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RESEARCH ARTICLE

IN VITRO ANTIBACTERIAL ACTIVITY OF COMBRETUM RACEMOSUM P. BEAUV (COMBRETACEAE) ON METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS STRAINS.

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Abstract

The purpose of this study was to evaluate the in vitro antibacterial activity of *Combretum racemosum* on 3 clinical strains of *Staphylococcus aureus* (*S. aureus* 1987C / 18, *S. aureus* 1989C / 18 and *S. aureus* 1994C / 18) and a strain of reference (*S. aureus* ATCC 29213). The effect of aqueous total extract and hydro-ethanolic 70% extract of the plant on the growth of the strains was evaluated by determining the antimicrobial parameters by the method of wells in agar medium and by the method of microdilution in liquid medium. The aqueous total extract showed a better efficacy with a MIC ranging from 0.19 ± 0 mg / mL to 0.78 ± 0 mg / mL followed by hydro-ethanolic 70% extract with a MIC of 0.39 ± 0 mg / mL at 1.56 ± 0 mg / mL. The BMCs of the extracts ranged from 0.39 ± 0 mg / mL to 1.56 ± 0 mg / mL (aqueous total extract) and from 1.56 ± 0 mg / mL to 3.12 ± 0 mg / mL (hydro-ethanolic 70% extract). Both extracts showed a bactericidal effect on all the strains tested. These results suggest that *C. racemosum* may be an alternative in the fight against *Staphylococcus aureus* infections.

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Introduction:-

Bacterial infections alone account for 45% of deaths in developing countries and almost one in every two premature deaths worldwide with 70% of deaths caused by microorganisms (Walsh, 2003). Among the microorganisms responsible for these infections, there are those of the genus *Staphylococcus* including *S. aureus*. These germs cause serious skin infections, pneumonia and septicemia. All these infections require long stays in hospitals for patients with high mortality (Cosgrove et al., 2003). These infections are treated with antibiotics. Indeed, antibiotics at the beginning of the twentieth century, have led to great advances in therapy and contributed to the rise of modern medicine. The introduction and clinical use of the first classes of antibiotics have significantly reduced mortality from previously incurable diseases. The effectiveness of antibiotic therapy in controlling and limiting the spread of pathogens has given rise to the hope of eradicating all infectious diseases (El amri et al., 2014). However, the massive and sometimes abusive use of antibiotics has significantly modified the microbial ecology thus increasing

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the rate of bacteria resistant to several families of antibiotics like the β -lactams they were previously sensitive to (Guessennd *et al.*, 2009). For example, half of all *staphylococcus aureus* in the United States are resistant to penicillin, tetracycline and erythromycin and especially to methicillin (Morrison *et al.*, 2007). Resistance to the latter leads to resistance to all β -lactams (Guignard *et al.*, 2005).

Faced with this phenomenon of antibiotic resistance and the many therapeutic failures, it therefore seems important to direct research towards other alternatives such as medicinal plant extracts to serve as a basis for obtaining new antibacterials (Zirih *et al.*, 2003). It is within this framework that we evaluated the antibacterial activity of extracts of the roots of *Combretum racemosum* P. Beauv. (Combretaceae) on methicillin-resistant *Staphylococcus aureus* strains. Indeed, plants contain a large source of bioactive molecules that are usually part of secondary metabolites (Haddouchi *et al.*, 2014). According to WHO (2002) estimates, more than 80% of the population in Africa still uses traditional medicine to meet their need for health care.

Material and methods:-

Equipment

Microbial material

The microbial material consists of 4 strains of *Staphylococcus aureus* including a reference strain (*S. aureus* ATCC 29213) and 3 clinical strains (*S. aureus* 1987C / 18, *S. aureus* 1989C / 18 and *S. aureus* 1994C / 18). All these strains were provided by the Institut Pasteur of Côte d'Ivoire.

Plant material

The roots of *Combretum racemosum* were harvested in June 2018 in the department of Anyama (Ivory Coast). The plant has been authenticated at the National Center of Floristry of the Felix Houphouët Boigny University of Abidjan-Cocody where it is kept since July 17, 1985 under the No. 16949.

