Antiplasmodial and antimicrobial potential of Canthium subcordatum extracts and isolates

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ABSTRACT

Phytochemical studies on the stem bark of Canthium subcordatum afforded eight known compounds: β-sitosterol (1), ursoic acid (2), ceribal (3), quinovic acid (4), Cerberinic acid (5), 3-O-β-D-glucopyranosylquinov acid (6), 3-O-β-D-glucopyranosyloleanolic acid (7) and Clemahexapetoside B (8). Interestingly, 3, 5 and 8 were isolated and characterized for the first time from the genus Canthium. Their structures were elucidated using their physical and spectroscopic data. Furthermore, antiplasmodial potency of Canthium subcordatum and antimicrobial activities of 6, 5 and 8 has not yet been investigated. Fractions and isolates were tested for their antiplasmodial potency. Only the methanol fraction inhibited the growth of P. falciparum with an IC₅₀ value of 3.044 µg/mL. The isolates were also tested against eight bacteria and fungi strains. 4 and 5 were the most active with inhibition diameter zones equal or above 12 mm and MIC equal or lower than 39 µg/mL.

Keywords: Canthium subcordatum; Rubiaceae; antiplasmodial; antimicrobial activity, macrocyclic glycoside.

INTRODUCTION

The genus Canthium is a genus of flowering plants in the Rubiaceae family. It is a poorly defined genus with about 50 species, including Canthium subcordatum in Africa and Asia. They are usually shrubs or small trees that are sometimes spiny. They can be recognized by their usually thorny stem, the leaves that are deciduous and usually placed opposite each other, the flowers that are hermaphroditic, small and usually green to white or pale yellow in colour and the fruits that are usually brown, yellow, or red; often drupaceous, subglobose, ellipsoid or discococcous when fully developed [1]. C. subcordatum is found in some countries in Africa. It is found in Southern Nigeria, Ivory Coast (Abidjan), Guinea (Mount Benna), Gambie, Ghana (Kumasi), Liberia (Kakatown), Sierra Leone, Angola, Sao Tomé and Cameroon (Mount Muanenguba, Nkongsamba-Loum, Idenau, Bomana and Mbalmayo) [2].

Plants of the Rubiaceae family have been shown to exhibit antimalarial, antimicrobial, anti-inflammatory and anti-inflammatory activities [3].

In folk medicine, the roots, leaves and stem bark of some species of Canthium are used for medicinal purposes including the treatment of malaria (Benin), fever, headache, conjunctivitis, diarrhoea (Burkina Faso), diarrhoea, fever, leucorrhoea, intestinal worms, and general debility (Ayurvedha), eczema (Ethiopia) sexually transmitted diseases (Guinea) and anti-venom in the treatment of snakebite and other wounds in some villages of Shimoga district in Karnataka, India. In Cameroon, the stem of Canthium sp. is used against ascariasis [4, 5].

The stem bark, leaves and roots of C. subcordatum have been exploited for their medicinal value: The stem bark Alcoholic extract has potential antidiabetic properties. Alcoholic extract of the roots is used for the treatment of fever, malaria, inflammation and cardiovascular diseases. Anti-inflammatory activity of the Petroleum ether and dichloromethane extracts of C. subcordatum has been reported [6]. Local traditional healers and some inhabitants of Mbalmayo, Centre Region, Cameroon reveal that the plant C. subcordatum is used by the local population to treat stomach disorders especially in the season when new crops are being consumed.

Malaria caused by the protozoan parasites of the genus Plasmodium remains one of the most important infectious diseases. P. falciparum is the species responsible for nearly all severe malaria cases and deaths [7]. History reveals that medicinal plants have always been considered as an important source of chemotherapeutic agents against malaria [8] such as quinine and artemisinin. Exploration of medicinal plants remains a promising strategy to identify new antimalarial agents.
To the best of our knowledge, very little phytochemical studies on C. subcordatum has so far been reported. Literature reports the isolation of iridoids and their derivatives. Also, C. subcordatum antiplasmodial potency has not yet been investigated. This study is aimed at investigating the antiplasmodial and antimicrobial activities of C. subcordatum extract as well as the isolated compounds.

**MATERIALS AND METHODS**

**General:** The masses of the isolates were taken using an electronic balance, mark MELTER PC 2000. A Büchi-540 apparatus was used to determine Melting points. Optical rotations (measured at room temperature) in CHCl₃ were realized on a Perkin Elmer Polarimeter (Model 241). ¹H-NMR and ¹³C-NMR spectra were recorded using a Bruker spectrometer containing 5 mm H and ¹³C probes, respectively, operating at 500 MHz and 125 MHz, using as internal standard, TMS. Flash and column chromatography was carried out on Silica gel 254-400 mesh (Merck) and silica gels (Merck 230-400 and 70-230 mesh), and TLC (with different eluent mixtures of ether, hexane, ethyl acetate, methanol and acetone) was done with pre-coated aluminium silica gel 60 F₂₅₄ sheets; spots viewed under UV lamps (254 and 365 nm) or sprayed with 50% H₂SO₄ reagent, heated and viewed.

