13±0,0	10±0,0
265C12	***C. koseri***	19±1,1	17,6±1,4	6±0,0	06±0,0	06±0,0
25922	***E. coli* ATCC**	13±0,6	11,3±0,3	6±0,0	30±0,0	34±0,0
35218	***E. coli* ATCC**	19,3±0,6	16,3±1,1	6±0,0	42±0,0	48±0,0

Included diameter of the wells (6mm), C: concentration, Tm: control, CRO: Ceftriaxone, CTX: Cefotaxim, C_3G: Third-generation cephalosporin

Table 4: Diameter (mm) of the zone of inhibition obtained with the 96% ethanol extract of *T. glaucescens* on strains.

		Concentration (mg/ml)			Beta-lactamin (C_3G)	
Code	**Germes**	**C_1=200**	**C_2=100**	**Tm=0**	**CRO**	**CTX**
75C12	***E. cloacae***	14±0,6	11±0,6	6±0,0	12±0,0	13±0,0
234C12	***E. coli***	15,3±0,6	13,6±0,6	6±0,0	09±0,0	12±0,0
244C12	***E. aerogenes***	14±1,0	13±1,0	6±0,0	20±0,0	20±0,0
252C12	***K. pneumoniae***	15±1,0	14±1,0	6±0,0	13±0,0	10±0,0
265C12	***C. koseri***	23±4,3	21,3±4,2	6±0,0	06±0,0	06±0,0
25922	***E. coli* ATCC**	16±1,0	14,6±1,5	6±0,0	30±0,0	34±0,0
35218	***E. coli* ATCC**	16,3±0,6	15±0,0	6±0,0	42±0,0	48±0,0

Included diameter of the wells (6mm), C: concentration, Tm: control, CRO: Ceftriaxone, CTX: Cefotaxim, C_3G: Third-generation cephalosporin

The results of this study showed that the ethanolic extract of the stem bark of *T. glaucescens* has demonstrated the largest values for zone of inhibition over that of the aqueous extract. According to the method of diffusion in a solid medium, an extract is considered active if it induces an inhibition zone greater than or equal to 10mm [17]. Indeed, the extracts gave diameters superior to 10mm. The 96% ethanol extract has given diameters of the inhibition zone ranging from 14±0.6 to 23±4.3mm for 200mg/ml on ESBLE. Diameters described at 200mg/ml were 16±1.0 and 16.3±0.6mm, respectively, for *E. coli* ATCC 25922 and ATCC 35218. The diameters of the inhibition zone of the aqueous extract ranged from 11.6 ± 0.3 to 19 ± 1.1mm for the strain ESBLE and 13±0.6mm and 19.3±0.6mm respectively for *E. coli* ATCC 25922 and 35218 at 200mg/ml. These results corroborate those of Adebayo et al. [7] and Bolou [8] which have shown that the aqueous extract of the leaves of *T. glaucescens* inhibits in vitro the growth of various bacterial strains. The antibacterial activity of the extracts could be explained by the richness of the Combretaceae family in terms of different constituents including tannins, polyphenols and alkaloids which confer an antimicrobial activity [18, 19]. At 200 mg/ml, the ethanolic extract has

been more active on the ESBL strain than the tested third-generation cephalosporins (ceftriaxone (30µg), cefotaxime (30µg)).

CONCLUSION

The study of antioxidant activity of extracts of *T. glaucescens* showed that both aqueous and ethanol extracts possess an antioxidant activity. These extracts may therefore constitute an alternative to some synthetics additives. However, even though this activity is distinctly lower than that of the vitamin C, the crude extracts contain a large number of different compounds.
With regard to antibacterial activity, it appears that these extracts have antibacterial properties on the ESBL comparable to marketed antibiotics..

The high proportion of polyphenolic compounds in aqueous and ethanolic extracts of the stem bark of *T. glaucescens* shows that these compounds possess both antibacterial and antioxidant activities.

It is very likely that they contain compounds that, once purified, can present significantly greater activity than that of the reference standard. Further research is necessary to identify, isolate and purify the constituents.

ACKNOWLEDGMENT

The authors thank the Pharmacodynamic-Biochemical laboratory of the Felix Houphouet Boigny University (Côte d'Ivoire) and the Institut Pasteur of Côte d'Ivoire for their technical support in this work.

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