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SCREENING PHYTOCHEMICAL AND ANTI-METHICILLIN RESISTANT (MRSA) ACTIVITY OF 70 % ETHANOLIC EXTRACT FROM THE STEM BARK OF *ALBIZIA LEBBECK* (L.) BENTH.(FABACEAE)

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ABSTRACT

The emergence of multi-drug resistant strains and limitations of present antimicrobial drugs have led to continuous search for natural products as curative agents for Anti-methicillin resistantinfections. The aim of this study was to evaluate antibacterial activity of an ethanolic extract from *Albizia lebbeck*stem bark against Anti-methicillin resistant. Methods and Results : The methods of dissemination swab on muller-hinton agar and double dilution were used to evaluate the antibacterial activity of 70 % ethanolic extract of stem bark of *Albizia lebbeck*.All multi-resistant strains of *Staphylococcus aureus* and the reference strain (ATCC 25923) were sensitive to 70 % ethanolic extract of the stem bark of *Albizia lebbeck*. The MBCvary from 0,49 mg/mL to 2mg/mL. Also, the phytochemical screening of this extract revealed the presence of Polyphenols, Gallic tannins, Catechin tanninsand Flavonoids. These findings confirm that an 70 % ethanolic extract from *Albizia lebbeck* stem bark inhibited growth of Anti-methicillin resistant at low concentration and could be utilised as an alternative Anti-methicillin resistant agent.

Keywords: *Albizia lebbeck*, Anti-methicillin resistant, 70 % ethanolic extract, MBC.

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INTRODUCTION

In the 1950s, methicillin was introduced to treat *S. aureus* infections. Unfortunately, after several years, resistance of *S. aureus* to methicillin was discovered [1]. Methicillin is a β-lactam antibiotic that interferes the penicillin-binding proteins needed for synthesis of peptidoglycans for *S. aureus* [2]. The emergence of MRSA infections cannot be underestimated, as treatments are ineffective and it is associated with increased morbidity, mortality, hospital admissions and healthcare costs [3]. MRSA also shows high resistance rates against tetracycline, clindamycin, cotrimoxazole, rifampicin, macrolides and fluoroquinolones [4].This antibiotic resistance in pathogenic microorganisms has caused a lot of premature deaths and has become a public health problem worldwide [5]. Many cases of multidrugresistance have been reported in Ivory Coast [6]. Therefore, various studies have been carried out to identify alternative treatments to curb the problem of MRSA resistance, especially the use of natural products. Plants offer a diverse reservoir of biologically active components as potential therapeutic agents. They could also have a significant clinical value in the treatment of infections caused by microbial resistant strains [7]. Thus an ethnobotanical sturdy was conducted in the Haut-Sassandra Region (Ivory Coast), we found *Albizia lebbeck*, a plant widely used in the treatment of skin diseases. Previous work has shown tha *Albizia lebbeck* is used in the treatment of various disseases : Furuncle,

cough, conjunctivitis, influenza, abdominal tumor[8], depression[9].The main objective of this study is to perform a phytochemical screening and to evaluate the anti-methicillin resistant activity of the 70% ethanolic extract of *Albizia lebbeck* stem bark on the *in vitro* growth of five clinical strains of *Staphylococcus aureus*.

MATERIAL AND METHODS

Figure 1 : *Albizia lebbeck* (L) Benth. (Fabaceae)

A: Pod; B: Leaf; C: Flower

Bacterial Material

Made up of a reference strain (ATCC 25923) and five multiresistant strains of *Staphylococcusaureus* obtained from biological products (Table 1). They are provided by the Antibiotics Unit, Natural Substances and Monitoring of Microorganisms for Anti-Infective (ASSURMI) and the Department of Bacteriology and Virology of the Pasteur Institute of Ivory Coast (IPCI).

Plant Material

Going by our ethnobotanical investigation in Haut-Sassandra Region (Ivory Coast), it appears that *Albizia lebbeck* plant is widely used in the treatment of microbial diseases. The stem bar kwas harvested, cut, washed with water and dried under the shade. These dried plant part, using a grinder were then reduced to a fine powder.