**Plant Material:** The bark of Canthium subcordatum was harvested on the 28th of August 2008 from Mbalmayo, Centre Region of Cameroon, along the Nyong River. Mr. Nana Victor of the National Herbarium of Cameroon (Yaoundé), where a voucher specimen (N¹1957/SRF/CAM) is deposited, identified the plant.

**Extraction and isolation:** C. subcordatum stem bark was chopped, dried and ground into fine powder (2.6 kg). The powdered plant material (2.6 kg) was extracted by soaking in 8 litters of methylene chloride/methanol (1:1) solvent mixture for 5 days. After filtration, the extract was concentrated with the use of a rotary evaporator under reduced pressure. A methylene chloride/methanol (1:1) crude extract was obtained. This crude extract was further concentrated to dryness under vacuum at low temperature (30°C) to afford 95 g CHCl₃/MeOH extract. This crude extract was further fractionated into three fractions [Hexane fraction (3.3 g), Ethyl acetate fraction (76 g) and Methanol fraction (12.1 g)]. Based on the thin layer chromatography (TLC) profiles of the three fractions, 75 g of the ethyl acetate fraction was made to undergo flash chromatography over silica gel and eluted with solvents of increasing polarity (n-hexane, n-hexane-EtOAc, EtOAc and EtOAc-Methanol) and grouped using their thin layer chromatography profiles in 5 sub-fractions. Sub-fractions 1 to 4 were mixed and eluted with n-hexane-EtOAc solvent mixtures of increasing polarity, on silica gel to furnish 1 (4.3 mg), Hex/EtOAc (90:10); 2 (3.9 mg), Hex/EtOAc (82:18); 3 (5.8 mg), Hex/EtOAc (85:15); 4 (3.2 mg), Hex/EtOAc (80:20); 5 (4.9 mg), Hex/EtOAc (80:20); 6 (3.6 mg), Hex/EtOAc (30:70); 7 (4.2 mg), EtOAc/MeOH (90:10) and 8 (4.7 mg), EtOAc/MeOH (88:12).

**In vitro antiplasmodial against Plasmodium falciparum W2:** The experiment was conducted as previously described by Tchokouaha and collaborators (2015) using a Chloroquine and others antimalarial resistant strain, Plasmodium falciparum W2 maintained at 37°C in RPMI 1640, pH 7.4 containing 25 mM HEPES, 10% heat inactivated human serum and 2% hematocrit under 5% CO₂, 3% O₂ and 91% N₂ atmosphere [12]. Serial treatment with 5% Sorbitol (Sigma, Germany) allowed for the synchronization of malaria parasite in the ring stage (Lambros and Vanderberg, 1979). The compound stock solution was prepared at 1 mg/ml in DMSO, serially diluted in supplemented medium. Culture at 1% parasitemia and 4% hematocrit was treated with compounds in triplicate with the highest dilution being 10 µg/mL and DMSO concentration did not exceed 0.1% in the final reaction medium [9]. Negative control consisted in equal amount of DMSO while artemisinin (Sigma, Germany) served as positive control. After 48h incubation, parasites were fixed by 1% formaldehyde in PBS after removal of the medium. A 50 µL of each culture was added to a tube containing 0.5 mL of PBS supplemented with 1 nM YOYO nuclear dye (Molecular Probes) and 0.1% Triton X-100 then added to the 5 mL round-bottom polystyrene tubes. Becton-Dickinson FACSsort flow cytometer that count nucleated (parasitized) erythrocytes was used to monitor the parasitemia of control and treated cultures by CellQuest software was used for data acquisition. The concentration that inhibit 50% of the parasites growth (IC₅₀) was determined by non-linear regression using Prism 5.0 software (GraphPad, CA, USA) [12].

**Antimicrobial assay using Paper disk diffusion and Microbroth dilution methods:** A total of 8 microorganisms; 3 Gram-positive, 3 Gram-negative bacteria and 2 yeast species were tested. The Gram- bacteria species were Staphylococcus aureus, Staphylococcus saprophyticus, Streptococcus faecalis; and the Gram- bacteria species Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa and two yeast strains of Candida (Candida albicans and Candida krusei). The strains were clinical isolates obtained from patients diagnosed with urogenital tract infections consulting in Tiko Hospital (Cameroon). Appropriate cultural and biochemical procedures were used for their isolation and identification. Isolated colonies of new cultures of the different germs were diluted in 5 ml Nutrient broth (NB, Oxoid), in well-labeled sterile bottles, and incubated for 24 hours at 37°C before antimicrobial susceptibility testing.

