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Short Communication *Pharmacology*

Phytochemical composition and in vitro effects of the ethyl acetate bark extract of *Distemonanthus benthamianus* Baillon *(Caesalpiniaceae) on Staphylococcus aureus* and *Streptococcus agalactiae*

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ABSTRACT

Distemonanthus benthamianus is a tree used in traditional African medicine to treat bacterial, fungal and viral infections. The pasty phase and the granular phase obtained from the ethyl acetate bark extract of *D. benthamianus* were tested for antimicrobial purposes on *Staphylococcus aureus* and on *Streptococcus agalactiae* alone and in combination with erythromycin. Only the pasty phase of the extract was active against the above bacteria (MIC = 1024 µg/mL ; MBC = 4096 µg/mL). The MIC of erythromycin against *S. aureus* and *S. agalactiae* was 8 µg/mL and 4 µg/mL, respectively. The MBC of erythromycin was 32 µg/mL against *S. aureus* and 8 µg/mL against *S. agalactiae*. The combination of that active phase (256 µg/mL) with erythromycin (1 µg/mL) was synergistic and the ratio MBC / MIC was 4 suggesting that the plant extract may be a bactericidal agent. Phytochemical analysis revealed the presence of flavonoids and phenolic compounds in the pasty phase of the plant extract and the absence of sterols, triterpenes and alkaloids in the same phase. In contrast, the granular phase contained all the above compounds except flavonoids. This result suggests that the active principle may belong to the class of flavonoids.

Key words: *Distemonanthus benthamianus*, MBC, MIC, *Staphylococcus aureus*, *Streptococcus agalactiae.*

RESUME

Distemonanthus benthamianus est un arbre utilisé en médecine traditionnelle africaine pour traiter les infections bactériennes et virales, ainsi que les mycoses. Les effets antibactériens des phases pâteuse et granulaire obtenues à partir de l'extrait à l'acétate d'éthyle de l'écorce de *D. benthamianus* ont été testés sur *Staphylococcus aureus* et sur *Streptococcus agalactiae* seule ou en association avec l'érythromycine. Seule la phase pâteuse a été active sur les bactéries évoquées (CMI = 1024 µg/mL ; CMB = 4096 µg/mL). La CMI de l'érythromycine vis à vis de *S. aureus* et de *S. agalactiae* a été de 8 µg/mL et de 4 µg/mL, respectivement. La CMB de l'érythromycine a été de 32 µg/mL sur *S. aureus* et de 8 µg/mL sur *S. agalactiae*. L'association de la phase pâteuse (256 µg/mL) avec l'érythromycine a été synergique et le rapport CMB / CMI a été de 4. Ceci montre que cet extrait de plante pourrait être un agent bactéricide. L'analyse phytochimique a révélé la présence des flavonoïdes et des composés phénoliques dans la phase pâteuse de l'extrait et l'absence des stérols, des triterpènes et des alcaloïdes dans cette même phase. Par contre, tous les composés sus-cités à l'exception des flavonoïdes ont été notés dans la phase granulaire. Ce résultat suggère que le principe actif appartiendrait à la classe des flavonoïdes.

Mots clés : *Distemonanthus benthamianus*, CMB, CMI, *Staphylococcus aureus*, *Streptococcus agalactiae.*

INTRODUCTION

Staphylococcus aureus and *Streptococcus agalactiae* are gram-positive cocci responsible for various human infections including skin diseases, urogenital tract infections, endocarditis and osteomyelitis. Although a large number of antibiotics have been manufactured to overcome these microorganisms, clinicians face the problem of drug resistance. Therefore there is need for development of new antimicrobial drugs with greater activity and stability. Some tropical plant species play an important role in the biochemical resistance mechanism of plants against pathogenic organisms [1]. *Distemonanthus benthamianus* is a tree moderately resistant to fungal attack. Thus it has the potential to be exploited as a source of new antibiotic. The bark of *D. benthamianus* is ground up and used for dressings and for other medicinal purposes such as the treatment of shingles and skin diseases [2]. In Cameroon, *D. benthamianus* is used in the treatment of uroginital tract infections and mostly female genital tract infections caused by numerous microorganisms.

