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In vitro Antiplasmodial and Antibacterial Activities of *Canthium multiflorum* Schum and Thonn (Rubiacea) Extracts

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Abstract: Because of the resistance of pathogens to actually available drugs, there is a continuous need for new agents to cure several diseases such as malaria and microbial infections. In the establishment of new drugs, plant are often a source of new leads, indeed aqueous, acetone and methanol extracts of *Canthium multiflorum* were tested for antiplasmodial and antibacterial activities. The antiplasmodial activity was performed on fresh clinical strains of *Plasmodium falciparum* using light microscopy. The results revealed that the methanol extract was the most active with IC_{50} of 4.69 μg mL^{-1}. The NCCLS microdilution method performed on clinical and reference strains of pathogenic bacteria yielded MIC and MBC values ranging from 312 to 1250 and 625 to 2500 μg mL^{-1}, respectively. The qualitative analysis of the extract revealed the presence of several chemical groups such as alkaloids, terpens and tannins that might be responsible for the activity of the plant. The issue of this study showed that *C. multiflorum* is a plant that many attention should be paid to because of it pharmacological potentials.

Key words: Canthium multiflorum, *Plasmodium falciparum*, pathogenic bacteria, plant extract

INTRODUCTION

Malaria is the most widespread parasitic disease in the world. It daily kills 3000 people approximately, 75% of the victims being children under 5 years (WHO, 2009). In the developing countries where are found more than 90% of the malaria-related deaths, the population continues to depend on traditional medicine for the treatment of the disease. Indeed, more than 80% of the population use products from traditional medicine for the treatment of their diseases (WHO, 2008). In addition to malaria, bacterial and fungal infections continue to cause many problems in health care systems, particularly in these developing countries where low levels of hygiene and sanitation expose the people to a wider array of microbial pathogens. It is reported that each year, 300,000 children die of diarrhoeal diseases, the most common microorganisms responsible being *E. coli*, *Shigella*, *Salmonella* and *Yersinia* (Simpore *et al*., 2009; Bonfiglio *et al*., 2002). In many regions, affected by these infections, local and indigenous plants are often the only available means of treating such infections. However, very few scientific data exist on the efficiency of these plants (Karou *et al*., 2007a, b).

Canthium multiflorum Schum and Thonn (Rubiacea) is a shrub or a tree widely distributed in Africa and wildely used in traditional medicine. The native area of *C. multiflorum* extends from Senegal to Soudan and from Gabon to Centrafric Republic. It grows preferentially on clay ground, laterite ground or near rivers. In folk medicine, leaves decoction of the plant is used to treat several diseases including fever, malaria, headache, oedema and rheumatism (Devineau, 1982). In Burkina Faso and Ivory Coast where the plants is locally called laagui fofana the leaves of the plant are used against diarrhoea, conjunctivitis, mycoses and other infectious diseases.

According to the several uses of the plant, it is being screened in the laboratories. Recently, Traoré *et al*. (2008) have isolated a new ursane derivative, 3-oxo-15-alpha, 19-alpha-dihydroxyursa-1,12-dien-28-oic acid from the roots of the plant, however there is a lack of scientific data about the antimalarial and the antibacterial activities of the plant. The present study aimed to described the *in vitro* antimalarial and antibacterial activities of the crude extracts of the plant.

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MATERIALS AND METHODS

Plant materials: The leaves of *C. multiflorum* were selected according to their traditional uses. The samples were harvested in January 2006 in Badara, a mountainous region in the Western Burkina Faso and were botanically authenticated at the Department of Plant Biology and Ecology of University of Ouagadougou where a voucher specimen (BC-cm07) was deposited.

Compound extractions and qualitative analysis: The harvested samples were dried in the laboratory at room temperature, afterwards they were pulverized with a mechanical crusher. The powder was used for several extractions. For aqueous extraction, 50 g of powder were boiled with reflux for 30 min in 300 mL water. After cooling at room temperature, the extract was filtered and lyophilised. For methanolic extraction, 50 g of powder were percolated in 500 mL of 70% methanol for 24 h. Methanol was evaporated with a rotary evaporator and the extract was lyophilised. For acetone extract, 50 g of powder were percolated with 500 mL of 70% acetone for 24 h. The extract was then washed with hexane in order to eliminate the chlorophyll and other pigments. The solvent were evaporated and the extract was finally lyophilised. Qualitative tests were performed on each extract for chemical groups such as alkaloids, steroids and triterpens, coumarins, flavonoids, tannins and anthocyanosides detection as described by Ciulei (1982).