**Reference antibiotics include Gentamycin (Sigma, USA) and Nystatin (Sigma, USA) for yeasts and bacteria species, respectively.**

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**Figure 1:** Isothermal curve of 1-8

A stock solution of pure compounds (1, 2, 3, 4, 5, 6, 7, 8, Gentamicin, Nystatin), was obtained with sterile distilled water (1 mg/mL) a volume of 50 µl was then imbibed on filter paper (Filter sterile papers 6 mm in diameter) at 120°C for 1 hr) to yield a 50 µg/disk. The discs of reference antibiotics were placed on the culture plates already inoculated with bacteria (Mueller Hinton Agar Plates) and and yeast (Sabouraud
Deaxuctose Agar Plates) and left at 37°C for incubation. The zones of inhibition were measured after 24 h [13, 14].

The minimum inhibitory concentration (MIC), which is the lowest concentration of the sample that inhibits visible growth of microorganisms, was determined by the micro broth dilution method [13, 14] in Mueller Hinton or Sabouraud broth containing 10% glucose and 0.5% phenol red supplements. A serial dilution was distributed from the first to the twelfth row on a 96 wells microplate of dry products (1, 2, 3, 4, 5, 6, 7, 8 and reference drugs (Gentamicin, Nystatin) initially in DMSO (20%) (100 μL) was made in microwells using supplemented Mueller Hinton or Sabouraud broth (50 μL). A total of 11 twofold dilution was made ranging from 2.4 to 2496 μg/mL.

After adding 50 μL of each dissolved sample successive dilutions were then carried out by transferring the mixture sequentially (50 μL) from the first to the eleventh well from which 50 μL was removed. The twelfth well well was used as growth control. The inoculum (50 μL, 10^5 colony forming units/mL (CFU/mL)) properly obtained were then added in each microwell. Tests were incubated aerobically at 37°C for 24 and 48 hours, for bacteria and fungi cultures, respectively. The Minimum Inhibitory Concentration was identified by monitoring any change in color from red to yellow due to the formation of acidic metabolites corresponding to microbial growth.

**RESULTS AND DISCUSSION**

Structures of the eight known isolates were established as β-sitosterol (4.3 mg) (1), ursolic acid (3.9 mg) (2), cerberin (an iridoid with Δ^5,7,8,9(14) -tetrane skeleton with 10-formyl and 11-carboxylic acid residues) (5.8 mg) (3) [15], quinovic acid (3.2 mg) (4), Cerberin acids (an iridoid with Δ^5,7,8,9(14) -tetrane skeleton with 10-formyl and 11-carboxylic acid residues) (4.9 mg) (5) [15], 3-O-β-D-glucopyranosylquinovic acid (3.6 mg) (6), 3-O-β-D-glucopyranosylsorboic acid (4.2 mg) (7) and Clemahexapetiside B (a symmetric macrocyclic glycoside) (4.7 mg) (8) [16](Figure 2).

![Figure 2: Structures of 1-8](image-url)
DMSO-δ6 δC 122.6(C-1a), 110.4 (C-2a), 146.4 (C-3a), 148.1 (C-4a), 112.3 (C-5a), 119.8 (C-6a), 164.8 (C-7a), 53.9 (C-3a-MeO), 96.4 (C-1’a), 71.1 (C-2’a), 75.5 (C-3’a), 69.2 (C-4’a), 72.3 (C-5’a), 64.2 (C-6’a), 122.1 (C-1b), 11.2 (C-2b), 147.1 (C-3b), 148.5 (C-4b), 112.3 (C-5b), 122.1 (C-6b), 164.4 (C-7b), 53.6 (C-3b-MeO), 96.8 (C-1’b), 71.2 (C-2’b), 69.5(C-3’b), 67.9 (C-4’b), 70.8 (C-5’b), 64.4 (C-6’b).

**Antiplasmodial assay:** Except for the methanol extract (C3) that inhibited the growth of *P. falciparum* with an IC50 value of 3.044 μL/mL, the hexane, ethyl acetate fractions and all affored compounds were inactive at the tested concentration.

The results of the antimalarial assay suggest that constituents of *Canthium subcordatum* might act in synergy to provide the antimalarial activity and fractionation will result in a loss of activity. Nevertheless, the antimalarial activity of its methanol extract supports the use of the stem bark and root of *C. subcordatum* in the traditional treatment of malaria and fever[17].

