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ANTIMICROBIAL AND ANTIPLASMODIAL ACTIVITIES OF A QUATERNARY COMPOUND FROM *RITCHIEA CAPPAROIDES* VAR. *LONGIPEDICELLATA*

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Abstract

The leaves of *Ritchiea capparoides* var. *longipedicellata* **(**Capparidaceae**)** is used in ethnomedicine in South-Western Nigeria to treat infectious and parasitic diseases. This study was aimed at identifying the compound(s) that are responsible for the antimicrobial and antiplasmodial activities of the leaves and also to contribute to the chemistry of the plant species. A 70 % aqueous ethanolic extract of the leaves of *R. longipedicellata* was subjected to repeated liquid chromatographic methods on silica gel, Lobar RP-18 column and Sephadex LH -20 to isolate a Draggendorf positive compound. The compound was identified by ^{1}H and ^{13}C NMR, ultra-violet spectroscopy and polarimetry. The antimicrobial activity of the compound was evaluated using the microbroth dilution method while the antiplasmodial activity was carried out according to Trager and Jenson (1976). The minimum inhibitory concentration (MIC) was expressed in mg/ml. The isolated compound, leavo-isomer of stachydrine, inhibited the growth of *Escherichia coli* NCTC 8196 and *Staphylococcus aureus* NCTC 6571 at the MIC of 5 mg/ml. In the anti-malaria assay, the compound had inhibitory activity with the concentration required to cause 100% lethality being 0.667 mg/ml.
Conclusion: The antibacterial and antiparasitic effects of quaternary ammonium compounds are well documented. However, this study is the first report of the presence and biological activities of this compound in this plant species which may justify the ethnomedicinal uses of the leaves.

Key words: Quaternary ammonium compounds, antimicrobial, antiplasmodial, Ritchea

Introduction

Ritchiea capparoides **(**Capparidaceae**)** formerly known as *R. longipedicellata* is an ornamental climbing shrub consisting of two varieties, var. *capparoides* (flower with four petals) and var. *longipedicellata* (flower consists of eight petals or more) (Burkill, 1985). Ethnomedicinally, the roots extract of *R. capparoides* var *longipedicellata* are used as an anthelmintic (Ajaiyeoba and Okogun, 1996). In the South-Western part of Nigeria, decoction of the leaves and root are widely used for the treatment of infectious and malarious conditions. Ogbunugafor et al., 2008; Anowi et al., 2012 reported the antiplasmodial and antimicrobial activities of the extracts of the plant. Previous chemical investigations have reported cleomin, a mustard oil as glycoside isolated from the root bark of the plant (Ogwuaka *et.al.*, 1981). In this paper, we report the isolation and characterization of a quaternary ammonium compound from the leaf, stem and root bark of the plant with antiplasmodial and antimicrobial activities.

Materials and methods

Identification of plant

The leaves, stem and root bark of the plant materials used for this study were purchased from Dugbe Herbal market, Ibadan, in South Western Nigeria in December 2008. The vegetative parts were identified by Mr G. Ademoriyo of the Department of Botany, Obafemi Awolowo University by comparison with the herbarium specimen. A specimen with herbarium no UHI 8220 was deposited in IFE herbarium, Obafemi Awolowo University, Ile Ife.

General experimental procedure

^{1}H- and ^{13}C-NMR were recorded on Varian 200 Spectrometer. Chemical shifts were expressed in ″ppm″. Ultra-Violet (UV) Spectra were recorded on a UV-160 (Shimadzu) UV-Visible spectrophotometer. Optical rotations were taken on a POLAX 2L (5223-E08). Extracts were fractionated on Silica gel (Merck 230-400 mesh, ASTM), Sephadex LH-20 and Lobar RP-18 column.