Technical material

The technical equipment used is composed of : a precision balance, a blinder, funnels, hydrophilic cotton, carded cotton, Whatman 3 mm filter paper to carry out the extractions. To this must be added the apparatus and glassware of use in a laboratory of Pharmacology and Microbiology.

Methods

Preparation of the vegetable powder

The roots of *C. racemosum* were harvested, washed with tap water, cut and dried under the sun at laboratory temperature (25-30 ° C) for three weeks. After drying, they were sprayed with an electric grinder and the dry powder obtained was used to prepare the various extracts of the plant.

Preparation of plant extracts

Preparation of the aqueous total extract

The aqueous total extract (ATE) was prepared according to the method described by Ackah *et al.*, (2008). In fact, 100 g of *C. racemosum* root powder were macerated in 1000 mL of distilled water by homogenization using a blinder. The homogenate obtained was first filtered on white fabric and then filtered twice on hydrophilic cotton and once on Whatman 3mm filter paper. The filtrate obtained was dehydrated with an oven at 50 ° C. for 48 hours to obtain the aqueous total extract (ATE).

Preparation of the hydroethanolic extract

The hydroethanolic 70% extract (HEE) was also prepared according to the method described by Ackah *et al.*, (2008). Again, 100 g of dry powder were macerated in 1000 ml of 70% ethanol (700 ml pure ethanol). + 300 ml of cold distilled water) by homogenization in a blinder. The homogenate obtained is first filtered on white fabric and then filtered twice on hydrophilic cotton and once on Whatman 3 mm filter paper. The filtrate obtained was dehydrated with an oven at 50 ° C. for 48 hours to obtain the hydroethanolic 70% extract (HEE).

Calculation of the yield of ATE and HEE extracts

The yield (R) of each plant extract was determined. Indeed, it corresponds to the ratio between the mass of the extract obtained after evaporation of the solvent on the mass of plant powder used for the extraction multiplied by 100 grams (Dinzedi, 2015) according to the following formula :

$$R (\%) = \frac{Me \times 100}{Mp}$$

R: yield in %; Me: mass in g of the extract obtained; Mp: mass in g of powder used for extraction

Study of the antibacterial activity of the extracts of the plant

Preparation of the bacterial inoculum

Two 18-hour young colonies of each strain of *S. aureus* resistant to methicillin were removed using a platinum loop and then homogenized in 10 mL of physiological saline (0.9% NaCl) and incubated during 3 hours at 37 °C to have a pre-culture. Then, 1 mL of the opalescent bacteria pre-culture broth was removed and diluted in a tube containing 9 mL of physiological saline (0.9% NaCl) to obtain the bacterial inoculum at approximately 10⁶ CFU / mL. In addition, a concentration range of each extract (ATE and HEE) ranging from 200 mg / mL to 1.56 mg / mL was prepared by the double dilution method in liquid medium for strain susceptibility testing (**Ouattara et al., 2007**).

Strains susceptibility test with different plant extracts

The sensitivity of the 4 strains of *S. aureus* was evaluated by the well method in Mueller-Hinton agar plate based on the diffusion of the antibacterial compound (plant extracts) with creation of a concentration gradient after a certain time of contact between the product and the target bacteria. For this, the Mueller-Hinton agar was uniformly seeded with 0.1 mL of the inoculum of each bacterial strain to be studied and then dried for 15 min at room temperature (22 °C). Then, wells approximately 6 mm in diameter were made in the agar plate in which 75 µL of each plant extract (200 mg / mL, 100 mg / mL, 50 mg / mL, 25 mg / mL, 12.5 mg / mL, 6.25 mg / mL, 3.12 mg / mL, 1.56 mg / mL) were deposited (**Koffi et al., 2014**). The agar was subsequently incubated in an oven at 37 °C for 18 to 24 hours. After incubation, the effect of the extracts was appreciated by measuring an inhibition zone around each well which indicates antibacterial activity of the extract. Oxacillin (6.25 mg / mL, 3.12 mg / mL, 1.56 mg / mL, 0.78 mg / mL, 0.39 mg / mL, 0.19 mg / mL, 0.09 mg / mL, 0.05 mg / mL) was used as the reference antibiotic (positive control).