**Antimicrobial assay:** Results of antimicrobial testing are presented in table 1. In general, it is observed that all the pure compounds tested presented an activity on at least three of the tested strains. Based on the inhibitory zones and MIC obtained, we noticed that 3, 4 and 5 presented comparable activities with the reference drug (Nystatin) on *Candida albicans*. *Candida krusei* was highly susceptible to 4, 5 and 6 with inhibitory zones of 12-14 mm and MIC ranging from 9.7 to 39 μg/mL. 9a was highly susceptible to 4 (ID=16 and MIC= 9.7 μg/mL) and 1 (ID= 12 mm and MIC 39 μg/mL) products. Ss on its part showed marked susceptibility to 4 products. These included 4 (ID=14; MIC=19.5), 5 (ID=12; MIC=39), NM15 (ID=12; MIC=39) and 8 (ID=14; MIC=19.5). Sf appeared to be markedly susceptible only to 41 product; 5 (ID=14; MIC=19.5) Ec and Kp were susceptible to two products 3 (ID=14; MIC=19.5) and 5 then 4 and 5 respectively. Pa was sensitive to 1, 4 and 5. 1 was active on two strains (Ss and Kp). 4 and 5 appeared to be the most active compounds on being able to inhibit the growth of six strains including fungi, Gram + and Gram -; with inhibition diameter zones equal or above 12 and MIC equal or lower than 39 μg/mL.

**Table 1:** Results of antimicrobial assay

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**Key 1:** ID in (mm) and MIC in (μg/mL). **Key 2:** NT: not tested; ND: Not determined; Sa: Staphylococcus aureus; Sc: Staphylococcus saprophyticus; Sf: Streptococcus faecalis; Ec: Escherichia coli; Kp: Klebsiella pneumonia; Pa: Pseudomonas aeruginosa; Ca: Candida albicans; Ck: Candida krusei. Genta: Gentamicin; Nyst: Nystatin.

The following ranges defined cut-off points for antimicrobial activities. For pure compounds: (MIC <10 μg/mL) for significant activity, (10 < MIC ≤ 100 μg/mL) for moderate activity, and (MIC > 100 μg/mL) for low activity.[18]

Apart from 3-O-β-D-glucopyranosylquinovic acid (6), 3-O-β-D-glucopyranosyleanolic acid (7) and Clemahexapetoside B (8) for which little is known about their antimicrobial properties, all isolated compounds have been previously reported for either antibacterial activity or antifungal activity, β-sitosterol (I), isolated from Vitex agnus-castus by bio-assay guided fractionation showed antibacterial activity by inhibiting the growth of *B. subtilis*, *S. aureus*, *S. epidermidis*, *E. faecalis* and *E. coli* [19]. It was suggested that beta-sitosterol act by inhibition DNA polymerase beta lyase activity [20]. Ursolic acid (2), antibacterial properties have been previously assayed against different bacterial species, and the results suggested the important antibiotic properties. Indeed, 2 showed activity against *Streptococcus mutans* and *S. sobrinus* with a MIC50 of 2.0 μg/mL, indicating their usefulness in inhibiting caries in teeth [21, 22, 23]. Additionally, 2 has also been reported to possess antimalarial activity [24]. Ursolic Acid (2) displayed some pleiotropic antibacterial mechanisms against methicillin-resistant *Staphylococcus aureus* (MRSA) [24]. Cerbinal (3) showed antifungal activity close to that of nystatin. In fact, Cerbinal is a well-known plant-derived antifungal compound previously isolated from *Gardenia jasminoides* leaves [25]. Quinovic acid (4), antimicrobial properties have been corroborated by many authors who indicated the inhibition of the growth of both Gram-positive and Gram – negative and *Aspergillus* species and *C. albicans* [26, 27]. Cerberinic acids (5), good antifungal activity is in line with previously reported antifungal and antibacterial activity of iridoids, [28].

**CONCLUSION**

In this study, antimalarial and antimicrobial potential of *Canthium subcordatum* extracts and isolates were investigated. Antiplasmodial activity testing on fractions and isolates revealed that the methanol fraction was active inhibiting the growth of *P. falciparum* with an IC50 value of 3.044 μg/mL. Antimicrobial activity testing on isolates against eight bacteria and fungi strains showed two compounds as the most active with inhibition diameter zones equal or above 12 mm and MIC equal or lower than 39 μg/mL.

Three known isolates were obtained and characterized from the genus *Canthium* for the very first time. Furthermore, antimalarial activity of *Canthium subcordatum* and antimicrobial activities of three isolates are herein reported for the first time.

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