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An Antisalmonellal Agent from the Leaves of *Glossocalyx brevipes* Benth (Monimiaceae)

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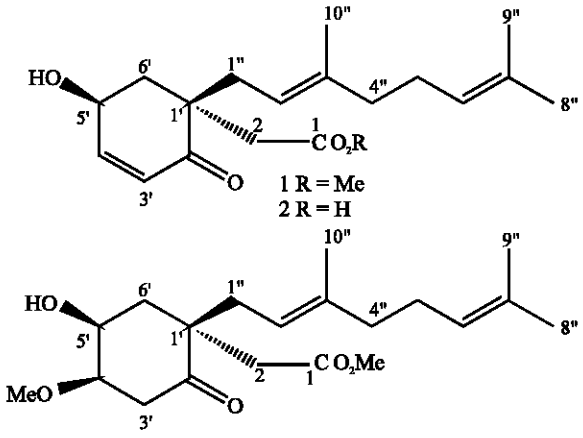
Abstract: Three homogentisic acid derivatives, namely methyl 2-(1'β-geranyl-5'β-hydroxy-2'-oxocyclohex-3'-enyl)acetate (**1**), 2-(1'β-geranyl-5'β-hydroxy-2'-oxocyclohex-3'-enyl)acetic acid (**2**), methyl 2-(1'β-geranyl-5'β-hydroxy-4'β-methoxy-2'-oxocyclohexyl)acetate (**3**), isolated from the leaves of *Glossocalyx brevipes*, were screened for antisalmonellal activity using both agar diffusion and broth dilution techniques. Only compound 2 showed significant antimicrobial activity against *Salmonella typhi* (26 mm), *Salmonella paratyphi* A (23 mm) and *Salmonella paratyphi* B (22 mm). The MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values were 10 and 80 μg mL^{-1}, respectively, against all the three bacteria strains used. These data support the use of *Glossocalyx brevipes* in anti-typhoid preparations.

Key words: *Glossocalyx brevipes*, homogentisic, antisalmonellal, anti-typhoid

INTRODUCTION

Glossocalyx brevipes Benth (Monimiaceae) is a shrub with hairy stems and leaves, growing in the humid rain forests of West and Central Africa[1]. In Cameroon folk medicine, the macerated leaves are added to antifever preparations[2]. A previous phytochemical study reported the isolation of three new derivatives of homogentisic acid, methyl 2-(1'β-geranyl-5'β-hydroxy-2'-oxocyclohex-3'-enyl)acetate(**1**), 2-(1'β-geranyl-5' β-hydroxy-2'-oxocyclohex-3'-enyl)acetic acid(**2**), methyl 2-(1'β-geranyl-5'β-hydroxy-4'β-methoxy-2'-oxocyclohexyl)acetate(**3**), isolated from the leaves of *G. brevipes*. Compounds 1 and 2 were reported to possess modest *in vitro* activity against *Plasmodium falciparum*[1]. Typhoid fever is caused by *Salmonella typhi*, whereas paratyphoid fevers are caused by *Salmonella paratyphi* A and *Salmonella paratyphi* B[3]. Typhoid fever continues to be a marked public health problem in developing countries in general and in Sub-saharan Africa in particular, where it is endemic. The greater prevalence of resistance to all three first-line antimicrobials (ampicillin chloramphenicol and co-trimoxazole) has been established[4].



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In a continuation of our search for therapeutic agents from natural sources with potential for the treatment of typhoid and paratyphoid fevers[5,6], the antimicrobial activity of the above three derivatives of homogentisic acid was investigated against *Salmonella typhi*, *Salmonella paratyphi* A and *Salmonella paratyphi* B.

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MATERIALS AND METHODS

Plant material: The leaves of *Glossocalyx brevipes* Benth (Monimiaceae) were collected in Kumba, South West Province of Cameroon, in the month of September and authentication was carried out at the Limbe Botanical Garden. A voucher specimen (UD 337) is deposited at the herbarium of the Botany Department, University of Dschang.

Test bacteria and culture media: The test microorganisms, *Salmonella typhi*, *Salmonella paratyphi* A and *Salmonella paratyphi* B, were obtained from the Medical Microbiology Laboratory of the Pasteur Centre, Yaounde, Cameroon. The culture media used, namely Salmonella-Shigella agar (SS agar) and Selenite Broth, were supplied by International Diagnostics Group PLC, Topley House, 52 Wash Lane, Bury, Lancashire BL9 6AU, UK.