Table 1 : List of strains sturdied

Strain	Codes	Profile	Biological products
Staphylococcus aureus	ATCC 25923	Sensitive to methicillin	-
	377 CA/15	Methicillin resistant	Pus
	310 CA/15	Methicillin resistant	Urine
	1541 C/14	Methicillin resistant	Urine
	412 C/14	Methicillin resistant	Pus
	505 PP/15	Methicillin resistant	Pus

Preparation of the aqueous total extract (ATE)

One hundred grams (100 g) of stem bark powder were homogenized in 1 liter of distilled water in a blender (mixer) for three times three minutes at room temperature. The homogenate was first squeezed in a square of clean white cloth (the mac was placed again in the blender to repeat the operation, it's an extraction by exhaustion) and then filtered through cotton wool, and finally on Whatman paper. Using an oven set at 50°C, the extraction solvent was removed. The evaporate was recovered in the form of dry powder and constituted the aqueous total extract (ATE) [10, 11].

Preparation of 70% Ethanolic Extract (70% FE)

The extract was obtained by dissolving 5 g of TAE in 100 mL of 70% ethanolic (67.2 mL of pure 96% ethanolic for 28.8 mL of distilled water) solution and then homogenised. After decantation and filtration of the alcoholic fraction on hydrophilic cotton and on Whatman filter paper (n°3), the filtrate collected is evaporated in an oven at 50°C. The powder obtained constitutes 70% ethanolic extract (70% EE) [11].

Effectivenes test

The strain sensitivity to 70 % ethanolic extract of *Albizia lebbeck* was performed using the agar diffusion method. Mueller Hinton agar were inoculated with a swab. A total of 4 wells of 6 mm in diameter were then made in the agar, of which 1 served as control well in the center of the agar and containing only sterile distilled water (TS). Each of the three wells received 50 µl of the test substance into the concentrations of 100, 50 and 25 mg/ml (C_1, C_2 and C_3 respectively). After 30 min diffusion at laboratory temperature, the plates were incubated at 37°C for 24 h. The presence or absenceof inhibition zone was observed and measured with a caliper or ruler in millimeters (mm). The results are expressed as the diameter of inhibition zone. Therefore, according to the sensitivity of strain, we have strains that are[12]:

- Not susceptible or resistant: diameter less than 8 mm;
- Susceptible: diameter between 9 and 14 mm;
- Very sensitive: diameter between 15 and 19 mm,
- Extremely sensitive: diameter greater than 20 mm.

Preparation of the concentration range of plant extracts

The range of concentration of plant extract was prepared in twelve test tubesnumbered T_1 to T_7 by the method of double dilution in geometrical ratio 1/2. The concentrations ranged from C_1 = 100 to C_7 = 1,56 mg/mL.

Preparation of the inoculum

The bacterial inoculum was prepared from colonies of less than 18-24 h in Mueller Hinton broth (BMH). A single colony of the bacterial culture was removed using a platinum loop and homogenized in 10 ml broth and incubated for 3 h at 37°C for a pre-culture. After incubation, a volume of 0.3 ml was taken and was added to 10 mL of sterile BMH. This made up bacterial suspension valued at approximately 106 cells/ml and constituted 100 dilusion or the pure inoculum.

Counting of bacterial inoculum

The counting of the inoculum was performed by dilution from 10 to 10 from the pure inoculum (100) until the 10^{-4} dilution. We obtained 4 dilutions 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. These various dilutions and the pure inoculum were inoculated with a calibrated loop of 2 µl per striae of 5 cm long on Mueller Hinton agar and incubated at 37°C for 24 h. This preparation constituted box A.

