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RESEARCH ARTICLE

PHYTOCHEMICAL STUDY AND ANTIBACTERIAL ACTIVITY OF ETHANOLIC AND AQUEOUS EXTRACTS OF BARKS OF MYRIANTHUS HOLSTII ENGL. (CECROPIACEAE)

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Abstract

The aim of this work is to study phytochemicals and evaluate the antibacterial activity of aqueous and ethanolic extracts of Myrianthus holstii bark on three reference and five clinical strains derived from biological products. The results obtained show that the gives the best extraction yield. Chemical screening revealed the presence of polyphenols, alkaloids, flavonoids, saponosides, quinones, anthocyanins, tannins, terpenoids and sterols in both extracts. The results obtained show that the strains tested have a variable sensitivity for the two extracts and their concentrations. Diffusion and dilution methods on Muller-Hinton were used to evaluate the antibacterial activity of the extracts. The diameters of the inhibition zones are between 8 and 16 mm for the ethanolic extract starting from 25 mg/mL and between 8 and 12 mm for the aqueous extract at 50 mg/mL. The results revealed that these extracts have a dose-dependent antibacterial activity on the bacterial strains used. However, the 70% ethanolic extract has a better antibacterial potential on the strains compared with the aqueous extract, namely on S. aureus (CMI=3.12 mg/mL), E. coli ATCC (CMI=12.5 mg/mL) and S. aureus Méti-R (CMI=12.5 mg/mL). Also, this extract is bactericidal on all strains studied and its MIC ranges from 3.12 to 100 mg/mL after 24 and 48 hours of incubation. This study showed that extracts of Myrianthus holstii could be used in the treatment of infectious diseases.

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Introduction:-

Medicinal plants have been used since ancient times to relieve and cure human diseases. In fact, their therapeutic properties are due to the presence of hundreds or even thousands of natural bioactive compounds called: secondary metabolites. The latter are then accumulated indifferent organs and sometimes in the specialized cells of the plant. Despite the progress of pharmacology, the therapeutic use of medicinal plants is very present in some countries of the world and especially in developing countries, in the absence of a modern medical system(Tabuti et al., 2003). According to OMS (2012), estimates, more than 80% of the population in Africa still use traditional medicine to

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meet their health care needs. Furthermore, the use of plant extracts and plant compounds is a valuable source for traditional medicine in the treatment and prevention of a wide spectrum of diseases; in particular infectious diseases (Al-Bayati &Khudir, 2008). Currently, the development of microbial resistance to antibiotics has led researchers to tap into the world of plants, particularly medicinal and culinary plants, in search of effective natural molecules that are devoid of any adverse effects. In fact, previous studies carried out on various extracts of medicinal plants have shown that they exhibit antibacterial activities (Bolou et al., 2011). Myrianthus holstii (Cecropiaceae) is one of the medicinal plants that could be used to fight infectious diseases. Scientific studies have shown that a decoction of leaves, stem bark, trunk of this species is mixed with twig for enema and helps fight against diarrhea, dysentery, cholera, fatigue, miscarriages and strengthens children (Baerts & Lehmann, 1989 ; Balagizi et al., 2005). And these studies have shown that bark is used in traditional pharmacopeia to treat these previously listed pathologies. The objective of this work is to carry out the phytochemical screening of certain chemical groups and to evaluate the antibacterial activity of the two extracts.

Materials and Methods:-

Plant material

The plant material consists of the bark of Myrianthus holstii (Cecropiaceae) harvested in October 2019 on the site of the University of Man, located 7 km from the city of Man, on the Man-Danane axis, (Ivory Coast).

Microorganisms

The bacterial carrier used in this study consists of three (3) reference strains namely Staphylococcus aureus CIP 7625 (ATCC 25923), Escherichia coli CIP 7624 (ATCC 25922) and Pseudomonas aeruginosa CIP 76110 (ATCC 27853) and five (5) Isolated clinical strains of biological products that are : ESBL E. coli (1087C / 13 isolated from urine sample), Salmonella typhi (1585C / 13, isolated from blood sample), Klebsiella pneumoniae ESBL (1942C/13, isolated from pus sample), S. aureus Met-R (1532C / 10, isolated from pus sample) and Klebsiella pneumoniae. These are the components of the Antibiotics Natural Substances and Monitoring Microorganisms for Anti-Infective (ASSURMI) of the Department of Bacteriology and Virology of the Pasteur Institute in Ivory Coast (IPCI).