Extraction and isolation: The extraction and isolation procedures have already been detailed by Mbah *et al.*[1] following the Mobah method[1]. In brief, the air-dried and powdered leaves (5 kg) of *G. brevipes* were macerated in a mixture of methylene chloride/methanol (1:1) for seven days. Filtration and concentration on a rotavapor afforded 1200 g of extract. Part of this extract (300 g) was treated with 0.1 M sulfamic acid (20 mL) followed by extraction with methylene chloride to yield the neutral fraction (230 g). The aqueous phase was basified to pH 7 with 5% aqueous ammonia and the crude alkaloid material (6 g) was obtained by extraction with methylene chloride. Vacuum liquid chromatography of the neutral fraction (150 g) on silica gel using a gradient of EtOAc in *n*-hexane (5:95, 10:90, 20:80, 40:60, 60:40 and 100:0) gave sixty fractions (500 mL each), which were concentrated and combined on the basis of TLC profiles. Chlorophyll was removed from fractions 29-35 (obtained with 20% EtOAc); 44-48 (obtained with 40% EtOAc); and 54-57 (with 60% EtOAc) by gel permeation through Sephadex LH-20 (50 g) (CH_2Cl_2/MeOH [8:2] 100 mL for each elution). The residue obtained from fractions 29-35 (6 g) was purified on a silica gel (125 g) column eluted with hexane/EtOAc (9:1; 150 mL for each fraction) to yield 1 (300 mg, colourless oil) and 3 (200 mg, colourless oil). Compound 2 (208 mg, colourless oil) was obtained from the residue of fractions 44-48 (7 g) under further purification by gel permeation through Sephadex LH-20 (40 g), eluted with CH_2Cl_2 (50 mL for each elution). The structures of these compounds were successfully determined using a combination of homo- and heteronuclear two-dimensional NMR techniques (^{1}H-^{1}H COSY, HETCOR, HMQC, HMBC spectra).

Antimicrobial assay: The antibacterial activity was determined using both agar diffusion and broth dilution techniques as previously described by Cheesbrough[3] and Gatsing *et al.*[6].

Agar diffusion susceptibility testing was done using the method of wells. On each plate containing Salmonella-Shigella agar (SS agar) medium already inoculated with the test organism, (100 μL of the bacteria suspension in selenite broth, at the concentration of 5.10^5 cfu mL^{-1}) equidistant wells (of 6 mm diameter) were bored using a cork borer. The bottom of each well was sealed with a drop of molten agar. Compounds 1, 2 and 3 were dissolved in DMSO. The wells were filled with 150 μL of the solution (of known concentration) of various compounds to be tested. Chloramphenicol (Sigma) was used as the standard drug. The petridishes were left at room temperature for at least 30 min to allow the compounds to diffuse from the wells into the medium. They were then incubated at 37°C for 24 h, after which the zones of no growth were noted and their diameters recorded as the zone of inhibition.

For the broth dilution susceptibility testing, the solution (maximum concentration) of the active compound (i.e. the compound that induced a zone of inhibition; compound 2) was prepared in DMSO, serially (2-fold) diluted and 0.4 mL of each dilution was introduced into a test tube containing 4.5 mL of selenite broth; then 0.1 mL of bacteria suspension (5.10^5 cfu mL^{-1}) was added and the mixture was homogenized. The total volume of the mixture was 5 mL, with the test-compound concentrations in the tube ranging from 160 to 1.25 μg mL^{-1} and those of chloramphenicol ranging from 64 to 0.5 μg mL^{-1}. After 24 h of incubation at 37°C, the MIC (minimum inhibitory concentration) was reported as the lowest concentration of antimicrobial that prevented visible growth. The MBC (minimum bactericidal concentration) was determined by sub-culturing the last tube to show visible growth and all the tubes in which there was no growth on already prepared plates containing SS agar medium. The plates were then incubated at 37°C for 24 h and the lowest concentration showing no growth was taken as the minimum bactericidal concentration.