Determination of antimicrobial parameters

Determination of Minimal Inhibitory Concentration (MIC)

The MIC of the different extracts was determined using the method described by **Koné et al., (2004)**. For this, in hemolysis tubes, 1 mL of each concentration of extract as well as oxacillin as a positive control, was added to 1 mL of the inoculum (10⁶ CFU / ml) leading to a halving of each concentration. Two other tubes, one without antibacterial agent served as growth control and the other without germ, for the control of sterility, were also prepared. All of these tubes were incubated at 37 °C for 24 hours. The MIC corresponds to the concentration of the first tube which does not allow to observe visible growth of the germ tested after 18 hours to 24 hours of incubation at 37 °C.

Determination of Minimal Bactericidal Concentration (MBC).

For the determination of the MBC of the different extracts and for each strain, the bacterial inoculum was homogenized and then diluted 1/10 to 10⁻⁴ dilution. Then, 2 µl of the initial bacterial inoculum and the 4 successive dilutions were used to seed a Mueller-Hinton agar plate (box A) using a sterile platinum loop, on streaks of 5 cm. long (**Diaha, 1999**). At the same time, 2 µL of the tube contents in which no haze was observed from the tube corresponding to the MIC were seeded on the surface of a Mueller-Hinton agar (box B) also on 5 cm streaks. The MBC is determined after 24 hours incubation at 37 °C by comparing the number of colonies of the box B with that of the box A. The concentration of the first experimental tube whose number of colonies was lower or equal to that of the 10⁻⁴ dilution of box A corresponds to the MBC. MBC is the lowest concentration of substance that leaves at most 0.01% of surviving germs after 24 hours of incubation at 37 °C. Finally, the antibacterial potential of the extracts was determined by calculating the MBC / MIC ratio. The extract is described as bactericidal if this ratio is less than or equal to 4 and bacteriostatic if this ratio is greater than 4 (**Berche et al., 1991**).

Statistical analysis of the results

The statistical analysis of the results was made from one-way ANOVA analysis followed by the Duncan test. All results obtained were expressed as mean ± standard deviation (n = 3). Probability values P <0.05 were considered significant. All results were analyzed using the STATISTICA 7.1 software.

Results:-

Yield of different extractions

The yield of the extractions varied according to the solvent used. In fact, for 100 g of plant powder used for extraction, the highest yield (18.50%) was obtained with the hydroethanolic 70% extract (HEE) followed by that of the aqueous total extract. (ATE) (5.61%) with a significant difference between these yields ($P < 0.05$).

Determination of the antibacterial activity of plant extracts

Sensitivity of strains to different extracts

Tables 1, 2 and 3 show the diameters of the zones of inhibition of the various extracts and of the reference molecule (Oxacillin). From these results, ATE and HEE had a strong inhibitory activity of 200 mg / ml to 6.25 mg / ml and 200 mg / ml to 12.5 mg / ml, respectively. In fact, the inhibition diameters varied from 18.33 ± 0.57 mm to 35 ± 1 mm (ATE) and from 10.33 ± 0.57 mm to 25 ± 1 mm (HEE) on all 4 bacterial strains. However, the inhibition of the strains by ATA remains greater than that of the HEE with a significant difference between the inhibition diameters of the two extracts.

As for Oxacillin (positive control), it also showed considerable areas of inhibition on the four bacterial strains studied with diameters between 16 ± 0 mm and 36 ± 0 mm for the different concentrations (6.25 to 0, 09 mg / mL). In addition, among all the bacterial strains studied, *S. aureus* strain 1994C / 18 seems to be the most sensitive to both extracts (ATE and HEE) with diameters of 35 ± 1 mm and 25 ± 1 mm respectively. In contrast, *S. aureus* 1989C / 18 was more sensitive (36 ± 0) than other strains with the reference antibiotic (oxacillin).

