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ANTIBACTERIAL ACTIVITIES OF THE EXTRACTS AND CONESSINE FROM
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Abstract

The methanolic extract and conessine isolated from the stem bark of *Holarrhena floribunda* (*Hf*) were tested for their antibacterial activities on *Bacillus*: *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megaterium* and *Bacillus stearothermophilus* using the disc diffusion method. Phytochemical analysis of the crude extract and fractions was also conducted. The inhibition parameters of the crude methanol extract and the total alkaloid fraction were determined using the macrodilution method. The results showed that the crude extract, the total alkaloid fraction and conessine exhibited a significant antibacterial effect against all the strains studied. The antibacterial effect of conessine is almost similar to that of chloramphenicol used as reference. The ratio of the minimal bactericidal concentration (MBC) over the minimal inhibitive concentration (MIC) indicates the bactericidal effect of the plant. These results support common use of stem bark of *Hf* and conessine isolated from *Hf* in the treatment of some infectious diseases.

Key words: Antibacterial activities, *Holarrhena floribunda*, *Bacillus*, conessine

Introduction

Holarrhena floribunda, a plant belonging to the *Apocynaceae* family (Letouzey, 1972), is widely distributed in the Centre and West provinces of Cameroon. Its stem bark is used in Cameroon to treat various ailments such as abdominal pains, nausea, indigestion, diarrhoea (Berhaut, 1971). However, little experimental studies have been done on the biological properties of this plant. The stem bark of *H. floribunda* is febrifuge and could be a quinine substitute, since it showed remarkable inhibitory activity against drug-resistant strains of *Plasmodium falciparum* (Fotie *et al.*, 2006). It contains conessine that is used for the destruction of amoeba without emetic effect. (Berhaut, 1971). However, there is no attempt to study the potential of *H. floribunda*'s antibacterial activities against microorganisms that cause food poisoning and food alteration. The present study was conducted to investigate the antibacterial properties of the crude methanol extract and conessine from the stem bark of *H. floribunda* against four strains of *Bacillus*.

Materials and methods

Plant material

The stem bark of *H. floribunda* was collected in Ndikinimeki, in the Centre Province of Cameroon, in January 2003. The biological identification of the plant was done by the National Herbarium in Yaounde, where the voucher specimen is conserved under the reference number, 20147/SFR/CAM.

Extraction and purification

The stem bark of *H. floribunda* was air dried and pulverized. The resulting powder (5.4kg) was macerated in methanol for 72h. The concentrated methanolic extract (224g) was treated with hydrochloric acid (5%). The aqueous solution was made alkaline with ammonia and extracted with ethyl acetate. The methanolic extract (A), the ethyl acetate extract (B) and the extract remain (C) were screened for antibacterial activity. The active fraction B was subjected to a bio-guided fractionation by flash chromatography on alumina column. Gradient elution using hexane (Hex) ethyl acetate (EA) to give eight fractions (F1-F8). The active fraction F1 (2.74g) obtained with Hex-EA (25-75%) was purified by flash chromatography on alumina column gradiently eluted with hexane-ethyl acetate to afford ten fractions (FA1-FA10). The active fraction FA1 was crystallised from acetone to yield 106 mg of shiny pink crystals identified as conessine on the basis of spectroscopic data and comparison of them with the data reported (Goutarel, 1964).

Microorganisms

The test microorganisms: *Bacillus cereus* F3748, *Bacillus subtilis* NCTC 3610, *Bacillus megaterium* 8174 were obtained from the Microbiology Laboratory, Institute of Food Research Reading, UK and *Bacillus stearothermophilus* CNCH 5781 from the Institute Appert, France.

Phytochemical tests

Phytochemical tests were carried out as described by Odebiyi and Sofowara, 1978. Each of the test was qualitatively expressed as negative (-) or positive (+).