Parasites: *Plasmodium falciparum* strains used were fresh clinical isolates obtained from symptomatic children, who did not take any antimalarial drug and coming for thick smear analysis in the primary health care center of Goupana, a village around Ouagadougou. Giemsa-stained blood smear were examined for the parasite identification and quantification. The parasite density was determined by counting the number of infected erythrocytes among 20,000 erythrocytes. From each patient with monoinfection due to *P. falciparum*, 4 mL of venous blood was collected in a tube coated with EDTA (Greiner Laboratechnik) for parasite culture.

Microbial strains: Microorganisms used in the present study involved reference and clinical strains. Reference strains were *Bacillus cereus* LMG 13569, *Enterococcus faecalis* CIP 103907, *Escherichia coli* CIP 105182, *Listeria innocua* LMG 1135668, *Salmonella enterica* CIP 105150, *Shigella dysenteriae* CIP 5451, *Proteus mirabilis* CIP 104588, *Staphylococcus aureus* ATCC 9244, *Staphylococcus aureus* ATCC 25293 and *Staphylococcus camorum* LMG 13567. Clinical strains were *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pyogenes*. These strains were isolated at the Laboratoire de Biologie Médicale Saint Camille in Ouagadougou. The identification of these bacteria was based on their biochemical profiles as recommended by the manual Bactériologie Médicale (Le Minor and Veron, 1984).

Antiplasmodial assay: Parasites were grown *in vitro* in a 96-well plate according to the methods described by Trager and Jensen (1976). Briefly, blood samples were washed three times with RPMI 1640 medium (Gibco-BRL). Then, RPMI supplemented with 4, 2 mM L-glutamine (Gibco-BRL), 10% bovin serum (Sigma St. Louis), 25 mM HEPES (Sigma St. Louis) 100 IU mL^{-1} streptomycin/ Penicillin (Gibco-BRL) was used to suspend washed erythrocytes. The haematocrit was 5%. The 96-well plates were filled with 200 μL per well of this preparation.

Aqueous and acetonic extracts and chloroquine phosphate were dissolved in distilled water. Methanolic extract was dissolved in dimethyl sulfoxide (DMSO) and a serial dilution was made in the wells to have a final concentration of 0.5% DMSO in first wells. Extracts concentrations ranged from 2000 to 3.2 μg mL^{-1} in the wells. Each concentration was made in triplicate. Plates were placed in a CO_2 incubator at 37°C with 5% CO_2 for a total period of 24-30 h. The antimalarial activity was determined by light microscopic method using Giemsa-stained smears as described by Le Bras and Deleron (1983). Parasite maturation was determined by counting mature schizonts among all asexual parasites for 20,000 erythrocytes. The concentrations causing 50% inhibition of the maturation (IC_{50} values) were determined from parasites growth percentages using regression equations.

Antibacterial assays

Agar disc diffusion: The *in vitro* susceptibility tests were performed with aqueous and methanolic extracts by the paper disc diffusion method (Ayandele and Adebiyi, 2007) using Mueller-Hinton agar plates. Briefly, microorganisms from growth on nutrient broth incubated at 37°C for 18 h were suspended in saline solution 0.85% NaCl and adjusted to a turbidity of 0.5 Mac Farland standards corresponding to 10^8 cfu mL^{-1}. The suspension was used to inoculate 9 cm diameter Petri plates with a sterile non toxic cotton swab. Six millimetre paper discs (Whatman No. 3) soaked with 10 μL of 10 mg mL^{-1} extract. Aqueous extract was disolved in distilled water, while methanolic extract was dissolved in DMSO and diluted with distilled water to have a final concentration of 1% DMSO. The plates were incubated aerobically at 37°C for 18 to 24 h. All tests were performed in duplicate and the antibacterial activity was expressed as the mean of Inhibition Zone Diameters (IZD) produced.

Broth microdilution assay: A microdilution broth susceptibility assay was used, as recommended by the National Committee for Clinical Laboratory Standards (2002) for the determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC). These tests were performed in 96 well-plates. The methanolic extract was properly prepared, sterilized by filtration with a micro filter (0, 22 μm) and transferred in plates to obtain a twofold serial dilutions ranging from 10 to 2500 μg mL^{-1}. Then plates were inoculated with diluted microbial suspensions to have 10^5 cfu mL^{-1} in each well. The final volume in each well was 200 μL. After 24 h incubation, bacterial growth was indicated by the presence of turbidity and pellet in wells. Minimum Inhibitory Concentration (MIC) was defined as the lowest extract concentration demonstrating no visible growth in the broth and the MBC was defined as the lowest concentration of the extract killing 99.99% of the initial inoculum. Minimum Bactericidal Concentration (MBC) values were determined by removing 100 μL of bacterial suspension from subculture demonstrating no visible growth and inoculating nutrient agar plates. Plates were incubated at 37°C for a total period of 48 h.