Preparation of plant extracts

Preparation of aqueous extract

100g powder of the bark of Myrianthus holstii were macerated for 24 hours in 1L of distilled water (Olakunle et al., 2005). The macerate has been wrung into a square of sterile tissue, filtered successively on cotton wool and one fold on filter paper (Whatman paper® 2 mm). 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(Zirihi et al., 2003).

Preparation of ethanolic 70% extract

It was carried out using modified method (Ouattara et al., 2007). A mass of 20g of plant powder was added in 100 mL of ethanol 70% and subjected to maceration for 72 hours. The macerate was treated according to the same procedure like the aqueous extract.

Preparation of bacterial inoculum

Two isolated colonies from each bacterial culture for 18 hours were homogenized in 10 mL of Muller-Hinton broth and incubated for 3 hours at 37°C for preculture. A levy of 0.1 mL of the preculture broth was diluted in a tube containing 10 mL of Mueller-Hinton (MH). This bacterial suspension was made consisting of 10^0 dilution of bacterial inoculum so as to obtain a bacterial load estimated to 10^6 Unit Format colonies per milliliter (CFU/mL).

Preparation of extracts concentration ranges

A range of concentration of each extract was prepared with a series of ten vice tubes through the method of double dilution an in medium liquid. This range of concentration is 200 mg / mL to 0.39 mg / mL numbered T1 to T10. For this, 10 mL of a mixture solution of DMSO / sterile distilled water (V / V) were placed in the tubes T1 and 5 mL in all the other tubes. Two grams (2g) of each extract were dissolved in the tubes T1 to obtain a concentration of 200mg/mL. A 5 mL volume of the tubes T1 was transferred into the tubes T2 and then homogenized. This operation was repeated until T10 tubes where 5 mL of T10 tubes are rejected. All tubes are kept refrigerated at 4°C(Bolou et al., 2011; Konan et al., 2014).

Determination of growth inhibition zones

The method of holes punch in the MH agar described by Ponce et al. (2003), has been accepted. Each pit or holes of 6 mm diameter was filled with 80 µL of extract concentrations of 200 and 100 mg / mL, taking care to separate two holes of at least 20 mm. A negative control wells was performed for each bacterial strain with 80 µL of the mixture of DMSO / sterile distilled water solution (V/V). After a pre-release of 45 minutes at laboratory temperature to 16 ° C, all the Petri dishes were incubated in an incubator at 37°C for 18-24h. Meanwhile, Ceftriaxone (CRO 30µg) for Enterobacteriaceae and oxacillin (OX 5µg) for staphylococci were used as positive controls. After incubation, the activities of the extracts were assessed by measurement of a growth inhibition area around the wells using a caliper. According to Dosso & Faye-kette (2000), a strain is called insensitive or resistant, sensitive and very sensitive if the diameters of inhibition are respectively less than 8 mm, between 9 and 14 mm and between 15 and 19 mm.

Determination of Minimum Inhibitory Concentration (MIC)

The macro dilution method in liquid medium described by Berché et al. (1991), was used to determine these antimicrobials parameters. Thus, in a series of 10 hemolysis tubes numbered C1 to C10 for each extract was introduced 1 mL of the bacterial inoculum. Then 1 mL of each extract concentration well known by the range of prepared concentration was added in the same tubes. This distribution of plant extract is made so that 1 mL of plant extract of 200 mg / mL was transferred in the tube C1, that of 100 mg / mL in the tube so C2 to C9 tube receive 1 mL plant extract of 0.78 mg / mL. C10 has been tube, received instead of plant extract, 1 mL of DMSO / Sterile distilled water (V/V), was used as a control. This distribution of plant extract concentration is well known in each tube already containing 1 mL of inoculum reduced the concentration of plant extract in medium at its half. Tube and the concentration of C1 increased from 200 mg / mL to 100 mg / mL. 100 mg / mL to 50 mg / mL for C2 so on until a concentration of 0.39 mg / mL for T9. This experiment was performed identically for each sample tested. The first nine (9) tubes (C1 to C9) are called "experimental tubes" and the last tube (C10) is rated "growth control tube or TC." The loaded tubes were incubated at 37 ° C for 24 h. The MIC is the concentration of the first tube where it finds no trouble visible to the naked eye.