Antibacterial activity

Disc diffusion method

The crude extract (A), total alkaloid fraction (B) and residue (C) were dissolved in the methanol to a final concentration of 10 mg/ml. Conessine was dissolved in methanol to a final concentration of 3 mg/ml. Antibacterial tests were then carried out by the disc diffusion method (Murray *et al.*, 1995) using 100 μ l of suspension containing 10^8 cfu/ml of bacteria spread on nutrient agar. Sterile paper discs (6mm in diameter) prepared from whatman number 1 filter paper were impregnated with A (1200 μ g/disc), B (500 μ g/disc), C (500 μ g/disc) and conessine (30 μ g/disc) placed on the inoculated agar. Negative control was prepared with methanol. Chloramphénicol (10 μ g/disc) was used as a positive reference standard for the susceptibility test. The inoculated plates were incubated at appropriate temperature for 24h. The antibacterial activity was evaluated by measuring the zone of inhibition against the test organisms. Each assay in this experiment was repeated twice. (Carbonnelle *et al.*, 1987).

Macrodilution assays

The minimal inhibitive concentration and the minimal bactericidal concentration values were also studied for the microorganisms that were determined as sensitive in the disc diffusion method. The bacterial strains were cultured overnight at appropriate temperature in nutrient agar. The test strains were suspended in nutrient broth to give a final density of 5×10^5 cfu/ml and these were confirmed by viable count. The crude extract and the total alkaloid in 10% DMSO were first diluted to the highest concentrations (1200 μ g/ml and 600 μ g/ml respectively), and then serial two-fold dilutions were made with nutrient broth in the concentration range from 37.5 to 1200 μ g/ml for the crude extract and 19 to 600 μ g/ml for the total alkaloid fraction in 10 ml sterile test tubes containing 1 ml of the inocula. The final volume in each tube was 5 ml (Zgodna and Porter 2001). The last tube containing 4 ml of nutrient broth without extract and 1ml of the inocula was used as a negative control. Tubes containing the same concentration of extracts without microorganism were also used as sterility control. Chloramphénicol at the concentration range of 5 to 80 μ g/ml was prepared in nutrient broth and used as positive control. The contents of each tube were mixed on a plate shaker at 300 rpm for 20s and then incubated at appropriate temperature for 18h. Bacterial growth was determined by absorbance at 600 nm using the spectrophotometer UV-120-01. These values of absorbance were used to draw a

concordance curve from which MIC, MBC and the dose that inhibits 50% growth after the incubation period (IC_{50}) were determined (carbonnelle *et al.*, 1987).

Results

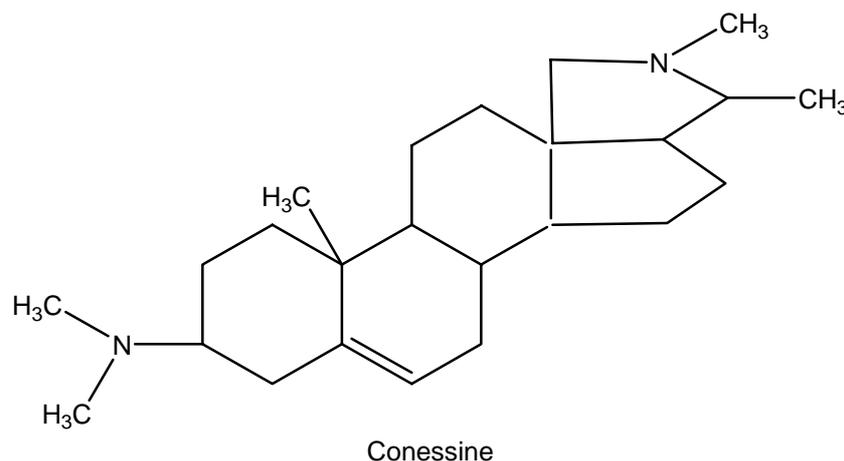
The results of our assay on the crude extract, the total alkaloid fraction and the residue are shown on Table 1. The crude extract showed the presence of alkaloids, saponins, tannins, polyphenols, cardiac glucosides, triterpenes, leucoanthocyanins and sugars; with the absence of phlobatannins, flavonoids, lipids, anthraquinones, sterols and glucosides. Saponins, tannins, polyphenols, cardiac glucosides, triterpenes, leucoanthocyanins, and sugars were detected in the residue.