Time-kill assay: Two reference strains (*Escherichia coli* CIP 105182 and *Salmonella enterica* CIP 105150) were chosen arbitrary for time-kill assay as described by Karou *et al.* (2006). For this test, the same 0.5 Mac Farland standards microbial suspensions were diluted in 50 mL of nutrient broth to have approximately 10^5 cfu mL^{-1}. The methanolic extract was added at the concentration of 2500 μg mL^{-1} for *E. coli* and 1250 μg mL^{-1} for *S. enterica*. These concentrations correspond respectively to 2 MBC of each bacteria. The cultures were incubated aerobically at 37°C in an incubator shaker (INNOVA™ 4000). At 0, 1, 2, 3, 4, 5, 7, 8, 9, 10, 11 and 12 h, an aliquot of 100 μL was removed and diluted with 10 mL sterile isotonic water. The obtained suspension was used to inoculate 90 mm diameter Petri plates previously filed with PCA (Plate Count Agar). After 48 h incubation at 37°C, The number of viable bacteria was evaluated by cfu counting. The experiment was carried out twice.

RESULTS

Phytochemical screening: The phytochemical screening of the extracts was first performed to detect the major chemical groups occurring in the extracts. The results of this screening are shown in Table 1. Among the extracts, the methanolic one was the extract that contained the most detected chemical groups.

Antiplasmodial activity: The antiplasmodial assay was performed on three fresh clinical isolates of *P. falciparum*. Both isolates were tested against chloroquine phosphate, aqueous, methanol and acetone extracts. The percentages of mature schizontes in the control wells were greater than 50% and DMSO did neither affect parasites growth nor their morphology as observed in control well with 0.5% DMSO only. Extracts showed different activities on parasite growth, with IC_{50} values ranging from 4.68 to 16.62 μg mL^{-1}. According to Table 2, methanol extract was the most active with IC_{50} value of 4.68 μg mL^{-1}.

Table 1: Chemical groups detected in *C. multiflorum* extracts

Extracts	Acetone	Methanol	Aqueous
Saponosids	+	-	+++
Tannins and polyphenols	+++	+++	+++
Reducing compounds	-	+++	-
Flavonoids	+++	+++	+++
Emodols	-	+	-
Alkaloids	-	+++	+
Proanthocyanidins	++	++	++
Anthracenosides	-	++	-
Coumarins	-	+	-
Triterpens and sterols	-	+++	-
Carotenoids	-	+++	+
Bial reaction	-	++	-
Foulger reaction	-	++	-

-: Not detected, +: Rare, ++: Abundant, +++: Very abundant

Table 2: IC_{50} values of *C. multiflorum* leaves extracts on *P. falciparum* strains

Extracts	Acetone	Aqueous	Methanol	CQP
IC_{50} (μg mL^{-1})	16.62±7.44	9.49±3.59	4.69±1.06	0.03±0.01

CQP: Chloroquine phosphate. Values are the means of different tests followed by SD

Table 3: Inhibition zone diameters (mm) recorded in agar disc diffusion with *C. multiflorum* extracts

Extracts	Methanol	Aqueous
Escherichia coli CIP 105182	15±1	13±1
Enterococcus faecalis CIP 103907	11±2	9±1
Bacillus cereus LMG 13569	18±1	13±1
Proteus misrabilis CIP 104588	17±1	11±1
Staphylococcus aureus ATCC 25293 BHI	15±1	12±1
Staphylococcus camorum LMG 13567	17±1	15±1
Proteus mirabilis 104588 CIP	14±1	12±1
Shigella dysenteria CIP 5451	15±1	13±1
Staphylococcus aureus ATCC 9244	15±1	14±1
Salmonella enterica CIP 105150	18±1	16±1
Pseudomonas aeruginosa (Clinical isolate)	0	0
Staphylococcus aureus (Clinical isolate)	15±1	12±1
Streptococcus pyogenes (Clinical isolate)	0	0

Antibacterial activity: The antibacterial assays in this study were performed by the disc diffusion and the broth microdilution methods. The activity was qualified and quantified by inhibition zone diameters, MIC and MBC values. According to Table 3, the susceptibility of bacteria to the extracts, on the basis of inhibition zone diameters varied. A positive result was defined as an inhibition zone diameter of 9 mm or more around the paper disc (Kitzberger *et al.*, 2006). According to our results, excepted *P. aeruginosa* and *S. pyogenes*, all bacteria were susceptible to both extracts. But globally, the highest IZD values were recorded with methanolic extract.