Determination of the minimum inhibitory concentration (MIC)

The MIC determination was made using two 96 well microplates for each series. In a series of wells numbered from C_1 to C_7 (in microplate) and each of the six strains (S_1-S_6) were added 0.2 mL of pure inoculum of each bacterial strain of *Staphylococcus aureus*. The TS (Sterility Control), contains 0.4 ml of sterile BMH. Then it was added to the wells (S_1 to S_6), 0.2 ml of plant extract according to the prepared concentration range. This distribution of plant extract was made such that 0.2 ml of plant extract (70 % EE) of 200 mg/mL was transferred into the wells C_1; C_2 wells received 0.2 mL of 100 mg/mL. TC well (Control well) received 0.2 ml of sterile BMH and 0.2 ml of sterile distilled water. Due to the volume / volume dilutionthus formed, the concentration in the wells were halved. Thus, the final concentrations in the wells were evolving C_1 = 100 mg/mL to C_7 = 1.563 mg/mL in the microplate. The plate was incubated at 37 ° C for 24 h. The MIC (Minimum Inhibitory Concentration) is the lowest concentration of extract for which no bacterial growth was observed after 24 hours of incubation time. It's determination was made by observing the turbidity induced by the growth of studied germs in each tube. The MIC was the lowest concentration value for which there was no germs growth visible to the naked eyes.

Determination of minimal bactericidal concentration (MBC)

The minimal bactericidal concentration (MBC) is the lowest concentration of antibactrial agent that leaves at most 0.01% of surviving germs. Using a calibrated loop 2 µl, the tube contents in which no germs was observed were collected and seeded on Mueller-Hinton agar starting with the MIC tube. Seeding was done by parallel stripes of 5 cm long on the surface of the agar. This constitute the box B. After 24 h of incubation in an incubator at 37 ° C the number of colonies on the streaks was compared to those of the box of the inoculum. Thus, the first experimental tube, of which germs count on the streak is less than or equal to 10^{-4} dilution represent the MBC.

Modalityof 70 % ethanolic extract action

The MBC / MICration is used to specify the modality of a substance [13].

If the result:

- MBC / MIC ≤ 2, the substance is said to be bactericidal
- MBC / MIC > 2, the substance is said to be bacteriostatic.

Phytochimical Screening

The identification of different chemicalcompounds in 70 % ethanolic was done by tubescharacterisation reactions. This method consistsof detecting the different families of chemicalcompounds that may exist in plant extracts onthe basis of characteristic colourations or precipitation reactions [14].

- **Alkaloids characterisation**

The characterisation of alkaloids was made usingBouchard (iodo-iodide) and Dragendorff(tetraiodo potassium bismuthate) reagent. 6 mLof 70% ethanolic extract solution was evaporatedto dryness. The residue was taken up in 6 mL ofalcohol at 60°C. The filtrate thus obtained wasdivided into two test tubes. In the first tube, twodrops Dragendorff reagent were added.The presence of alkaloids was characterisedby observing orange-coloured precipitates.In the second tube, two drops of Bouchardreagents was added. The appearance of areddish-brown colour indicates the presenceof alkaloids. A control test was made withquinine.

- **Characterisation of polyphenols**

The polyphenols colorimetry forms colouredprecipitate with a solution of ferric chloride(FeCl3). Thus, one drop of alcoholic solution of2% ferric chloride and 2 mL of solution of 70 %ethanolic extract was added. The formation ofblue-black or green colouring more or less darkconfirms to the presence of polyphenols. Acontrol test was performed with a solution ofphenol.

- **Characterisation of flavonoids**

Flavonoids have been characterised by thereaction to cyanidin. Thus, 2 mL of 70% ethanolicextract were evaporated to the dry sand bath.The residue thus obtained was mixed with 5 mL dilute hydrochloric acid 2 times. The mixture wascollected in a test tube, in which pink-orange orviolet colouration will appear. The addition of 3drops of isoamyl alcohol intensifies this colouringand confirms the presence of flavonoids. Analcoholic solution of quercetin was used as acontrol.

- **Tannins characterisation**

The Stiasny reagent (Formalin 30%,concentrated HCl 1/0.5) helped to distinguish thecatechin tannins (by precipitation) of gallictannins (by saturation). Tannins cathéchiques: to10 mg of 70% ethanolic extract, were added 10mL of Stiasny reagent. The mixture was heatedin a water bath at 80°C for 30 minutes. Aftercooling in a stream of water, observation ofprecipitate in the form of clear-brown flakescharacterises catechin tannins. An alcoholicsolution of catechin was used as a control. Gallictannins: For this test, the filtrate obtained fromthe reaction of catechol tannins characterisationwas saturated with sodium acetate. To thismixture was added a few drops of a diluteaqueous solution of FeCl3 at 1% (approximately1 mL). The appearance of an intense blue-blackcolouration indicates the presence of gallictannins not precipitated by Stiasny reagent. Analcoholic solution of gallic acid was used as acontrol.