The purpose of this work was to evaluate the in vitro antimicrobial effects of the ethyl acetate bark extract of *D. benthamianus* on *S. aureus* and *S. agalactiae* with a view to assessing the therapeutic value of the extract that might prove valuable for management of the above mentioned human diseases.

MATERIALS AND METHODS

Plant sample

Fresh bark of *Distemonanthus benthamianus* was collected at Eloumden (Yaounde-Cameroon) in February 2001 at about 4.00 p.m. and identified by Tsabang Nole from the institute of medicinal researches and studies of

medicinal plants of Cameroon under voucher s specimen n° TN 275.

Bacteria strains and culture media

Bacteria used in this study were *Staphylococcus aureus* isolated from a patient's pus and *Streptococcus agalactiae* isolated from vaginal smears in the laboratory of bacteriology of the CHU Yaounde. Pathologic samples were cultured on chapman medium for *S. aureus* or on 5% sheep agar for *S. agalactiae*. *S. aureus* was grown at 37°C with aeration for 24h and *S. agalactiae* was grown at 37°C in a closed jar system for 24h. *S. aureus* colonies appeared yellow surrounded by a yellow area indicating mannitol fermentation. *S. agalactiae* appeared greenish and surrounded by a clear zone of haemolysis. Identification of bacteria was achieved using Gram stain reaction, catalase test and latex test agglutination (Pastorex biorad). One colony of each bacterium was then subcultured on trypticase soy agar (TSA) for *S. aureus* or on blood agar for *S. agalactiae*. For susceptibility testing bacteria were cultured in broth media at 37°C for 18h (Trypticase soy broth for *S. aureus*, and buffered dextrose broth for *S. agalactiae*). Trypticase soy broth (TSB) and TSA were purchased from Biomerieux. Buffered dextrose broth (BDB) from Sanofi or on blood agar from Biomerieux.

Preparation of the plant extract

D. benthamianus bark was sun-dried and ground into a powder. The powder obtained (4 kg) was macerated in ethyl acetate for 4 days at room temperature. The residue was also extracted three times as described above. The filtrate from different extractions was concentrated under reduced pressure to obtain 800g of ethyl acetate bark extract with a yield of 20% that presented two distinct phases; a dark pasty phase and a yellow granular phase. Both phases were separated by decantation and used in our study. 225.28 mg of each phase of the plant extract was dissolved in 5% dimethylsulfoxide (DMSO) and the solution was adjusted with sterile distilled water to obtain a final extract solution concentration of 45.056 mg/mL.

Phytochemical analysis

The test of Lieberman Buchard for sterols and triterpenes detection was conducted as follows: 2 mL of methylene chloride were poured on 1 mg of each phase of the plant extract. A few drops of acetic anhydride and a few drops of concentrated sulphuric acid were then added. The presence of sterols and triterpenes was noted by the appearance of green and violet colours, respectively.

The test of flavonoids consisted in adding 1g of the extract to 5 mL of pure methanol. After that, a few drops of concentrated hydrochloric acid and magnesium chips were introduced in the medium. The test was considered positive when a red or a violet colour was observed.

The test of alkaloids: 1 mg of extract was dissolved in 3 mL of methanol and 5 drops of Dragendorf reagent were added to the medium. The formation of a precipitate meant that the reaction was positive.

The test of phenols: in a test tube, 5 mg of the plant extract were dissolved in methanol and a few drops of iron chloride were added. The test was positive when a blue or a violet colour appeared.

