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ANTICOCCIDIAL CONSTITUENTS FROM THE STEM BARK OF *Turraeanthus africanus*

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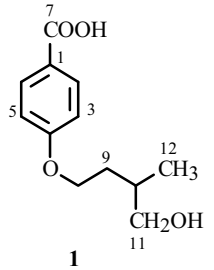
In order to study some biological active products, phytochemical investigation of the stem bark of Turraeanthus africanus *have led to the isolation of a novel compound **1**, a new benzoic acid derivative, named turraeanthin C, and two known compounds sesamin (**2**) and stigmasterol. The structures of these compounds were established by spectral analysis, including two-dimensional nuclear magnetic resonance. The extract and the isolated compounds **1** and **2** showed noteworthy activity against* Toxoplasma gondii *intracellular parasite in mammals.*

Key words: anticoccidial activity, *Turraeanthus africanus*, benzoic acid derivative, Meliaceae.

The evaluation of medicinal plants used in the preparation of folk remedies has provided modern medicine with effective pharmaceuticals for the treatment of diseases caused by various parasites. Parasites are a very important problem in medical and veterinary fields. They are widespread in tropical regions and in other part of the world where HIV infections are high as opportunist parasites. The discovery and development of anticoccidial compounds are effective methods for the prevention and treatment of these problems. *Toxoplasma gondii* is an obligate intracellular parasite that is able to infect a wide range of mammalian and avian species [1]. In humans, *Toxoplasma* infections are widespread and can lead to severe disease in individuals with immature or suppressed immune systems. Consequently, toxoplasmosis became one of the major opportunistic infections of the AIDS epidemic [2]. Toxoplasmosis can also affect *T. gondii* negative women during pregnancy, and so it is a serious threat for embryos.



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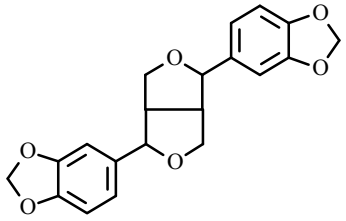

2

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TABLE 1. ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) Data for Turraeanthin C (**1**)

Position	δ_H (m, J/Hz)	δ_C	HMBC correlation
1	-	124.0	-
2/6	7.85 (d, J = 8.8)	131.5	4, 6/2, 7
3/5	7.01 (d, J = 8.8)	114.5	1, 4, 5/3
4	-	160.0	-
7	-	172.0	-
8	4.05 (td, J = 8.2, 4.0)	66.4	4, 9, 10
9a	1.48 (m)	32	8, 10, 11, 12
9b	1.85 (m)		
10	1.70 (m)	32.5	8, 9, 11, 12
11	3.25 (d, J = 6.8)	66.0	9, 10, 12
12	0.88 (d, J = 7.4)	17.0	9, 10, 11
COO**H**	12.5		

Turraeanthus africanus is a tree belonging to the family of Meliaceae which grows in tropical and subtropical regions from Sierra Leone to the Congo and Angola. This tree has a higher economic value as timbers and is also used for the treatment of diseases such as asthma, stomachache, intestinal worms, and inflammatory diseases [3]. Previous phytochemical studies carried out on stem barks and seeds of *Turraeanthus africanus* have led to the isolation and characterization of several secondary metabolites belonging to the diterpenes, triterpenes, limonoids, and alkaloids [4, 5]. Some of these compounds exhibited interesting biological activities [6]. As part of our continuing investigation of Cameroonian medicinal plants, we wish to report herein the isolation and structural elucidation of a new benzoic acid derivative, named turraeanthin C. The biological activity of turraeanthin C (**1**), sesamin (**2**), and the extract of this plant were evaluated against *Toxoplasma gondii* (Apicomplexa).

A sample of the air-dried stem bark of *Turraeanthus africanus* was extracted by maceration in methanol. After concentration; the obtained extract was defatted with hexane, and fractionation was achieved by column chromatography over silica gel, leading to turraenthin C, sesamin and stigmasterol.