Table 1:-Diameters (mm) of zones of inhibition with aqueous total extract (ATE)

Bacterial strains	Concentrations (mg/mL)							
	200	100	50	25	12,5	6,25	3,12	1,56
<i>S. aureus</i> ATCC 29213	34 $\pm 1^a$	31 $\pm 1^a$	29,33 $\pm 0,57^a$	27 $\pm 1^a$	23,66 $\pm 1,52^a$	20 $\pm 1^a$	0 \pm 0	0 \pm 0
<i>S. aureus</i> 1987C/18	34,66 $\pm 0,57^b$	33 $\pm 1^b$	31,33 $\pm 1,52^b$	28 $\pm 1^b$	24,66 $\pm 0,57^b$	22,66 $\pm 0,57^b$	0 \pm 0	0 \pm 0
<i>S. aureus</i> 1989C/18	33,33 $\pm 0,57^c$	29 $\pm 1^c$	26 $\pm 1^c$	24,33 $\pm 0,57^c$	22,66 $\pm 0,57^c$	18,33 $\pm 0,57^c$	0 \pm 0	0 \pm 0
<i>S. aureus</i> 1994C/18	35 $\pm 1^d$	33,66 $\pm 0,57^d$	31,66 $\pm 0,57^d$	28,66 $\pm 1,52^d$	24,66 $\pm 1,52^d$	19,33 $\pm 1,15^d$	0 \pm 0	0 \pm 0

The averages are expressed with the standard deviations (\pm) values with the same letter with exponent in each of the columns are not significantly different ($p < 0.05$).

Tableau 2:-Diameters (mm) of inhibition zones with hydro-ethanolic 70% extract (HEE)%

Bacterial strains	Concentrations (mg/mL)							
	200	100	50	25	12,5	6,25	3,12	1,56
<i>S. aureus</i> ATCC 29213	24,33 $\pm 0,57^a$	20 $\pm 2^a$	16,33 $\pm 2,08^a$	13,33 $\pm 0,57^a$	10,33 $\pm 0,57^a$	0 \pm 0	0 \pm 0	0 \pm 0
<i>S. aureus</i> 1987C/18	24,66 $\pm 1,15^b$	22,66 $\pm 0,57^b$	18,66 $\pm 0,57^b$	13,66 $\pm 0,57^b$	11,33 $\pm 0,57^b$	0 \pm 0	0 \pm 0	0 \pm 0
<i>S. aureus</i> 1989C/18	23 $\pm 1^c$	21,33 $\pm 0,57^c$	17,33 $\pm 1,52^c$	13 $\pm 1^c$	11 $\pm 1^c$	0 \pm 0	0 \pm 0	0 \pm 0
<i>S. aureus</i> 1994C/18	25 $\pm 1^d$	21,66 $\pm 1,52^d$	19,33 $\pm 0,57^d$	15 $\pm 1,73^d$	12 $\pm 1^d$	0 \pm 0	0 \pm 0	0 \pm 0

The averages are expressed with the standard deviations (\pm) values with the same letter with exponent in each of the columns are not significantly different ($p < 0.05$).

Table 3:-Diameters (mm) of inhibition zones obtained with oxacillin (control)

Bacterial strains	Concentrations (mg/mL)							
	6,25	3,12	1,56	0,78	0,39	0,19	0,09	0,05
<i>S. aureus</i> ATCC 29213	33 \pm 0 ^a	30 \pm 0 ^a	28 \pm 0 ^a	24 \pm 0 ^a	21 \pm 0 ^a	18 \pm 0 ^a	0 \pm 0	0 \pm 0
<i>S. aureus</i>	32 \pm 0 ^b	28 \pm 0 ^b	27 \pm 0 ^b	24 \pm 0 ^b	20 \pm 0 ^b	18 \pm 0 ^b	16 \pm 0 ^a	0 \pm 0

1987C/18								
<i>S. aureus</i> 1989C/18	36±0 ^c	33±0 ^c	29±0 ^c	25±0 ^c	22±0 ^c	16±0 ^c	0±0	0±0
<i>S. aureus</i> 1994C/18	34±0 ^d	31±0 ^d	27±0 ^d	24±0 ^d	20±0 ^d	20±0 ^d	18±0 ^b	0±0

The averages are expressed with the standard deviations (\pm) values with the same letter with exponent in each of the columns are not significantly different ($p < 0.05$).