Susceptibility testing

Minimal inhibitory concentrations (MICs) were determined by broth dilution method. Several drops of a 18h broth culture were diluted in 30 mL of TSB for *S. aureus* or BDB for *S. agalactiae* to obtain a final inoculum of 10^5 to 10^6 CFU/mL. The concentration of the inoculum was determined by means of a spectrophotometer Lamubda 1 Perkin-Elmer. 1 mL of that inoculum was distributed in many tubes. Immediately after, 100 µL of 2-fold serially diluted erythromycin (Maneesh pharmaceuticals) solution was introduced at the concentration range of 0 to 128 µg/mL and at the concentration range of 0 to 4096 µg/mL for both phases of the extract. An equivalent volume of either 5% DMSO solution or sterile distilled water was introduced into control tubes. MICs were read after a 24 h incubation period at 37°C. Minimal bactericidal concentrations (MBCs) defined as the lowest concentration that kills at least 99.9% of the initial inoculum were determined by subculturing 10 µL of each clear tube on TSA (*S. aureus)* or on blood agar *(S. agalactiae)* and incubated for 24 h at 37°C. Five replicates were done for each dose.

***Time* killing studies**

For time killing studies, antibiotics were used at ¼ of their MIC [3] and only the pasty phase of the extract (256 µg/ml) was used alone and in combination with erythromycin (2 µg/mL for *S. aureus*, 1 µg/mL for *S. agalactiae*). The extract was combined with erythromycin in view of determining the activity of the plant material. The granular phase could not be used here because it was not active. Samples of 100 µL taken after 0, 3, 6, and 24 h of antibiotic exposure were serially diluted and cultured onto TSA for *S. aureus* or onto blood agar for *S. agalactiae*. Colonies were counted after incubation for 24 h.

RESULTS

Phytochemical composition

Table 1 gives chemical composition of both phases of the extract. Phytochemical analysis revealed the presence of phenolic compounds and flavonoids in the pasty phase of the extract and the absence of sterols, triterpenes and alkaloids in the same phase, whereas the granular one contained sterols, triterpenes, alkaloids and phenolic compounds but lacked flavonoids.

Table. 1: Chemical composition of the different phases of the ethyl acetate bark extract of *Distemonanthus benthamianus*.

	Pasty phase	Granular phase
Sterols	–	+
Triterpenes	–	+
Flavonoids	+	–
Alkaloids	–	+
phenol	+	+

+ : Present – : absent

Susceptibility testing

Tubes that received either 5 % DMSO solution or sterile distilled water presented a cloudiness indicating bacterial growth. This means that neither 5% DMSO nor distilled water inhibited bacterial growth.

Time killing curves

D. benthamianus (256 µg/mL) did not inhibit *S. aureus* growth during the experiment compared to the initial inoculum and to the growth control. Erythromycin (2 µg/mL) had a bacteriostatic effect on staphylococcal growth after a 24 h incubation period. That bacteriostatic effect was enhanced in the presence of the plant extract (Figure.1).
D. benthamianus (256 µg/mL) was more active against *S. agalactiae* than against *S. aureus* with a decrease in the initial inoculum of 1.4 after 24h of incubation. Erythromycin (1 µg/mL) provoked a progressive fall in *S. agalactiae* population. The association of the extract with erythromycin was synergistic (Figure.2).

Table 2: MICs and MBCs of *Distemonanthus benthamianus* and erythromycin against *S. aureus* and *S. agalactiae*.

	S.aureus			*S. agalactiae*		
	MIC (µg/mL)	MBC (µg/mL)	MBC/MIC	MIC (µg/mL)	MBC (µg/mL)	MBC/MIC
Pasty phase of *D. benthamianus*	1024	4096	4	1024	4096	4
Granular phase of *D. benthamianus*	>4096	>4096		>4096	>4096	
Erythromycin	8	32	4	4	8	2