Test Organisms

The malaria parasites were clinical isolates obtained from the Department of Pathology, Obafemi Awolowo University Teaching Hospital Complex, Ile Ife, Nigeria. The microorganisms used for the study were comprised of *S. aureus* NCTC 6571, *E. coli* NCTC 8196, *B. subtilis* NCTC 8326, and *P. aeruginosa* ATCC 19429. They were obtained from the culture collections at the Microbiology Laboratory of the Department of Pharmaceutics, Obafemi Awolowo University, Ile-Ife, Nigeria.

Extraction and Isolation

The dried leaves were ground to powder (600 g). The powdered plant material was extracted at room temperature for two days using 70 % aqueous ethanol. The mixture was filtered and the filtrate obtained was concentrated *invacuo* using rotatory evaporator to obtain 68 g of the

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crude extract. The crude extract was suspended in 300 ml of water and partitioned successively with dichloromethane (6 X 200 ml), ethyl acetate (8 X 150 ml) and butanol (4 X 250 ml). The fractions were each concentrated on rotary evaporator to give 17.5, 9.8 and 5.3 g for dichloromethane, ethyl acetate and *n*-butanol fractions respectively. The fractions and eluates from subsequent column separations were analyzed on Thin Layer Chromatography (TLC) using 100% methanol on silica gel (normal phase) and spots were detected by spraying with Draggendorf's reagent. Only ethyl acetate and butanol fractions showed Draggendorf positive spots. Co-TLC of both the *n*-butanol and ethyl acetate fractions showed similar TLC profile of a Draggendorf positive spot. Thus, the ethyl acetate fraction was subject to repeated chromatography on silica gel using a gradient of solvents in increasing polarity, from Hexane: EtOAc (v/v, 100:0, 90:10, 80:20, 60:40, 20: 80, 0:100) to EtOAc : MeOH, (v/v., 100:0, 95:5 90:10, 80:20, 60:40, 20: 80) to MeOH (v, 100) to give sub-fractions **I-VII** . **VII** (1.34g), with the Draggendorf's positive spot extracted with MeOH-EtOAc (v/v, 80:20 to 100:0 v/v) was subjected to reversed phase chromatographic separation on a Lobar RP-18 column using 500 ml of isocratic solvent gradient of H_2O-MeOH (v/v, 40:60). Final purification on Sephadex LH-20 with isocratic elution with 300 ml of EtOAc-MeOH (v/v, 60:40,) afforded **1** (158 mg). The extract of the stem bark, as well as that of the root bark, were spotted along with compound **1 (**isolated from the leaves) on silica gel plate and the plate was developed using 100% methanol as the mobile phase. The spots were later detected by spraying the dry plate with Draggendorf solution.

In-vitro antiplasmodial activity of 1 on *P. falciparum*

The *in vitro* antiplasmodial evaluation of compound **1** was performed according to Trager and Jenson (1976). The reference drug used was Artemether (625µg/ml). A template was prepared by introducing 300 µl of compound **1 and the reference drug** into separate wells of the series "A" of a 96-well flat-bottomed plate, and serial dilutions were done with the RPMI-HEPES medium. Then, daughter plates were prepared from this by pipetting 25 µl of each corresponding test sample into 25 µl of the medium. Blood samples containing *Plasmodium falciparum* strains (200 µl of 20 parasites per field) was then inoculated into the wells. The preparation was incubated at 37^{o}C for 36 hours, periodically checking for schizonts. The wells were then harvested as thick smears on microscope slides, dried and stained with Giemsa staining fluid. The slides were observed under X100 objective and the number of schizonts per 200 leucocytes counted.