Turraeanthin C (**1**), mp 120–122°C, was isolated by silica gel column chromatography of the methanol extract of *T. africanus*. Its high-resolution electrospray-TOF mass spectrum (HR-ES-IMS) showed a pseudomolecular ion peak $(M + H)^+$ at *m/z* 225.1120 (calcd for $C_{12}H_{17}O_4$, 225.1122). Although the ^{13}C NMR spectrum revealed only 10 resonances, two sets of signals (δ 114.5 and 131.5) were ultimately assigned to degenerate carbons on the basis of symmetry, accounting for all 12 carbons. The absorption maxima at 229 and 287 nm in the UV spectrum and at 3400 and 1715 cm^{-1} (free carboxyl group) and 1517 cm^{-1} (aromatic) in the IR spectrum indicated the presence of a benzoic acid functionality [7 – 10]. The presence of the carboxyl group was confirmed in the ^{13}C NMR spectrum with the signal at δ 172 ppm and in the ^{1}H NMR spectrum by a large signal at δ 12.5 (Table 1). In the ^{1}H NMR spectrum of **1**, four aromatic protons were observed as two doublets at δ 7.01 and 7.85 in the AA′BB′ system, indicating the presence of a *para*-disubstituted benzene ring. Compound **1** has also characteristic signals due to the 4-hydroxy-2-methylbutanol unit with a doublet at δ 0.88 ppm corresponding to the methyl group, a multiplet at δ 1.48 and 1.85 attributable to the diastereotopics protons of the methylene group, a multiplet at δ 1.77 corresponding to the methine group, a doublet at δ 3.25 assignable to the methylene of an hydroxyl group, and a triplet dedoubled at δ 4.25 attributable to the oxymethylene. Results of HMBC experiments are in excellent agreement with the identities of the structural units. In addition, an HMBC correlation observed between the oxygenated methylene protons (CH_2O-) and the oxygenated aromatic carbon C-4 allowed connection of 4-hydroxy-2-methylbutanol unit via an ether linkage. The remaining COOH unit is situated in the *para* position of the 4-hydroxy-2-methylbutanol unit. All the above spectroscopic data led us to the structure elucidation of compound **1**, turraeanthin C, characterized as a novel 4-hydroxy-(2-methylbutanol) benzoic acid.

The compounds reported in this study **1** (TA 10), sesamin (TA 1), and the crude extract (EETA) were evaluated on embryonic fibroblastic MRC5 cells by cytotoxic assay (sulforhodamine B [11]; a cytotoxicity profile was established with significant IC_{50} values at 10 μg/mL.

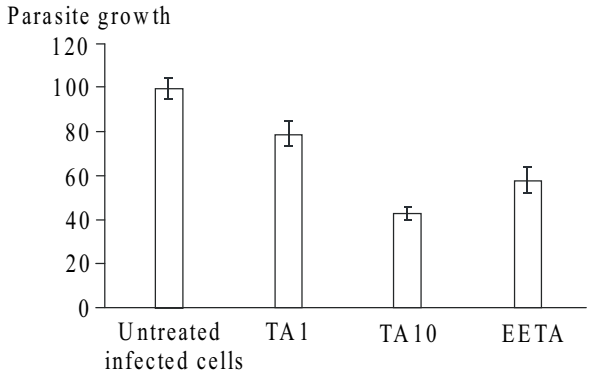

Fig. 1. *Toxoplasma gondii* growth in presence of the different tested products. Parasites were incubated for 5 days in the presence of the products.

HFF cells were infected by tachyzoites of recombinant *T. gondii* (β-galactosidase has been inserted). The molecules and extracts to be assayed were added after 1 h of contact between *T. gondii* and HFF cells. β-Galactosidase assays were monitored at 540 nm/420 nm, which indicated the parasite growth.

The experiments have been made in triplicate. We obtained an inhibition of parasite growth of around 55% for compound **1** (TA 10) at 10 μg/mL, 20% for sesamin (TA 1) at 10 μg/mL, and around 40% for crude extract (EETA) at 10 μg/mL (Fig. 1). These compounds appear to be responsible for the observed activity of the extract of the stem bark of this plant.

For toxoplasmosis, most clinical experience with atovaquone, a hydroxy-1,4-naphthoquinone, which is a structural analog of ubiquinone, has been with HIV-infected or AIDS patients. *T. gondii* is usually obtained from a reactivation from the central nervous system (CNS) in persons who have advanced immunosuppression [2]. CNS toxoplasmosis affects 7% of AIDS patients [12]. Medications for immunocompetent adults include pyrimethamine plus either trisulfapyrimidines or sulfadiazine. In pregnancy, spiramycin is usually given. Although these molecules are the drugs of choice for therapy and secondary prophylaxis of toxoplasmosis [13], some patients are intolerant of one of these regimens. This point added to the emergence of chemoresistance shows the need for alternative therapies. *T. gondii* is also studied as a model for coccidia, because it is easier to handle. Coccidiosis is one of the most detrimental diseases in poultry [14]. In order to avoid the potential for coccidiosis outbreak and the resulting financial loss, broiler chickens are continuously medicated with coccidiostatic drugs. However, concern has been expressed regarding the emergence of resistant coccidial strains [15]. In addition, the use of antibiotic feed additives in general is being phased out in Europe and only a few coccidiostatic drugs remain as non-prescription feed additives [16]. If all coccidiostatic feed additives are withdrawn from use, alternative feeding strategies must be set up, although vaccines are already available. There is, therefore, a need for intensive research into the identification and evaluation of alternatives to traditional coccidiostatics that would satisfy consumer demands and would be closer to environmentally friendly farming practices.