Table 1: Phytochemical screening of the crude methanol extract, the total alkaloid fraction and the residue of *Holarrhena floribunda*.

Extracts	Chemical Groups													
	Alk	Sap	Tan	Glu	Pph	Cgl	Tri	Leu	Su	Phl	Fla	Lip	Anth	Ster
Crude Extract	+	+	+	-	+	+	+	+	+	-	-	-	-	-
Total alkaloid	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Residue	-	+	+	-	+	+	+	+	+	-	-	-	-	-

Alk: Alkaloids **Pph:** Polyphenols **Su:** Sugars **Anth:** Anthraquinones **Sap:** Saponins **Cgl:** Cardiac glucosides **Phl:** Phlobatannins **Ster:** Sterols **Tan:** Tannins **Tri:** Triterpenes **Fla:** Flavonoids **Glu:** Glucosides **Leu:** Leucoanthocyanins **Lip:** Lipids

The bio-guided purification of the total alkaloid fraction from the crude extract led to the isolation of the active compound. This compound was identified as conessine.



The results in Table 2 indicated that the crude extract, the total alkaloid fraction and conessine possessed significant antibacterial activities against all the microorganisms tested. The residue was inactive.

Figure 1 illustrates the zone of lysis by the disc diffusion method using the crude methanol extract, the total alkaloid fraction, the residue and conessine against four *Bacillus* species, compared to chloramphenicol. The antibacterial effect of these extracts and conessine was found to be comparable to the antibacterial activity exhibited by chloramphenicol.

The MIC, MBC, and IC_{50} were determined for the microorganisms that were identified as being sensitive to the extracts in the disc diffusion method. The results are shown in Table 3.

Table 2: Antibacterial activity of *Holarrhena floribunda* extracts from the disc diffusion method.

Extracts and compound tested	Inhibition zone diameters (mm)			
	Bacterial stains			
	<i>B. cereus</i>	<i>B. subtilis</i>	<i>B. megaterium</i>	<i>B. stearothermophilus</i>
Chloramphenicol (10 µg)	22	26	21	29
Crude extract (1200 µg)	17	18	19	22
Total alkaloid fraction (500 µg)	18	22	23	23.5
Conessine (30 µg)	18	23	23	24.5
Residue (500 µg)	-	-	-	-
Negative control	-	-	-	-