Antibacterial activity Assay

The typed culture organisms were streaked on nutrient agar plates and incubated at 37 ^{o}C for 24 hours. Colonies obtained were suspended in sterile distilled water to obtain concentrations of approximately 10^6 CFU/mL, by comparison with the McFarland tube number 0.5. The MIC values of the isolated compound against the bacteria strains were determined based on a micro-well dilution method (Zgoda and Porter, 2001) with some modifications. The compound was tested at an initial concentration of 20 mg/ml. Sterile nutrient broth (100µl) was added to 96-well microtitre plates followed by the addition of 100 µl of compound **1** to the 1st wells and serially diluted two folds to the 10th well. Then, 100 µl of the above mentioned test micro-organisms were added to each microtitre plates in the 1st to 10th well and the 12th well to give the final volume of 200 µl. The 11th well containing only the nutrient broth and the 12th well containing both the nutrient broth and the inoculum without the tested compound were used as no-growth and growth control respectively. Streptomycin (Shijiazhuang, China) was used at the serial concentration range of 1000-1.95 µg/ml in nutrient broth as positive control. The prepared microtitre plates were sealed so that they do not dry and incubated overnight at 37 ^{o}C. Microbial growth was determined by absorbance at 630 nm using the DNM-9602 universal micro plate reader (Perlong New Technology, England) and confirmed by plating 5 µl samples from clear wells on nutrient agar medium. The compound tested in this study was screened in duplicate against each organism. The MIC was defined as the lowest concentration of the compound to inhibit the growth of microorganisms.

Results

Spectra data of compound 1

Compound **1;** UV λ^{MeOH}_{max} nm (log ε):218.5 (2.0). ; $[\alpha]_D^{25} = -13^0$ [MeOH]. **^{1}H NMR, CDCl3** ppm 3.13 [3H, s, (N methyl gp)], 3.35 [3H, s, (N- methyl gp)], 3.96[1H, t, (H-2) 2.75-3.70 ppm[overlapping signals, m, H-3, H-4 and H-5], **^{13}C NMR; CDCl3 ppm:** 169.0 (C=O), 76.6 (C-2) 66.9, (C-5) 52.1 (N-methyl), 45.7, (N-methyl), 25.4 (C-3), 18.9 (C-4).

Bioassay results

The results of the antiplasmodial and antimicrobial activities are as shown in Tables 1 and 2 below.

Discussion

Compound **1** had R_f value of 0.30 on a normal phase silica gel plate using 100% methanol as mobile phase. The ^{1}H NMR spectrum of compound **1** featured two deshielded methyl signals (integrating for three protons each) at δ: 3.13 (3H, s, N-CH_3) and 3.35(3H, s, N-CH_3). The signal at δ 3.96, a triplet integrating for one proton is attributable to H-2 while the multiplet centered around 2.75-3.70 integrating for six proton will account for the three methylene groups present. The ^{13}C NMR showed 7 signals in all. Attached Proton Test (APT) showed that there are two methyl groups attached to a quaternary nitrogen (at 52.1 and 45.9 ppm), one methine carbon (at 76.6 ppm) and a quaternary carbonyl carbon at 169.0 ppm. Of the three methylene groups present, the most deshielded (66.9 ppm) is attributable to C-5. The other two methylene groups at 25.41 and 18.86 ppm were assigned to C-3 and C-4 respectively. In comparison with literature values (Chambers and Kunin, 1987), compound **1** was suggested to be stachydrine. The optical rotation $[\alpha]^{25}_D$ was observed to be -13^0, therefore compound **1** was confirmed to be leavo-stachydrine.

The result of the co-TLC of the crude extracts of the stem as well as the root bark with compound **1** showed a deep orange coloured spot at R_f R_f = 0.30 (mobile phase was 100% Methanol) when sprayed with Draggendorf reagent, indicating that compound **1** is the alkaloidal compound present in all the vegetative parts tested.

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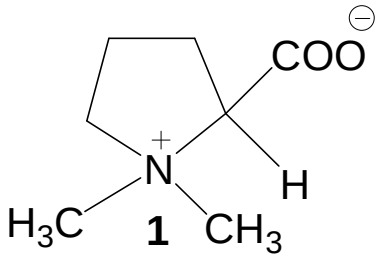