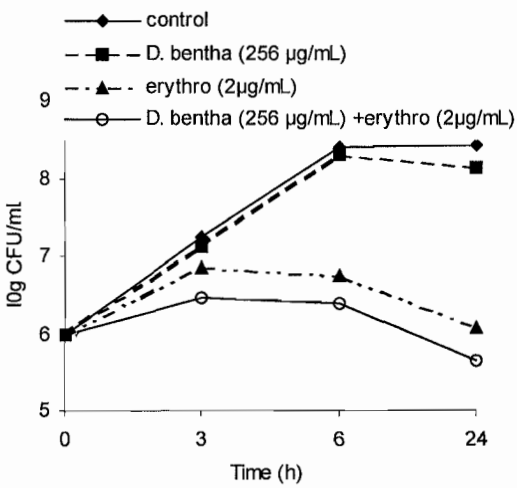

Figure. 1: Time killing curves of *Distemonanthus benthamianus* (D. bentha), erythromycin (erythro), and *D. benthamianus* plus erythromycin

DISCUSSION

The present study was undertaken to evaluate the *in vitro* antibacterial effects of *Distemonanthus benthamianus*. To achieve this aim the dilution method was preferred to the diffusion method, which is strongly recommended for assessing antimicrobial activity of a plant material. *Staphylococcus aureus* was chosen because it has already developped resistance to almost all antibiotics. *Streptococcus agalactiae* was mostly isolated from women who were consulted for genital tract infection when we started this study. The results of susceptibility testing indicate that the plant material possesses antimicrobial effects depending on the ethyl acetate phase of the extract tested. The pasty phase was active against *S. aureus* and *S. agalactiae* whereas the granular phase was not. So, the pasty phase of *D. benthamianus* ethyl acetate bark extract contains substances capable of inhibiting staphylococcal and streptococcal growth. This pharmacological difference could be explained by the difference in the chemical composition of the two phases. The potency of the active phase might be due to flavonoids which are a type of phenolic compounds that were present only in the active pasty phase. It has been reported that some phenolic compounds such as griseofulvin possess antimicrobial properties [4]. The pasty phase ratio MBC / MIC was 4, suggesting a bactericidal effect. In order to verify that hypothesis time killing curves have been constructed.

Time killing curves showed a bactericidal effect of the pasty phase of the extract on *S. agalactiae* at a sub-inhibitory concentration whereas at this same concentration the pasty phase of *D. benthamianus* was not active against *S. aureus*. The combination of the pasty phase of *D. benthamianus* with erythromycin was synergistic in both *S.*

aureus and *S. agalactiae*. It is known that the combination of two bactericidal antibiotics may be synergistic [5, 6]. It has been observed that the extract, like erythromycin was able to inhibit streptococcal growth even at a sub-inhibitory concentration. Exposure of some bacteria strains to sub-inhibitory concentrations of erythromycin affected the composition of their cell walls [7]. In our own study, it is impossible at this level to determine by which mechanisms *D. benthamianus* at ¼ of its MIC lowers *S. agalactiae* growth. The above results confirmed that the active phase of the ethyl acetate bark extract of *D. benthamianus* acts as a bactericidal agent. Compared to erythromycin the pasty phase of the plant extract that contains a variety of active principles appeared to be less potent.

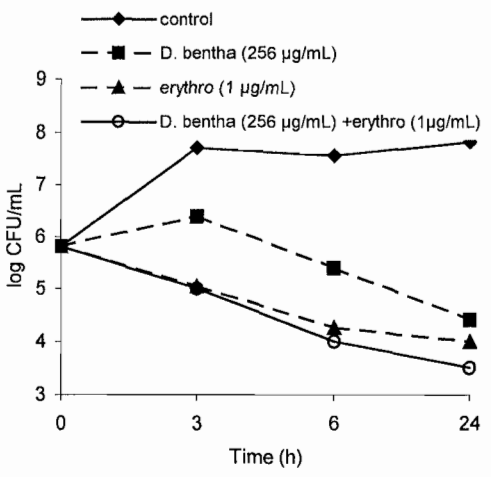

Figure. 2: Time killing curves of *Distemonanthus benthamianus* (D. bentha), erythromycin (erythro), and *D. benthamianus*.

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