Table 4: MBC and MIC values determined by microdilution method

Extracts	MIC	MBC
	----------(μg mL^{-1})----------	
Escherichia coli CIP 105182	625	1250
Enterococcus faecalis CIP 103907	1250	2500
Bacillus cereus LMG 13569	625	2500
Listeria innocua LMG 1135668	625	1250
Staphylococcus aureus ATCC 25293 BHI	1250	>2500
Staphylococcus camorum LMG 13567	625	1250
Proteus misrabilis CIP 104588	1250	>2500
Shigella dysenteria CIP 5451	625	>2500
Staphylococcus aureus ATCC 9244	1250	2500
Salmonella enterica CIP 105150	312	625
Pseudomonas æruginosa (Clinical isolate)	2500	>2500
Staphylococcus aureus (Clinical isolate)	312	625
Streptococcus pyogenes (Clinical isolate)	2500	>2500

Table 5: Viability of microorganisms after 11 h exposure to the *C. multiflorum* methanolic extract

Time (h)	0-3	4	5	6	7	8	9	10	11
E. coli CIP 105182	uc	uc	8.10^4	6.10^4	7.10^3	3.10^3	2.10^3	2.10^3	0
S. enterica CIP 105150	uc	7.10^4	8.10^3	4.10^3	2.10^3	10^3	0	0	0

uc: Uncountable, the viability is expressed as number of cfu mL^{-1}

Since, the methanolic the highest inhbition zones were recorded with the methanolic extract this extract was used for the microdilution assay. The assay gave MIC values ranging from 312 to 1250 and 625 to 2500 μg mL^{-1} for MBC (Table 4). The lowest MBC was obtained with *Salmonella enterica* CIP 105150 and *Staphylococcus aureus* (Clinical isolate). In order to appreciate the effect of the extract on bacteria, MBC/MIC ratios were evaluated, ratios greater than 1 were considered as microbiostatic, while others were microbicide. On this basis, the extract was bactericid on *E. coli*, *E. faecalis*, *S. camorum*, *S. aureus*, *S. enterica* and bacteriostatic on *B. Cereus*.

In order to follow the bactericidal effect of the methanolic extract on bacteria in an inoculum as a function of the time, time-kill assay was performed with *E. coli* CIP 105182 and *S. enterica* CIP 105150 arbitrarily selected among the test microorganisms. The results showed that after 8 and 10 h exposition for *E. coli* CIP 105182 and *Salmonella enterica* CIP 105150, respectively. There were no viable microorganism in the initial inoculum (Table 5) and the effect of extract was faster on *S. enterica* than it was on *E. coli*.

DISCUSSION

The aim of this study was to evaluate the antiplasmodial and the antimicrobial activities of leaves extracts of *C. multiflorum*. Indeed, a phytochemical screening was first performed on the extracts. The results showed that several chemical groups such as tannins or alkaloids often encountered in plant extracts occurred in *Canthium multiflorum*.

The antiplasmodial assay revealed that methanol extracts was the most active on the parasite strains. According to the phytochemical screening, this extract contains several chemical groups that may be responsible for the antimalarial activity. Amongst theses groups alkaloids and terpenoids can be cited. Their antiplasmodial activity is well documented. An alkaloid is a plant-derived compound that is toxic or physiologically active, contains nitrogen in a heterocyclic ring, is basic, has a complex structure and is of limited distribution in the plant kingdom. Many plants such as *Sida acuta*, *Pavetta crassipes* or *Mytragina inermis* were found to exert antimalarial activity related to their alkaloids contents (Karou *et al*., 2003; Sanon *et al*., 2003a, b). Alkaloids exert their antimalarial activity trough different mechanisms including DNA sequence intercalation. For terpnoids compounds many authors have reported their antimalarial properties. For example, a tritepenoid compound named azadirachtine with a good antimalarial activity has been isolated from *Azardirachta indica* (Khalid *et al*., 1989) and the most efficient antimalarial drug today, artemisin is a terpenoid compound isolated from *Artemisia annua* a plant previously used in chineese traditional medicine.

These chemical group may also be responsible for the recorded antibacterial activity in the present study in addition to phenolic compounds. As example for alkaloids, their antibacterial properties have been demonstrated by Karou *et al*. (2006). Various studies have previously reported the antibacterial activity of phenolic compounds such as flavonoids coumarins and tannins (Karou *et al*., 2005; Ouattara *et al*., 2007; Shan *et al*., 2007). Tannin is a general descriptive name of polymeric phenolic substances capable of tanning lather or precipitating gelatine from solution, a property known as astringency. Their molecular weights range from 500 to 3000. The inhibition of microorganisms by phenolic compounds may be due to iron deprivation or hydrogen bounding with vital proteins such as microbial enzymes (Scalbert, 1991).

The results of the present study confirm the traditional use of the leaves of *C. multiflorum* for the treatment of malaria and several diseases such as diarrhoea and suggest that a great attention should be paid to this plant which is found to have the potential source of pharmacological leads. The study represents the preliminary report on antiplasmodial and antimicrobial activity of the crude extracts of the plant. Further investigations are undergoing for the isolation and the identification of active principles.

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