Figure 1: Structure of compound 1

The results of the antiplasmodial activity of **1** on *Plasmodium falciparum* showed that the Minimum Inhibitory Concentrations (MIC) was 0.667 mg/ml with IC_{50} calculated as 0.0063±0.00 mg/ml (6.3 µg/ml), with IC_{50} calculated as 0.0068 mg/ml (6.8 µg/ml) and 0.0057 mg/ml (5.7 µg/ml) respectively. According to Rukunga and Simons (2006), **1** with IC_{50} for the *in vitro* antiplasmodial activity less than 10 µg/ml falls into the category of the highly active antiplasmodial natural products. Athemeter on its part exhibited MIC and IC_{50} values of 0.008 µg/ml and 0.0021±2.7µg/ml respectively. Quaternary ammonium compounds have been reported to have potent antimalarial activity based on their ability to inhibit de novo phosphatidylcholine synthesis. This has been found to lead to the inhibition of membrane biogenesis in the malaria parasite, *Plasmodium falciparum* (Wengelnik *et. al.* 2002). It is therefore not surprising that **1** possessed antimalarial activity. Phospholipid (PL) metabolism is an attractive target for new malaria chemotherapy due to its vital importance to the parasite. PL metabolism is absent from normal mature human erythrocytes, but the erythrocyte PL content increases by as much as 500% after infection, specifically due to the metabolic machinery of the parasite. Phosphatidylcholine is the major PL of infected erythrocytes, representing about 45% of the total PL. In this pathway, choline transport which regulates the supply of polar head precursors to the parasite is a regulatory rate-limiting step (Ancelin *et al.*, 2003). However, lipophilicity was required to inhibit *P. falciparum* phospholipids metabolism, leading to parasite death. For mono quaternary ammonium salts, an increase in the lipophilicity around nitrogen has been found to be beneficial for antimalarial activity (Calas *et. al.*, 2000). Attempt would be made to evaluate the influence of increasing the lipophilicity around the nitrogen atom of stachydrine on the potency of the antimalarial activity.

Table 1: Antiplasmodial activities of Compound 1 on *Plasmodium falciparum*

Conc. (mg/ml)	Mean No of Schizonts/200 WBC	% Inhibition
2.000	0 ±0.00	100
0.667	0 ± 0.00	100
0.222	15 ± 0.18	95.07
0.074	34 ± 1.02	88.82
0.025	89 ± 2.07	70.72
0.008	127 ± 0.08	58.22
0.003	255 ± 0.11	16.12
0.000	304 ± 1.02	0

MIC: **1** (0.667 mg/ml), AR T Artemeter (ART) (0.008 µg/ml). Result is presented as the Standard error of the mean.

Antibacterial bioassay

.As shown in Table 3, compound **1** showed activities against the two Gram positive bacteria, *S. aureus* and *B. subtilis*, and *E. coli*, one of the Gram negative bacteria at MIC values of 10, 5 and 5 mg/ml respectively. Compound **1** showed activity against *S. aureus* and *E. coli* only, both at the MIC of 5 mg/ml. The compound did not inhibit the growth of *P. aeruginosa* at the concentration of 20 mg/ml which was the highest concentration tested. The membrane-disruptive and antimicrobial activities of cationic surfactants are well recognized. These agents are often active against a broad range of bacteria and other cells and can also inactivate certain viruses (Lindstedt *et. al.*, 1990). Stachydrine has been reported in other species of the family Capparidaceae and some Lamicaeae and is of chemotaxonomic significance in the Capparidaceae. The presence of stachydrine, a quaternary ammonium compound in the leaves, the stem and root bark justifies why the plant parts mentioned above are popular in the treatment of infections and malaria in ethnomedicine.

Table 2: MIC in mg/ml of compounds 1 against typed culture microorganisms

Compound	***S. aureus*** NCTC 6571,	***B. subtilis*** NCTC 8326	***E. coli*** NCTC 8196	***P. aeruginosa*** ATCC 19429
1	5	>20	5	>20
Streptomycin (positive control)	0.125	0.125	0.125	0.125

Conclusion

This study has demonstrated the presence of stachydrine in **the leaves, stem and root bark of** *Ritchea capparoides var longipedicellata*. The compound was found to be active in the antimalarial and antimicrobial assay used.

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