- **Terpenes characterisation**

Sterols and terpenes characterisation was madeby the Liebermann-Burchard reaction. To 0.2 g of70 % ethanolic extract, were added 5 mL of ethylether, then the mixture was macerated for 30minutes. The solution obtained after themaceration was filtered and then evaporated todryness. The residue was then dissolved in 0.5mL of acetic anhydride. Using a pipette, 2 mL ofconcentrated sulfuric acid were laid down at thebottom of the test tube without stirring. Theappearance of brownish red or purple ringreflects the two liquid contact zone. The upperliquid turns green or purple to green or purpleindicating the presence of sterols and terpenes.A control test was performed with progesterone.

- **Coumarins characterisation**

For the detection of coumarins, 2 mg of 70 %ethanolic extract was added to 2 mL of warmwater and then homogenised. The homogenatethus obtained was divided into two test tubeThere after, 0.5 mL of diluted ammonia at 25%was added to the contents of one of the tubes.After observation under UV 365 nm, thepresence of fluorescence in the tube whereammoniac was added indicates the presence ofcoumarins.

RESULTS

Effectiveness test

The diameters of the inhibition zones are reported in figure 2, figure 3 and figure 4. It is noted that 70 % ethanolic extract had a good inhibitory activity, with different concentrations tested on bacterial strains. Having diameter of inhibition ranging from 9 ±0.57 to 17,0±0.57 mg/mL.

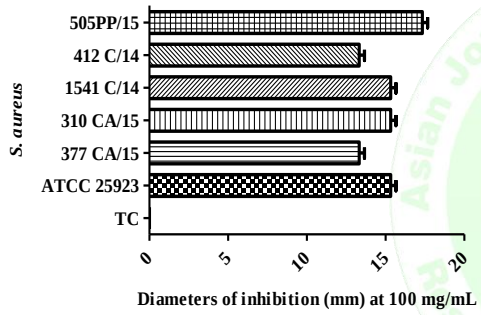

Figure 2: Dose-response action of 70%ethanolic extract of *Albizia lebbeck* stem bark*on Staphylococcus aureus.* Data **expressed as mean ± ecart-type (n=3)**

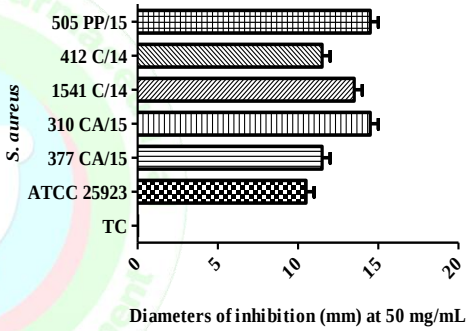

Figure 3: Dose-response action of 70%ethanolic extract of *Albizia lebbeck* stem bark*on Staphylococcus aureus.***Data expressed as mean ± ecart-type (n=3)**

TC : witness control

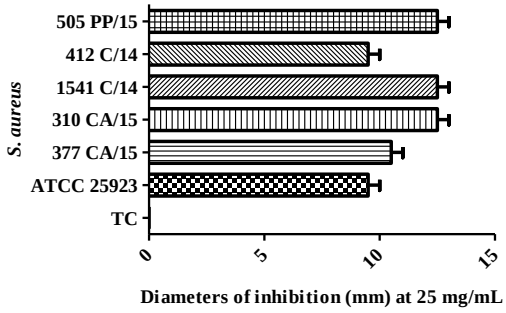

Figure 4: Dose-response action of 70%ethanolic extract of *Albizia lebbeck* stem bark*on Staphylococcus aureus.***Data expressed as mean ± ecart-type (n=3)**

Effect of 70 % ethanolic extract of *Albizia lebbeck*on multi-resistant strains.