Determination of Minimum Bactericidal Concentration (MBC)

From the MIC, the lowest concentration that leaves no more than 0.01% survival of bacteria suspended starting 24 hours corresponds to the CMB. It is determined by plating by a streak on Mueller-Hinton agar by streaking 5 cm using a loop, beginning with the first and incubated undisturbed at 37°C for 24 h tube.

Antibacterial activity of the extracts tested

The antibacterial effect of different extracts tested was considered bactericidal or bacteriostatic depending on the MBC / MIC ratio. According Yéo et al. (2014), when this ratio is greater than 4, the extract has bacteriostatic and bactericidal, if the ratio is less than or equal to 4.

Phytochemical screening

Test for sterols and polyterpenes (reaction LIEBERMANN)

After evaporation to dryness 5 mL of each solution in a capsule on a sand bath without charring, the residue was dissolved in hot acetic anhydride and 1 mL in a test tube, we poured cautiously with 0.5 mL of concentrated sulfuric acid along the tube wall to the solution. The applications to the interphase of a purple or purple ring, turning blue to green, indicate a positive reaction(Kpemissi, 2007).

Test for alkaloids (reactions Dragendorff and Bouchardat)

Six milliliters of plant extract were evaporated. The residue was taken up in six milliliters of alcohol at 60 °C and the alcoholic solution thus obtained was divided into two test tubes.

In the first tube was added two drops of Dragendorff reagent. The appearance of a precipitate or an orange color indicated the presence of alkaloids. In the second tube was added two drops of reagent Bouchardat. The appearance of a reddish brown color indicated a positive reaction to the presence of alkaloids(Kpemissi, 2007).

Test for 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(Bidie et al., 2011).

Test for 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 hydrochloric alcohol half. The successive addition of three magnesium shavings and three drops of isoamylic alcohol showed an intense pink or violet in the presence of flavonoids(Zakia et al., 2015).

Test for saponosides
A volume of two milliliters of each extract was 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(Munmi et al., 2013).

Test for catechol or condensed tannins (reaction Stiasny)
A volume of five milliliter of each extract was evaporated and an amount of 10 mL 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 was cooled to room temperature. Positive feedback had resulted in the formation of large flakes brown clear or dirty precipitates(Yéo et al., 2014).

Quinonic substances research
For this research, 2 mL of each extract solution is first evaporated to dryness in a sand-bath capsule without charring, then the residue is triturated in 5 mL of 1: 5 hydrochloric acid. Then the solution obtained is brought to the boiling water bath for half an hour. Finally, after cooling on a current of cold water, the hydrolyzate is extracted with 20 mL of chloroform and the chloroform phase is collected in another test tube supplemented with 0.5 mL of ammonia diluted by half. The appearance of a color ranging from red to purple indicates the presence of quinones(Yéo et al., 2014).

Search anthocyanins
The presence of anthocyanin in an extract solution is indicated by a red color which increases with the addition of dilute HCl and turns purplish-blue-green by the addition of ammonia (Kpemissi, 2007).

Test for Gallic tannins
The above solution was saturated and one or two drops of alcoholic solution of iron chloride to 2 % have been added. The positive response has led to the appearance of blue-black coloration characteristic intense tannins Gallic (Yéo et al., 2014).

Statistical analysis
Variance analysis (ANOVA-one-way), followed by the Turkey test, were used to compare the variations of the MICs between them and the CMBs between them, and to verify whether the activity of extracts of the bark of Myrianthus holstii was statistically influenced by the phenotypes (sensitive and resistant) of the bacteria. The results obtained were expressed as means ± Standard deviation. $P < 0.05$ probability values were considered statistically significant. Graph Pad Prism 6 software was used for this statistical test.

