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Antioxidant and Antibacterial Activities of *Terminalia superba* Engl. and Diels (Combretaceae) Bark Extracts

Kougnimon Fifamè Espérance Elvire[1]*, Akpovi Dewanou Casimir[1], Dah-Nouvlessounon Durand[2], Boya Bawa[2], Baba Moussa Lamine[2] and Loko Frédéric[1]

[1]Laboratoire de Recherche en Biologie Appliquée (LARBA/ EPAC/UAC) 01BP 2009 Cotonou, Bénin
[2]Laboratoire de Biologie et de Typage Moléculaire en Microbiologie, Faculté des Sciences et Techniques, Université d'Abomey-Calavi, 05 BP 1604 Cotonou, Bénin

**Corresponding author*

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A B S T R A C T

Terminalia superba (*T. superba*) is locally used for the treatment of various diseases, including diabetes mellitus, gastroenteritis, female infertility, abdominal pains, bacterial, fungal and viral infections. This study aimed to ascertain the antioxidant and antimicrobial activities of ethanolic and hydro-ethanolic extracts of *T. superba* barks. Total phenols, flavonoids, and tannins were measured in the extracts by a spectrophotometry method. The DPPH method was used to evaluate extracts antioxidant activity. The antibacterial activity was evaluated using broth micro-dilution method *in vitro* on *Staphylococcus aureus* isolated clinical strains from three skin infections (buruli ulcer, furuncles and abscesses) and a *Staphylococcus aureus* reference strains (*Staphylococcus aureus* ATCC 29213*)*. Clinical strains were multi drug resistant with or without a virulence factor (Panton-Valentine Leukocidin (PVL).The phytochemical screening of ethanolic and hydro-ethanolic extracts of *T. Superba* barks revealed the presence of tannins (catechic and gallic), flavonoids, saponins, free anthracene derivatives, reducing compounds, mucilage. Ethanolic and hydro-ethanolic extracts showed antioxidant activities. Both extracts had antibacterial activities on *Staphylococcus aureus* ATCC 29213 and *S. aureus* isolated from skin infections. This study shows that *T. superba* has high antibacterial and antioxidant activities.

Introduction

Medicinal plants are recognized as the most popular form of alternative medicine (Ogbonnia *et al.*, 2011). Herbal prescriptions and natural remedies are commonly employed in developing countries for the treatment of various diseases, this practice being an alternative way to compensate for some perceived deficiencies in orthodox pharmacotherapy (Zhu *et al.*, 2002). In Benin, they offer a wider available and affordable alternative to pharmaceutical drugs and natural food supplements.

To face the increase in many microbial pathogens resistance against conventional antibiotics, it is essential to seek other drugs with wide spectrum anti-microbial activities. Therefore, research must be directed to biologically active extracts and compounds from plant species to fight microbial diseases (Chanda *et al.,* 2011). A high number of medicinal plants have been recognized as an important resource of natural antimicrobial compounds (Mahady, 2005). Moreover, many medicinal plants have an antioxidant activity that is attracting more and more the attention of several research teams for its role in the fight against several diseases such as cancer, atherosclerosis, cerebro-vascular condition, diabetes, hypertension, and Alzheimer's disease (Vârban *et al.,* 2009). Numerous physiological and biochemical processes produced oxygen-centered free radicals and other reactive oxygen species (Stankovic *et al.,* 2011). Antioxidants are capable of scavenging free radicals, which can oxidize many biological macromolecules (DNA, proteins, and lipids) in cells and tissues. Phytochemical compounds exhibit several activities such as antioxidant, anti-inflammatory, anti-hepatotoxic, anti-tumoral and anti-microbial (Zengin *et al.,* 2011).

This study is focused on *Terminalia superba* Engl. & Diels (Combretaceae) *which is* used by various traditional healers for the treatment of bacterial, fungal and viral infections. The main objective of this work is to perform a phytochemical screening in order to evaluate the phenolic composition and determine the potential anti-radical activity and antibacterial activity of two extracts of *T.superba*.