RESULTS

The Three compounds isolated from the leaves of *G. brevipes*, namely methyl 2-(1'β-geranyl-5'β-hydroxy-2'-

Table 1: Diameters of inhibition of *S. typhi*, *S. paratyphi* A and *S. paratyphi* B by the compounds isolated from the leaves of *Glossocalyx brevipes*

Compound	Concentration (mg mL^{-1})	Bacteria and diameters of zones of inhibition (mm)		
		S. typhi	*S. paratyphi* A	*S. paratyphi* B
1	0.5	NA	NA	NA
2	0.5	26	23	22
3	0.5	NA	NA	NA
Chloramphenicol (standard)	0.1	28	25	27

Key: NA = Not Active
1: Methyl 2-(1'β-geranyl-5'β-hydroxy-2'-oxocyclohex-3'-enyl)acetate,
2: 2-(1'β-geranyl-5'β-hydroxy-2'-oxocyclohex-3'-enyl)acetic acid,
3: Methyl 2-(1'β-geranyl-5'β-hydroxy-4'β-methoxy-2'-oxocyclohexyl)acetate.

Table 2: Inhibition parameters (MIC, MBC) of compound 2, isolated from the leaves of *Glossocalyx brevipes*, against *S. typhi*, *S. paratyphi* A and *S. paratyphi* B

Compound	Parameters	Bacteria Strains		
		S. typhi	*S. paratyphi* A	*S. paratyphi* B
2	MIC (μg mL^{-1})	10	10	10
	MBC (μg mL^{-1})	80	80	80
	MBC/MIC	8	8	8
Chloramphenicol (standard)	MIC (μg mL^{-1})	2	2	2
	MBC (μg mL^{-1})	16	16	16
	MBC/MIC	8	8	8

Key: 2: 2-(1'β-geranyl-5'β-hydroxy-2'-oxocyclohex-3'-enyl)acetic acid

oxocyclohex-3'-enyl)acetate (1), 2-(1'β-geranyl-5'β-hydroxy-2' -oxocyclohex-3' -enyl)acetic acid (2), methyl 2-(1'β-geranyl-5'β-hydroxy-4'β-methoxy-2'-oxocyclohexyl)acetate (3), were tested against three *Salmonella* species (i.e., *S. typhi*, *S. paratyphi* A and *S. paratyphi* B) at the concentration of 0.5 mg mL^{-1}, using agar diffusion technique. The data obtained showed that compounds 1 and 3 were not active against the bacteria strains used, whereas compound 2 showed significant antibacterial activity against *S. typhi* (26 mm), *S. paratyphi* A (23 mm) and *S. paratyphi* B (22 mm). Chloramphenicol (0.1 mg mL^{-1}), used as standard, produced inhibition diameters of 28 mm against *S. typhi*, 25 mm against *S. paratyphi* A and 27 mm against *S. paratyphi* B (Table 1).

Compound 2, which showed antibacterial activity against all the three bacteria strains used, was further studied using broth dilution technique and the following results were obtained: the MIC and MBC values were 10 μg mL^{-1} and 80 μg mL^{-1}, respectively, against all the three bacteria strains tested. For chloramphenicol the MIC and MBC values were 2 and 16 μg mL^{-1}, respectively, against the same bacteria strains (Table 2).

DISCUSSION

Among the three homogentisic acid derivatives isolated from *G. brevipes,* 2-(1'β-geranyl-5'β-hydroxy-2'-oxocyclohex-3'-enyl)acetic acid (2) was the only compound found to exhibit antimicrobial activity against the three bacteria used. Compounds 1 and 3 did not show any antibacterial activity against these bacteria strains. From the structures of these compounds it can be seen that compound 1, methyl 2-(1'β-geranyl-5'β-hydroxy-2'-oxocyclohex-3'-enyl)acetate, differs from compound 2 only by the methyl group attached to the carboxyl end of the molecule. That is, the carboxyl group in compound 1 is methylated (-COOMe), whereas it is free (-COOH) in compound 2. Also, that carboxyl group is methylated in compound 3 (-COOMe). Therefore, the antisalmonellal activity of compound 2 may be attributed to the free carboxyl group (-COOH) in the molecule.

Antimicrobial substances are considered as bactericidal agents when the ratio MBC/MIC≤4 and bacteriostatic agents when the ratio MBC/MIC>4[7]. For compound 2, the ratio MBC/MIC>4, suggesting that it may be classified as bacteriostatic agent. Compound 2 was five times less active than chloramphenicol; also it was found to be more active against *S. typhi* than against *S. paratyphi* A and *S. paratyphi* B. The isolation of this active antisalmonellal principle supports the use of *Glossocalyx brevipes* in anti-typhoid preparations.

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