Results:-

Yield
The values of the yields were calculated with respect to the initial mass of M. holstii powder for one test. The extraction yields of the plant are 7.95% for the ethanolic extract (EE) and 6.40% for the aqueous extract (EA). (Table 1)

Table 1:- Extraction Yield of Extracts.

Extracts	Mass (g)	Yield (%)	Colors and Aspects
EEec	19.40	6.40	chestnut (powder)
EAec	15.90	7.95	chestnut clear (spangle)

Phytochemical study
Table 2 shows the large groups of chemical families contained in the two extracts of Myrianthus holstii. Phytochemical screening of Aqueous Extract (EA) and Ethanol Extract (EE) of Myrianthus holstii bark shows the

presence of several families of chemical compounds. These are sterols/polyterpenes, alkaloids, gallic tannins and catechins, quinones, saponosides, anthocyanins, flavonoids and total polyphenols.

Table 2:- Phytochemical analysis of ethanolic and aqueous extract of M. holstii bark

Extracts	Alcaloids		Saponosides	Anthocyans	Tannins		Flavonoids	Polyphenols	Quinones	Sterols and polyterpenes	
	B	D			Gal	Cat					
EAec	-	++	+++	-	-	-	+	++	-	-	
EEec	-	+	+	-	+++	+	++	+++	+++	+	

- : Absence + : Presence ++ means + + +: abundant.;
EE : Ethanol Extract, EA : Aqueous Extract; Gal : Gallic ; Cat : catechetical ; ec : bark B : Bouchardaf ; D : Dragendorff

Antimicrobial Activity

In Table 3, the results show that S. aureus ATCC (16 mm), S. aureus Méti-R, and K. pneumoniae BLSE (14 mm) strains are highly sensitive to Eec. The strains P. aeruginosa ATCC, E. coli and Salmonella Typhi are less sensitive to the same extract with diameters of 12 mm and 10 mm respectively. All of these tested germs showed sensitivity at concentrations of 100 and 200 mg/mL.

Table 3:- Inhibition zone diameters (mm) with Ethanol Extract (70%) of Myrianthus holstii barks and antibiotics on the strains tested (n = 3).

Tested strains	Concentrations (mg/mL)					Antibiotics	
	C1=200	C2=100	C3=50	C4=25	Ts	AMC/CRO/ IPM	OXA/ FOX
Salmonella Typhi	10±0.1	8±0.0	6±0.0	6±0.0	6±0.0	6	Nd
K. Pneumoniae	12±0.2	10±0.1	8±0.2	6±0.0	6±0.0	6	Nd
K. Pneumoniae BLSE	14±0.0	12±0.2	10±0.1	8±0.1	6±0.0	8	Nd
E. coli BLSE	10±0.3	10±0.0	8±0.0	6±0.1	6±0.0	10	Nd
S. aureus Méti-R	14±0.3	12±0.1	10±0.2	8±0.1	6±0.0	Nd	6
E. coli ATCC 25922	10±1.0	8±0.2	6±0.1	6±0.3	6±0.0	8	Nd
S. aureus ATCC 25923	16±0.0	14±0.1	12±1.0	10±0.4	6±0.0	Nd	8
P. aeruginosa ATCC 27853	12±0.1	10±0.0	8±0.1	6±0.1	6±0.0	Nd	6

T = 0: Including Control = the diameter of the wells (6mm) with DMSO / water (0.5: 0.5, V/V); CRO: Ceftriaxone (30µg), OXA: oxacillin (5µg); Meti-R: Methicillin -resistant; IMIP -I: Intermediate imipenem; ESBL: extended spectrum beta-lactamase.

In Table 4, EAec showed the lowest antibacterial activity on most strains tested compared to ethanol extract. It should be noted that the highest inhibition diameters were recorded with the strains S. aureus ATCC, Klebsiella pneumoniae, E. coli and S. aureus with diameters of 12 mm and 10 mm respectively. In contrast, Pseudomonas aeruginosa ATCC and Klebsiella pneumoniae BLSE (6 mm) showed no sensitivity. The majority of the germs tested were sensitive to the concentration of 100 mg/mL.

Table 4:- Inhibition zone diameters (mm) with Aqueous Extract (EA) of Myrianthus holstii bark and antibiotics on the strains tested (n = 3).