Materials and Methods

Plant material

The *T.superba* barks were collected in Itchèdé, Toffo Forest at Adja-Ouèrè (Benin). The identification of the plant was confirmed at the National Herbarium in Benin. The barks dried at 25°C were ground into a fine powder.

Microorganisms

The antimicrobial activity of *T. superba* extracts was tested against a standard strain (*Staphylococcus aureus* ATCC 29213) and *Staphylococcus aureus* strains isolated from various types of skin infections such as buruli ulcer, furuncles and abscesses. Sixteen (16) clinical strains were Multi Drug Resistant and produced Panton Valentine Leukocidin (PVL) virulence factor. Twenty-three (23) clinical strains were Multi Drug Resistant without Panton Valentine Leukocidin (PVL) virulence factor (Sina *et al.,* 2013). The tested strains were initially cultured in Muller-Hinton broth containing 20% glycerol and were stored at -80˚C.These microorganisms were obtained from the Laboratory of Biology and Molecular Typing in Microbiology (University of Abomey-Calavi, Benin, West Africa).

Preparation of crude extracts

The extracts were prepared according to the method described by Talbi *et al.,* (2015). Fifty grams (50 g) of powder were dissolved in 500 ml ethanol 96% (ethanolic extract) and 500 ml ethanol 70% (hydro-ethanolic extract). Seventy-two hours (72h) after, the macerate was filtered with hydrophilic cotton and Whatman filter paper and was evaporated to dryness at 40°C using a Rotavapor. The resulting powder was stored in a refrigerator at 8°C until use.

Phytochemical analysis

Qualitative phytochemical screening of *T.superba* was carried out on the extracts (ethanolic and hydro-ethanolic), using the standardly employed precipitation and coloration reactions as described by Houghton and Raman (1998).

Antioxidant activity: DPPH free radical scavenging assay

The quantitative evaluation of antioxidant activity was based on the methodology proposed by Brand-Williams *et al.,* (1995). DPPH nitrogen radical scavenging assay was performed based on reduction of 2, 2-diphenyl-1-picrylhydrazl (DPPH) recorded at 517 nm according to a standard method. Ascorbic acid was used as standard. Percentage of inhibition/ scavenging (% AA) was calculated by the following formula:

$$(\%\ AA) = \frac{\text{Awhite} - \text{A sample}}{\text{A white}} \text{X100}$$

Where, A white is the absorbance of the white reaction mixture, and

A sample is the absorbance of the sample.

The percentage of inhibition of DPPH was plotted against the extracts concentration to obtain IC_{50}. IC_{50} is the concentration of extracts which can decrease the initial DPPH concentration by 50 %. Lower IC_{50} value shows higher radical scavenging activity.

Antibacterial activity of ethanolic and hydro-ethanolic extracts of *T. superba*

Antimicrobial susceptibility testing

Antimicrobial susceptibility was evaluated using the Kirbye-Bauer disk diffusion method on agar Mueller-Hinton (bioMérieux, Marcy l'Etoile, France) in accordance with The Clinical and Laboratory Standards Institute [CLSI] (2015), the following 18 antimicrobial agents were tested: Penicllin G (6µg), oxacillin (5µg), Ofloxacin (5µg), cefoxitin (30µg), gentamicin (10µg), tobramycin (10µg), kanamycin (30µg), vancomycin (5µg), teicoplanin (15µg), fusidic acid (10µg), fosfomycin (50µg), rifampicin (5µg), trimethoprim/ sulfamethoxazole (1.25/ 23.75µg), erythromycin (15µg), lincomycin (30µg), pristinamycin (15µg), linezolid (30µg) and tetracyclin (30 µg).

Panton-Valentine Leukocidin (PVL) identification

All *S. aureus* isolates were investigated for the carriage of PVL. For the phenotypic detection of toxins radial gel immunodiffusion was performed. The production of Panton-Valentine Leukocidin (PVL) were evidenced from culture supernatants after 18 h of growth in Yeast Casamino-acid Pyruvate (YCP) medium (Gauduchon *et al.,* 2001) by radial gel immunodiffusion in 0.6% (wt/vol) agarose with component-specific rabbit polyclonal and affinity-purified antibodies (Prévost *et al.,* 1995 Gravet *et al.,* 1998).

Determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of *T.superba* stem bark extracts

Hydro-ethanolic and ethanolic extracts of *T. superba* bark were reconstituted in distilled water at 80 mg/ml. The prepared solutions were sterilized by filtration using filter-syringes on 0.22 µm Millipore membrane. The sterility of the stock solutions was verified by culturing aliquots of each solution on Mueller Hinton media, incubated at 37°C for 24 up to 48 hours.

The microdilution method (quantitative activity) was used for the determination of MIC. The MIC was defined as the lowest concentration of extracts inhibiting visible bacterial growth after 18 or 20 h incubation at 37°C (Kouitcheu *et al.,* 2013). Into each well, 100 µL of broth Muller Hinton enriched with glucose solution 1% and 5% red phenol solution was added. Then, 100 µL of each extract was added in every first well of the microplate.

Geometric dilutions ranging from 80 to 0.078 mg/ml were carried out and subsequently, 100µL of media containing 10^6UFC/ml of the indicator strain was added to all wells to yield 40 to 0.039 mg/ml of concentration. The plates were then incubated at 37°C for 24 h. The experiment was done in triplicate. A color change from red to yellow was indicative of bacterial growth.

To obtain the MBC, 20 µL of each well colored red was spotted on Muller Hinton agar and incubated at 37°C for 24 h. The MBC were determined by the minimum concentration that allowed less than 0.01% of bacterial growth.

The antibiotic power (AP) of each extract was thereafter calculated with the formula CMB/CMI. According to the values obtained in inhibition tests, the extracts was classified as bactericidal when MBC/MIC $\leq$ 4 and bacteriostatic when MBC/MIC >4

Statistical analysis

The data were analyzed with excel and SPSS 17 software. Calibration curves were carried out with Excel. Means and standard deviations were determined. For the comparison of the variances and means of the different modalities, the Student Levene and *t* tests were used respectively after verification of normality (Skewness and Kurtosis).

In the case where the normality hypothesis is not verified, a non-parametric test has been carried out in order to compare the modalities of variables. *P values*<0.05 were significant.

Results and Discussion

Phytochemical screening

Table 1 illustrated the phytochemical screening of *T. superba* bark crude extracts (ethanol, hydro-ethanol).

Qualitative analysis of *T.superba* barks crude extracts (ethanol, hydro-ethanol) reveals various phytochemicals compounds: tannins (Cathetic, Gallic), flavonoids, saponins, free anthracenics, reducing compounds and mucilage.

Previous phytochemical studies on *T.superba* bark identified several compounds, including tannins, flavonoids (Dongmo *et al.*, 2006; Kouakou *et al.*, 2013; Goze *et al.*, 2014; Ahon *et al.*, 2011), saponosides (Kouakou *et al.*, 2013; Goze *et al.*, 2014; Ahon *et al.*, 2011) and many reducing compounds (Kouakou *et al.*, 2013; Goze *et al.*, 2014).

Contrary to our findings, coumarins and quinone (Kouakou *et al.*, 2013; Goze *et al.*, 2014), triterpenoids and sterols (Kouakou *et al.*, 2013; Goze *et al.*, 2014; Ahon *et al.*, 2011), alkaloids (Ahon *et al.*, 2011) were reported in *T. superb* bark. The environment, periods of harvest of organs, stocking conditions of organs and extract solvents may influence the synthesis and expression of phytochemical components in the plant (Sauvion *et al.*, 2013).

Antioxidant activity

Table 2 shows IC_{50} of *T.superba* extracts and standards.