After the incubation time at 37 ° C, increasing concentrations of 70 % ethanolic extract have led to a gradual reduction of bacterial growth and a dose-dependent turbidity of the culture medium and that for each bacterial strain studied. The antibacterial parameters values obtained for each bacterial strain are reported in Table 2.

Table 2: Antifungal Parameters

		70 % ethanolic extract (mg/mL)			action
Strain	Codes	MIC	MBC	MBC/MIC	
Staphylococcus aureus	ATCC 25923	3,12±0,0	3,12 ± 0, 0,0	1	bc
	377 CA/15	6,25±0,0	6,25± 0, 0,0	1	bc
	310 CA/15	3,12± 0,0	6,25± 0, 0,0	2	bc
	1541 C/14	6,25±0,0	6,25± 0, 0,0	1	bc
	412 C/14	3,12±0,0	6,25± 0, 0,0	2	bc
	505 PP/15	6,25±0,0	3,12± 0, 0,0	0,49	bc

bc : Bactericidal

Phytochimical sorting

The phytochemical sorting performed with the extracts of 70 % ethanolic extracallowed to detect the presence of various chemical groups (Table 3). They are the polyphenols, tannins, and flavonoids, in both 70% ethanol extract .

Table 3 : Results of the phytochemical screening of 70%ethanolic extract

Molecules sought	Sample tested (70 % EE)
Alkaloids	-
Polyphenols	+
Gallic tannins	+
Catechin tannins	+
Flavonoids	+
Terpene / sterol	-
Coumarins	-

+: presence of the chemical group,-: absence of the chemical group,70%EE: 70%ethanolic extract

DISCUSSION

Recently, a number of plants have been reported for antimicrobial properties across the world [15]. Several workers have reported that many plants possess antimicrobial properties. In this study all multi-resistantstrains of *Staphylococcus aureus* are sensitive to 70 % ethanolic extract of stem bark of *Albizia lebbeck* compared to controls in a dose-response relationship.We observed progressive increase of the inhibition zone as the concentration of the 70 % ethanolic extract increases. The diameters of the zones of inhibition ranged from 9 ±0.57 to17.0±0.57 mm for multi-resistant strains. A bacterial strain is said to be sensitive to an extract when the inhibition diameter of the extract is between 9 and 14 mm [12]. The inhibition zone diameters are all greater than 9 mm, it could be said that the 70 % ethanolic extract of the stem bark of *Albizia lebbeck* is active. The MBC / MIC ratio gives values that are less than or equal to two, so it can be said that the 70% ethanolic extract of *Albizia lebbeck* is bactericidal on the five clinical strains of *Staphylococcus aureus* [13].This property might be due to direct action of bioactive compounds on membrane resulting in its lysis and cell death. Our results are comparable to those of [16]which showed that *Albizia bernieri*, another species of *Albizia*, had a bactericidal activity on *Staphylococcus aureus*. The phytochemical composition of the stem bark of *Albizia lebbeck* revealed thepresence of Polyphenols, Flavonoids and tanninswhose antibacterial properties are known [17, 18]. The antibacterial activity of 70% ethanolic extract of *Albizia lebbeck* is therefore linked to the presence of these molecules.Tannins for example, are known for their ability to inhibit the growth of manymicroorganisms including bacteria [19]. In addition to this property, the tannins areendowed with astringent and healing power. This provides a scientific rational for the use of this plantin the treatment of skin diseases.The biological properties of these compound justify the antimicrobial activities expressed in this study and to link these activities and the traditional use of *Albizia lebbeck*in the treatment of bacterial diseases.

CONCLUSION

Our study has shown that the 70 % ethanolic extract of the stem bark of *Albizia lebbeck* has antibacterial activity. Allmulti-resistant strains of *Staphylococcus aureus* as well asthe reference strain studied were susceptible to the 70 % ethanolic extract of the stem bark of *Albizia lebbeck*. 70 % ethanolic extract exhibit a bactericidal activity on various strains. This study justifies the traditional use of stem bark of *Albizia lebbeck* in treating skin diseases. From the outcome of

our study, the 70 % ethanolic extract of *Albizia lebbeck*opens a new path with respect to the search for new natural substances that can neutralize multi-resistant strains.

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