Tested strains	Concentrations (mg/mL)					Antibiotics	
	C1=200	C2=100	C3=50	C4=25	Ts	AMC/CRO/ IPM	OXA/ FOX
Salmonella Typhi	8±0.2	6±0.0	6±0.0	6±0.1	6±0.0	6	Nd
K. Pneumoniae	12±1.0	10±0.1	8±0.3	6±0.0	6±0.0	6	Nd
K. Pneumoniae BLSE	6±0.3	6±0.3	6±0.1	6±0.3	6±0.0	8	Nd
E. coli BLSE	12±0.3	10±1.0	8±0.2	6±0.1	6±0.0	10	Nd

S. aureus Méti-R	10±0.1	8±0.2	6±0.0	6±0.2	6±0.0	Nd	6
E. coli ATCC 25922	12±0.3	10±0.0	8±0.3	6±0.2	6±0.0	8	Nd
S. aureus ATCC 25923	12±1.0	10±0.3	8±0.3	6±0.0	6±0.0	Nd	8
P. aeruginosa ATCC 27853	6±0.0	6±0.0	6±0.3	6±0.1	6±0.0	Nd	6

T = 0 : Including Control = the diameter of the wells (6mm) with DMSO / water (0.5: 0.5, V/V) ; CRO : Ceftriaxone (30µg), OXA: oxacillin (5µg); Meti-R: Methicillin-resistant; IMIP -I: Intermediate imipenem; ESBL: extended spectrum bêta-lactamase.

Antibacterial Parameters

Table 5 presents the Minimum Inhibiting Concentrations (MICs) of the ethanolic and aqueous extracts of the bark (EC and ECec. Ethanol extract 70% had minimum inhibitory concentrations (MICs) of between 100 and 3.12 mg/mL. Ethanolic extract from Myrianthus holstii stem barks had a bactericidal effect on 100% of bacterial strains, unlike aqueous extract. The S. aureus ATCC strain was the most sensitive to plant extracts with an IMC of 3.12 mg/mL.

Table 5:- Antibacterial Parameters of Ethanol and Aqueous Extract Fractions of M. holstii bark on in Vitro Growth of Test Organisms.

		BACTERIAL STRAINS							
Extracts	**Antibacterial parameters (mg/mL)**	**Salmonella Typhi**	**K. pneumoniae BLSE**	**K. pneumoniae**	**E. coli BLSE**	**S. aureus Méti-R**	**E. coli ATCC**	**S. aureus ATCC**	**P. aeruginosa ATCC**
EEec	**MIC**	100	12,5	100	50	12,5	12,5	3,12	50
	MBC	100	25	100	50	50	12,5	3,12	50
	MBC/MIC	1	2	1	1	4	1	1	1
	Effect	bactericidal	bactericidal	bactericidal	bactericidal	bactericidal	bactericidal	bactericidal	bactericidal
EAec	**MIC**	>100	>100	>100	50	12,5	50	12,5	>100
	MBC	>100	>100	>100	50	50	50	25	>100
	MBC/MIC	Nd	Nd	Nd	1	4	1	2	Nd
	Effect	-	-	-	bactericidal	bactericidal	bactericidal	bactericidal	-

MIC: Minimum Inhibitory Concentration; CMB : Minimum Bactericidal Concentration Nd : Not determined

Discussion:-

Based on the results obtained, the production of the crude extracts showed that the extraction yields vary considerably from one extract to another. Calculating yields allows quantitative assessment of the extractable values that can be derived from each species. These yields also make it possible to envisage the quantity of organs to be removed if necessary for a possible similar study, which would make the use of medicinal plants more rational and therefore sustainable of the species concerned. The results obtained may be lower than those ofKanoun et al. (2014), for the same organic solvent (70% ethanol) with the bark of Punica granatum which is of the order of 37%. The extracts were then subjected to a qualitative phytochemical analysis. Certain compounds are present in the ethanolic extract and absent in the aqueous extract of the same plant. And the presence of the majority of these metabolites sought was detected at the level of the extract EE. On the other hand, there is an absence of saponosides and alkaloids with the Dragendorff reagent in EA. This could be explained by the fact that ethanol concentrates more compounds than water. According to Turkmen et al. (2007), the absence of a compound could be explained by the nature of the solvent used, the study area or the quality of the soil. The study of antibacterial activity depends on several factors, namely the species of the plant, the method chosen for the preparation of the extract, the solvent used and the sensitivity of the bacterial species (Loziene et al., 2007). Antibacterial activity varies depending on the solvent used, the strain and the plant itself. In this study, the antibacterial activities of the two extracts (EEec and EAec) of the bark of M. holstii, yielded significant inhibiting zone diameters on the growth of all the germs tested:

S. aureus ATCC, S. aureus Méti-R, P. aeruginosa ATCC, Salmonella Typhi, Klebsiella pneumoniae BLSE and Klebsiella pneumoniae, Escherichia coli ATCC and Escherichia coli BLSE. With the exception of the aqueous extract bark (EAec) on the strains Pseudomonas aeruginosa ATCC and Klebsiella pneumoniae BLSE. Ethanolic Extract (Eec) also provided good inhibitory activity on the growth of the resistant, reference and sensitive strains tested. The inhibition diameters are between 8 and 16 mm for the ethanolic extract and between 08 and 12 mm for the aqueous extract. These diameters are comparable to those obtained by Sharmila & Gomathi (2011) on clinical strains producing betalactamine with total extracts of Terminalia glaucescens Planch ex. Benth. They are also comparable to those of Yéo et al. (2014), in roots of Cochlospermum planchonii used in the treatment of various infections with some of the same strains.

In addition, the CMB/CMI ratio made it possible to determine the bactericidal and bacteriostatic powers of the plant extracts. According to Berché et al. (1991), when this ratio is greater than 4, the extract is termed bacteriostatic and bactericidal when it is less than or equal to 4. These results have therefore made it possible to assert that each extract has demonstrated a bactericidal power against Staphylococcus aureus ATCC, Staphylococcus aureus Méti-R, Escherichia coli ATCC and Escherichia coli BLSE, since the values of the CMB/CMI ratios are less than or equal to 4. Taking into account the antibacterial parameters presented inTable V, it is found that on Staphylococcus aureus ATCC, the EE possesses the smallest CMB/MIC value (3.12 mg/mL). This means that this extract is the most active. The high EA activity may be due to a difference in concentration of the different chemical groups present in this extract. The aqueous extract of this plant had no activity on Klebsiella Pneumoniae BLSE, Salmonella Typhi, Staphylococcus aureus and Pseudomonas aeruginosa at concentrations of 100; 50 and 25 mg/mL. A similar finding was made by Ponce et al. (2003), in Indonesia in extracts of Crossandra infundibuliformis. The analysis of these results also shows that the combination of solvents (ethanol 70%, water 30%) is the method which makes it possible to better concentrate the active ingredients of M. holstii. Pseudomonas aeruginosa, Salmonella Typhi, and Klebsiella pneumoniae strains have increased resistance to amino penicillins, which remains of particular concern (Golstein, 2000 ; Bourjilat et al., 2009). The explanation lies in the ineffectiveness of the active molecules in this plant and in relation to the membrane structure and the origin of the strains. This observation could be explained by the nature or complexity of the structure and the nature of the bacteria (Gram- and Gram+). In fact, the wall of Gram+ bacteria consists almost exclusively of peptidoglycans, with teichoic acid polymers associated with them (Guinoiseau, 2011). This medicinal plant contains numerous bioactive principles which can provide a therapeutic response to the emergence of bacteria resistant to many conventional antibiotics.

Conclusion:-

The study of the antibacterial activity of plant extracts from the bark of this plant revealed that these phenolic compounds are active on the reference strain tested, Staphylococcus aureus. On the other hand, we note that the aqueous extract from the bark showed no detectable antibacterial activity against the strains Pseudomonas aeruginosa ATCC and Klebsiella pneumoniae tested. The results obtained in this work have shown that the antibacterial effect of the plant Myrianthus holstii on the deferent strains tested is significant. This efficacy is due to the presence of secondary metabolites known for their antibacterial effects. The tri-phytochemical analysis was used to demonstrate the antibacterial activity of polyphenols especially tannins and flavonoids.

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