The hydro-ethanolic extract (IC_{50} = 11.60 µg/ml) has a higher DPPH free radical-scavenging activity than the ethanolic extract (IC_{50} = 34.72 µg/ml); however, the scavenging activity of the hydro-ethanolic extract is lower than that of the antioxidant standard ascorbic acid (9.62 µg/ml).The IC_{50} of the hydro-ethanolic extract is 2.99-fold lower than that of ethanolic extract and 1.20-fold higher than that of ascorbic acid. The free radical-scavenging capacity of the hydro-ethanolic extract of *T. superba* bark is higher than that of the ethanolic extract. This can be attributed

to the higher concentration of phenolic compounds in the hydro-ethanolic extract of *T. superba* barks. Previous reports showed a correlation between the antioxidant activity and the amount of total phenolic content (Negro *et al.*, 2003). Phenolic compounds are hydrogen donors capable of directly scavenging free radicals and reducing oxidative damage (Wintola *et al.*, 2015). In other medicinal plant extracts, these compounds also activated endogenous antioxidant systems and inhibited the lipid per oxidation of human erythrocytes (Ribeiro *et al.*, 2015). The antioxidant activity of *T. superba* bark was also determined by Momo *et al.*, (2009).

Table.1 Phytochemical analysis *T. superba* bark extracts (ethanolic and hydro-ethanolic)

Chemical groups			EE	HEE
Alkaloids			-	-
Tannins	Catechin tannins		++	+++
	Gallic tannins		++	+++
Flavonoids			+	++
Anthocyanins			-	-
Leucoanthocyanins			-	-
Coumarin			-	-
Quinone derivatives			-	-
Mucilages			++	++
Reducting compound			++	++
Saponins			+ H= 1.5 cm IM= 4/10	+ H = 1.9cm IM= 4/10
Triterpenes			-	-
Steroids			-	-
Cyanogenic derivate			-	-
Anthracenics derivate	Free anthracenic		+	+
	Combine anthracenic	o-hétérosides	-	-
		c- hétérosides	-	-
Cardiotonic glycoside			-	-

-: absence; +: present in low concentration; ++: present in moderate concentration; +++ present in high concentrations; EE: ethanolic extract; HEE: Hydro-ethanolic extract, H: Height of the foam; IM: Foam Index.

Table.2 IC_{50} of the ethanolic and hydro-ethanolic extracts of *T.superba barks* compared with the antioxidant standards ascorbic acid

	IC_{50} (µg/ml)	Calibration curve	R^2
EE	34.72	Y= 18,80 ln (x) -16,69	0.947
HEE	11.60	Y= 14,27 ln (x) + 14,63	0.935
AA	9.62	Y= 14,16 ln (x) -17,91	0.885

EE = Ethanolic extract; HEE= Hydro-ethanolic extract; AA = Ascorbic acid; IC_{50}: Concentration of extracts can inhibit 50% of DPPH radicals

Table.3 Antimicrobial susceptibility

Drug(s)	**PVL + (n = 16)**	**PVL – (n= 23)**	**Total (n= 39)**	**P**
	Number (%)	Number (%)	Number (%)	
Fusidicacid (10µg)	0	0	0	-
Cefoxitin (30µg)	5 (31,25)	6 (26,09)	11 (28,21)	0,725
Erythromycin (15µg)	12 (75)	14 (60,87)	26 (67,67)	0,357
Fosfomycin (50µg)	0	0	0	-
Gentamicin (10µg)	6 (37,5)	16 (69,57)	22 (56,41)	0,047
Kanamycin (30µg)	12 (75)	8 (34,78)	20 (51,28)	0,013
Lincomycin (15µg)	5 (31,25)	7 (30,43)	12 (30,77)	0,957
Linezolid (10µg)	0	0	0	-
Ofloxacin (5µg)	4 (25)	6 (26)	10 (26)	0,939
Oxacillin (5µg)	13 (81,25)	6(26,09)	19 (48,71)	0,001
PenicllinGG (6µg)	16 (100)	23 (100)	39 (100)	-
Pristinamycin (15µg)	4 (25)	5 (22)	9 (23)	0,812
Rifampicin (5 µg)	4 (25)	11 (47,83)	15 (38,46)	0,150
Teicoplanin (15µg)	2 (12,5)	4 (17,39)	6(15,38)	0,677
Tetracyclin (30 µg)	3 (18,75)	5(21,74)	8 (20,51)	0,820
Tobramycin (10µg)	9(56,25)	4(17,39)	13(33,33)	0,011
Trimethoprim/sulfamethoxazole (1,25/23,75 µg)	7 (43,75)	10 (43,48)	17 (43,59)	0,987
Vancomycin (5µg)	0	0	0	-