(-): absence of inhibition zones

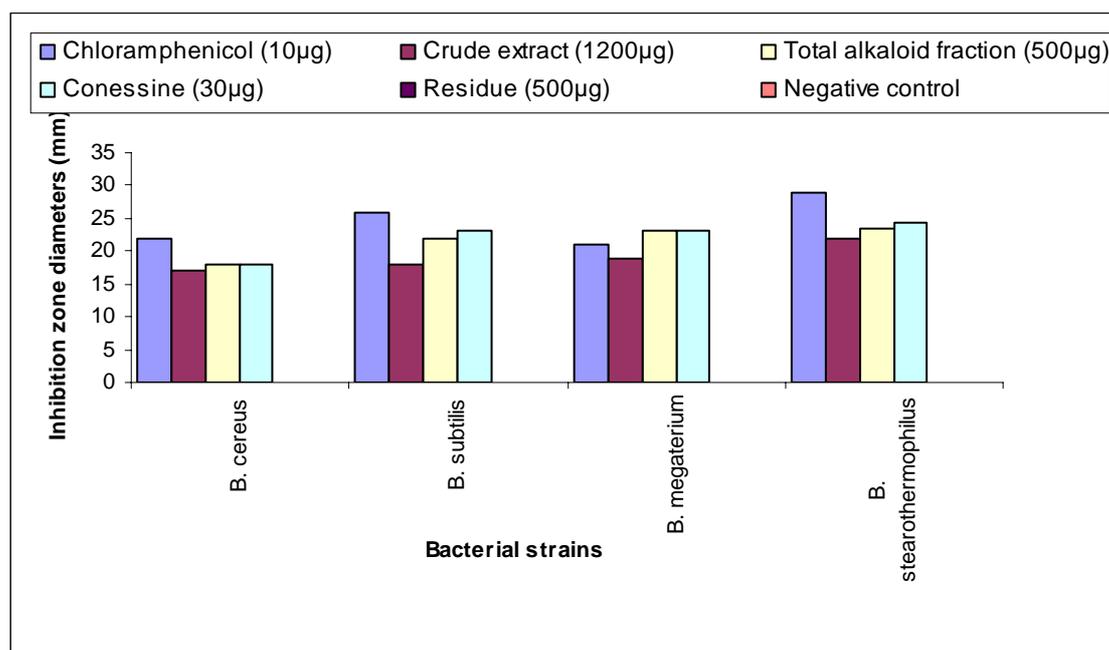


Figure 1: Zone of lysis measured in mm by disc diffusion method for the methanol crude extract, the total alkaloid fraction, the residue and conessine from *Holarrhena floribunda* on four *Bacillus* species compared to chloramphenicol.

Discussion

The extracts of *H. floribunda* have significant antibacterial properties against all the microorganisms tested. However, greater and remarkable antibacterial activities of the crude extract, the total alkaloid fraction and conessine were observed on *Bacillus stearothermophilus*. The inhibition zone values were in the range of 22 mm, 23.5 mm and 24.5 mm respectively at a concentration of 10 mg/ml (Table 2). Maximum MIC values were recorded with *Bacillus cereus* and were 215 µg/ml for the crude extract and 150 µg/ml for the total alkaloid fraction (Table 3). In the diffusion method in solid

medium, 30 µg of conessine and 10 µg of chloramphenicol showed almost similar antibacterial effects.

These results together with the phytochemical analysis, suggest that the biological activity obtained is attributed to conessine. The antibacterial activity of this compound is due to the formation of pores in the cell walls and the leakage of cytoplasmic constituents by this active compound present

Table 3: MIC, MBC and IC₅₀ values (µg/ml) of *Holarrhena floribunda* extracts in the macrodilution assays compared to chloramphenicol.

Inhibition parameters Bacterial strains	Crude extract				Total alkaloid fraction				chloramphenicol			
	MIC	MBC	IC ₅₀	$\frac{MBC}{MIC}$	MIC	MBC	IC ₅₀	$\frac{MBC}{MIC}$	MIC	MBC	IC ₅₀	$\frac{MBC}{MIC}$
<i>B. cereus</i>	215	390	110	1.8	150	280	70	1.8	18.5	33	9.5	1.8
<i>B. subtilis</i>	185	320	100	1.7	95	170	45	1.8	9	16	4.5	1.8
<i>B. megaterium</i>	175	317	90	1.8	90	160	40	1.8	36	70.5	18	1.9
<i>B. stearotheophilus</i>	115	210	55	1.8	50	105	30	2	8.5	14.5	4.5	1.7

in this plant (Gnanamani *et al.*, 2003). In all the cases, the ratio $\frac{CMB}{CMI}$ suggested that *H. floribunda* has a bactericidal effect.

Conclusion

Bacillus stearotheophilus, *B. cereus*, *B. subtilis* and *B. megaterium* were found to be sensitive to *H. floribunda* extracts. The results therefore, suggest that *H. floribunda* extracts possess antibacterial activity. The bio-guided purification of this plant showed that the antibacterial activity is attributed to conessine. This active compound can be exploited as an antibacterial agent for the production of new drugs against food poisoning.

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