Antibacterial parameters of plant extracts

The antibacterial parameters (MIC, MBC) and the MBC / MIC ratio of the different extracts (ATE and HEE) of *C. racemosum* and the reference antibiotic (oxacillin) on the *in vitro* growth of the different strains are shown in Table 4. These results show that plant extracts and oxacillin have an inhibitory action on the growth of all strains of *S. aureus* tested. However, with the total aqueous extract (ATE), the clinical strains (*S. aureus* 1987C / 18, *S. aureus* 1989C / 18 and *S. aureus* 1994C / 18) were the most sensitive recording the same MIC values ($0,19 \pm 0$ mg / mL) and MBC ($0,39 \pm 0$ mg / mL) compared to the reference strain (*S. aureus* ATCC 29213) with MIC = $0,78 \pm 0$ mg / mL and MBC = $1,56 \pm 0$ mg / mL. Furthermore, the ratio of MBC / MIC of the ETA on all the strains was 2 thus showing a bactericidal effect of this extract on these strains.

For HEE, the determined MIC was identical to MBC for *S. aureus* strains ATCC 29213 ($1,56 \pm 0$ mg / mL) and *S. aureus* 1987C / 18 ($0,39 \pm 0$ mg / mL). With the *S. aureus* 1989C / 18 and *S. aureus* 1994C / 18 strains, the MIC values were 0.78 mg / mL and 1.56 mg / mL, respectively, while the MBC values were $1,56 \pm 0$ mg / mL for *S. aureus* 1989C / 18 and $3,12 \pm 0$ mg / mL for *S. aureus* 1994C / 18. The MBC / MIC ratio for all of these strains was less than 4 also showing a bactericidal effect of HEE.

As for the reference antibiotic (oxacillin), the results showed that the strains of *S. aureus* 1989C / 18 and *S. aureus* 1994C / 18 recorded the same MICs ($0,78 \pm 0$ mg / mL) and MBCs ($1,56 \pm 0$ mg / mL). For the *S. aureus* strain ATCC / 29213, the observed MIC value was identical to that of MBC ($1,56 \pm 0$ mg / mL). In contrast, the MIC ($1,56 \pm 0$ mg / mL) and MBC ($3,12 \pm 0$ mg / mL) values recorded were greater with the *S. aureus* 1987C / 18 strain. The MBC / MIC ratios being less than 4 for all strains, oxacillin also had a bactericidal effect.

Table 4:-Parameters and antibacterial powers of plant extracts

Bacterial strains	Extracts	Antibacterial parameters (mg / mL)		Efficiency report (MBC/MIC)	antibacterial power
		MIC	MBC		
<i>S. aureus</i> (ATCC/29213)	ATE	0,78±0,00 ^a	1,56±0,00 ^a	2	Bactéricide
	HEE	1,56±0,00 ^b	1,56±0,00 ^a	1	Bactéricide
	Ox	1,56±0,00 ^b	1,56±0,00 ^a	1	Bactéricide
<i>S. aureus</i> 1987C/18	ATE	0,19±0,00 ^c	0,39±0,00 ^b	2	Bactéricide
	HEE	0,39±0,00 ^d	0,39±0,00 ^b	1	Bactéricide
	Ox	1,56±0,00 ^b	3,12±0,00 ^c	2	Bactéricide
<i>S. aureus</i> 1989C/18	ATE	0,19±0,00 ^c	0,39±0,00 ^b	2	Bactéricide
	HEE	0,78±0,00 ^a	1,56±0,00 ^a	2	Bactéricide
	Ox	0,78±0,00 ^a	1,56±0,00 ^a	2	Bactéricide
<i>S. aureus</i> 1994C/18	ATE	0,19±0,00 ^c	0,39±0,00 ^b	2	Bactéricide
	HEE	1,56±0,00 ^b	3,12±0,00 ^c	2	Bactéricide
	Ox	0,78±0,00 ^a	1,56±0,00 ^a	2	Bactéricide

ATE : aqueous total extract ; HEE : hydro-ethanolic 70% extract ; Ox: Oxacillin, MIC: minimal inhibitory concentration ; MBC: Minimal Bactericidal Concentration. Averages are expressed with standard deviations (\pm). Values with the same superscript letter in each of the columns are not significantly different ($p < 0.05$).