Table.4 Comparative study of the bactericidal activity of ethanolic and hydroethanolic extracts on reference strain

	EE			HEE		
	MIC	MBC	AP	MIC	MBC	AP
S.aureus ATCC 29213	0.078	0.078	1	0.078	0.078	1

S. aureus ATCC 29213= *Staphylococcus aureus* ATCC 29213; MIC = Minimal Inhibitory Concentration (mg/ml); MBC = Minimal Bactericidal Concentration (mg/ml);AP (CMB/CMI) = antibiotic power; EE: ethanolic extract; HEE: hydro-ethanolic extract.

Table.5 Comparative study of the bactericidal activity of ethanolic and hydroethanolic extracts on LPV + strains

	EE			HEE		
S. aureus PVL+	MIC	MBC	AP	MIC	MBC	AP
1	1.25	2.5	2	0.625	0.625	1
2	1.25	2.5	2	0.625	0.625	1
3	1.25	5	4	0.625	0.625	1
4	1.25	10	**8***	0.625	1.25	2
5	1.25	10	**8***	0.312	0.625	2
6	1.25	10	**8***	0.312	0.625	2
7	1.25	2.5	2	0.312	0.625	2
8	1.25	5	4	0.312	0.625	2
9	0.625	5	**8***	0.312	0.625	2
10	0.625	5	**8***	0.312	0.625	2
11	0.625	5	**8***	0.312	2.5	**8***
12	0.625	2.5	4	0.312	0.625	2
13	2.5	20	**8***	0.312	1.25	4
14	1.25	20	**16***	0.625	5	**8***
15	2.5	10	4	0.312	0.312	1
16	1.25	20	**16***	0.312	2.5	**8***
p= 0.028						

MIC = Minimal Inhibitory Concentration (mg/ml); MBC = Minimal Bactericidal Concentration (mg/ml); AP (CMB/CMI) = antibiotic power; AP without* = bactericidal power; AP With * = bacteriostatic power; *S. aureus*P VL^{+} =PVL positive *Staphylococcus aureus*; EE: ethanolic extract; HEE: hydro-ethanolic extract.

Table.6 Comparative study of the bactericidal activity of ethanolic and hydroethanolic extracts on LPV- strains

S. aureus MR/PVL-	EE			HEE		
	MIC	MBC	AP	MIC	MBC	AP
1	1.25	10	**8***	0.312	2.5	**8***
2	1.25	1.25	1	0.312	0.625	2
3	1.25	2.5	2	0.312	0.625	2
4	0.625	5	**8***	0.312	0.625	2
5	0.625	1.25	2	0.312	0.625	2
6	0.625	1.25	2	0.312	0.625	2
7	0.625	5	**8***	0.312	2.5	**8***
8	0.625	5	**8***	0.312	0.625	2
9	0.625	5	**8***	0.312	0.312	1
10	0.312	0.625	2	0.312	0.312	1
11	0.312	0.625	2	0.312	0.312	1
12	0.312	2.5	**8***	0.312	0.312	1
13	0.312	5	**16***	0.312	2.5	**8***
14	0.312	2.5	**8***	0.312	0.312	1
15	0.312	0.625	2	0.312	0.312	1

16	0.312	0.625	2	0.312	0.312	1
17	0.312	2.5	**8***	0.156	0.312	2
18	0.312	2.5	**8***	0.156	1.25	**8***
19	0.312	1.25	4	0.156	0.312	2
20	0.312	1.25	4	0.156	0.312	2
21	0.312	1.25	4	0.156	0.312	2
22	0.312	2.5	**8***	0.156	1.25	**8***
23	0.312	2.5	**8***	0.156	0.312	2
p= 0,032						

MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration; AP (CMB/CMI)= antibiotic power; AP without* = bactericidal power; AP with* = bacteriostatic power;*S. aureus*MR/PVL$^-$ = PVL negative multi-resistant *Staphylococcus aureus*; EE: ethanolic extract; HEE: hydro-ethanolic extract.