Discussion:-

The purpose of this study was to evaluate the antibacterial activity of aqueous and ethanolic *C. racemosum* extracts on the *in vitro* growth of four methicillin-resistant strains of *S. aureus*. The results show that the HEE (18.50%) yield was higher than the ATE (5.61%). The variation in yields could be explained by the chemical content of the plant, which can be extracted by each solvent used (Adjou & Soumanou, 2013). Indeed, the amount of extractable bioactive molecules in the plant depends on the extraction method and the ability of the solvent to dissolve the

endogenous compounds (Sultana *et al.*, 2009, Kamarudin *et al.*, 2016). This confirms the results of work on the leaves of *Annona senegalensis* (Traore *et al.*, 2012), *Terminalia arjuna* (Sultana *et al.*, 2009) and *Clerodendrum splendens* (Kouadio *et al.*, 2013).

Regarding the antibacterial potential, the results showed that root extracts of the plant inhibited strains of *S. aureus*, indicating that this plant has significant *in vitro* antibacterial properties. In fact, the inhibition diameters recorded varied from 18.33 ± 0.57 to 35 ± 1 mm for the ATE and from 10.33 ± 0.57 to 25 ± 1 mm for the HEE. According to Ponce *et al.*, (2003), an extract is considered active if it induces an inhibition zone greater than or equal to 9 mm. As a result, both extracts used were active on *S. aureus* strains tested at concentrations of 6.25 ± 0 mg / mL for ATE and 12.5 ± 0 mg / mL for HEE. However, of the two extracts, ATE was more effective on bacterial strains inducing higher inhibition diameters between 33.33 ± 0.57 and 35 ± 1 mm on the *S. aureus* at the 200 concentration. mg / mL. Analysis of the results shows that the sensitivity of each strain tested remains related to the concentration of the extract used in a dose-response relationship.

Regarding the antibacterial power, the evaluation of the extracts of plants tested by the dilution method revealed that these extracts have a significant antibacterial capacity on all the strains studied with MICs ranging from 0.19 ± 0 mg / mL to 1.56 ± 0 mg / mL for ATE and from 0.39 ± 0 mg / mL to 1.56 mg / mL for HEE. Moreover, the ratio of MBC / MIC activity expressing the bacterial potency of the two extracts of *C. racemosum* varied from 1 to 2. According to Berche *et al.* (1991), when this report concerning a substance is less than or equal to 4, this substance is considered bactericidal and when it is greater than 4, it is said to be bacteriostatic. If we stick to these authors, all extracts tested have a bactericidal action on all strains studied. The antibacterial activity of *C. racemosum* extracts is related to the presence of bioactive molecules in these extracts. Moreover, several studies have been conducted in this direction. Indeed, a phytochemical sorting of the extracts of this plant by Onocha *et al.* (2005); Okwuosa *et al.* (2012) and Kamou *et al.* (2016) revealed that it contains alkaloids, phenolics, saponins, cardiotonic glycosides, sterols and polyterpenes in abundance. All these classes of molecules are known for their antibacterial properties (Bruneton, 1993, Bssaibis *et al.*, 2009, Tiwari *et al.*, 2011). These molecules could therefore be responsible for the inhibitory activity of *C. racemosum* on different strains of *S. aureus* resistant to methicillin. Therefore this plant could be recommended in the fight against infections caused by these strains.

Conclusion:-

At the end of this study, which evaluated the effect of the roots of *Combretum racemosum*, we note that the results showed that the two extracts tested inhibited all bacterial strains in a dose-dependent relationship with a bactericidal effect on all of these strains. In addition, the aqueous total extract was the most active compared to the hydro-ethanolic 70% extract. It would be important to optimize the process of extraction and identification of the active ingredients of this plant for use *in vivo* against infections caused by strains of *S. aureus*.

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Conflict of interest statement

The authors declare no conflict of interest in this article.

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