Antibacterial activity of ethanolic and hydro-ethanolic extracts of *T. superb* on the clinical *S. aureus* strains and the reference strains

Table 3 shows the antimicrobial Susceptibility Panton-Valentine Leukocidin is present in 16 S. aureus isolates.

There is a wide range in the susceptibility of the isolates to the various antibiotics examined. All of the strains are resistant to benzyl penicillin, while other antibiotics (vancomycin, fusidic acid, fosfomycin, and linezolid) are active against some of the strains (Table 3).

Tables 4, 5 and 6 show the comparative study of the antibacterial activity of ethanolic and hydro-ethanolic extracts on the clinical and reference strains. The minimum inhibitory concentration (MIC) values of both extracts against *Staphylococcus aureus* ATCC 29213 (reference strain) is 0.078 mg/ml.

The minimum bactericidal concentration (MBC) values of both extracts observed against *Staphylococcus aureus* ATCC 29213 (reference strain) is 0.078 mg/ml. AP (MBC/MIC)=1 for both *T. superb* extracts. Results shows that both ethanolic extract and hydro-ethanolic extract are bactericidal on the reference strain (Table 4). The minimum inhibitory concentration (MIC) values of ethanolic extract against *Staphylococcus aureus* PVL$^+$ strains ranged from 0.625 to 2.5 mg/ml. The minimum inhibitory concentration (MIC) values of hydro-ethanolic extract against *Staphylococcus aureus* PVL$^+$ strains ranged from 0.312 to 0.625 mg/ml.

Ethanolic extract is bacteriostatic on 9 (56.25%) *S.aureus* PVL + strains and is bactericidal on 7 (43.75%) *S.aureus* PVL+ strains.

Hydro-ethanolic extract is bacteriostatic on 3 (18.75%) *S.aureus* PVL+ strains and is bactericidal on 13 (81.25%) *S.aureus* PVL+ strains. Hydro-ethanolic extract is more significantly more bactericidal than ethanolic extract **(**p=0.028) (Table 5).

The minimum inhibitory concentration (MIC) values of ethanolic extract observed against *Staphylococcus aureus* PVL$^-$ strains ranged from 0.312 to 1.25 mg/ml. The minimum inhibitory concentration (MIC) values of hydro-ethanolic extract observed against *Staphylococcus aureus* PVL$^+$ strains ranged from 0.156 to 0.312 mg/ml.

Ethanolic extract is bacteriostatic on 12

(52.17%) *Staphylococcus aureus* PVL - strains and is bactericidal on 11 (47.83%) *Staphylococcus aureus* PVL -strains. Hydro-ethanolic extract is bacteriostatic on 5 (21.74%) *Staphylococcus aureus* PVL- strains and is bactericidal on 18 (78.26%) *Staphylococcus aureus* PVL- strains. Hydro-ethanolic extract is significantly more bactericidal than ethanolic extract (p= 0.032) (Table 6).

T. superba bark extracts (ethanolic and hydro-ethanolic) possessed bactericidal activity. Flavonoids, saponins and tannins were reported to have antimicrobial activity. Phenolic compounds also have antimicrobial activity via several mechanisms, including adsorption and disruption of microbial membranes, ion deprivation, enzyme interaction, and interaction with membrane transporters (Favela-Herna Ândez *et al.*, 2015;Cowan, 1999).Previous studies have indicated that *T.superba* has antimicrobial activities against several microorganisms (Kuete *et al.*, 2010; Tabopda *et al.*, 2009; Kra *et al.*, 2015; Ahon *et al.*, 2011).

In conclusion, *T. superba* contains chemical molecules such as tannins (catechics, gallic), alkaloids, flavonoids, saponosides, mucilages, reducing compounds, free anthracenics. These active ingredients are used in the treatment of certain human pathologies. We showed here that *T. superb* extracts had antibacterial activities on *Staphylococcus aureus* ATCC 29213 and *S. aureus* isolated from skin infections indicating that *T. superba* is a potential source of natural antioxidants and antimicrobials compounds.

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