EXPERIMENTAL

Methods and Instruments. Melting points were determined on a Koeffler hot-stage apparatus and are uncorrected. IR spectra were recorded on a FT-R Shimadzu 408 spectrophotometer in KBr disks. UV spectra were obtained on a Beckman model 25 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker spectrometer equipped with a 5 mm ^{1}H and ^{13}C probe operating at 300 and 75 MHz, respectively, with TMS as internal standard. Jmod and two-dimensional NMR spectra (HMBC and COSY) were measured with the usual pulse sequence, and data processing was performed with standard software. Mass spectra were recorded on an API Q-STAR PULSAR I of Applied Biosystems and on a Q-TOF Waters, Electrospray Z-spray for low and high resolution, respectively.

Plant Material. The stem bark was collected from *T. africanus* (Welw. ex C.D.C.) Pellegr. at Bonepoupa, in Cameroon, in August, 2003. The plant material was identified by Dr. L. Zapfack, from the Botanic Department of University of Yaounde I, where a voucher specimen is deposited.

Extraction and Isolation of Compounds. The stem bark, dried and ground (8 kg), was extracted by maceration at room temperature in 24 L of methanol for 48 h, yielding 150 g of a brown viscous residue, and the extract was concentrated to dryness. This residue was subjected to column chromatography over silica gel (230 to 400 mesh), using hexane-ethyl acetate of increasing polarity. A total of 200 fractions of ca. 200 mL each was collected and combined on the basis of TLC analysis, leading to 12 series (A-L). The pure compounds were obtained either by direct crystallization or after further purification on column chromatography. Series B (2.40 g) eluted with *n*-hexane-EtOAc (4:1) was further purified on silica gel using *n*-hexane with a gradient of EtOAc to give 43 fractions of ca.150 mL each. Fractions 12-28 eluted with *n*-hexane-EtOAc (19:1) crystallized in room temperature to afford sesamin (200 mg). Series C (6.40 g) eluted with *n*-hexane – EtOAc (4:1) was further chromatographed on a silica gel column using *n*-hexane with a gradient of EtOAc to give 69 fractions of ca.150 mL each. Fractions 18–42 crystallized in room temperature to yield stigmasterol (100 mg). Series D (8.70 g) eluted with *n*-hexane–EtOAc (7:3) was subjected to column chromatography on silica gel eluting with a gradient system of hexane-EtOAc mixture of increasing polarity. A total of 300 fractions of ca. 50 mL each was collected. Finally, the combined fractions 230–235 eluted with *n*-hexane – EtOAc (4:1) afforded turraeanthin C (50 mg).

Aluminum sheets pre-coated with silica gel 60 F254 nm (Merck, Darmstadt, Germany) were used for thin layer chromatography, the isolated spot being visualized with ultra-violet light and detection accomplished by spaying with 10% $CeSO_4 – H_2SO_4$ followed by heating at 100°C.

Turraeanthin C (1). White solid, mp 120–122°C. UV (MeOH, λ_{max}, nm, log ε) 229 (3.5), 287 (3.6). IR (ν_{max}, cm^{-1}): 3400, 1715, 1517. ^1H and ^{13}C NMR see Table 1. HR-ES-IMS *m/z* 225.1120 $[M + H]^+$ (calcd for $C_{12}H_{17}O_4$, 225.1122).

Sesamin (**2**) and stigmasterol were identified by spectroscopic data. These data were consistent with those previously reported [17, 18].

Assays of Bio-activity.

Cytotoxicity. The method of sulforhodamine B was used to assay the toxicity of TA 10 (**1**) and TA 1 (**2**) on embryonic fibroblastic MRC5 cells (Biomerieux, France) according to Fricker and Buckley (1996).

Quantification of *Toxoplasma gondii* by β-Galactosidase Assay. HFF cells were infected by 2×10^4 tachyzoites of recombinant *T. gondii* (the gene of β-galactosidase was inserted) in 96 well plates in 200 µL for one hour. The wells were washed in MEM medium, and 200 µL of dilutions of turraeanthin C (TA 10), sesamin (TA 1), or EETA were added. Two controls were made: uninfected and infected nontreated cells. The plates were centrifuged at 500 g for 5 min. The cells were lysed in HEPES 100 mM pH 8, $MgSO_4$ 1 mM, Triton X 100 1 %, DTT 5 mM buffer for 1 h at 50°C. Lysis is controlled by microscopy. The reaction buffer (phosphate buffer100 mM pH 7.3, β-mercaptoethanol 102 mM, $MgCl_2$ 9 mM) was added in each well, and the plate was incubated 5 min at 37°C. Forty µL of chlorophenol red-β-D-galactopyranoside 6.25 mM in solution in phosphate buffer pH 7.3 was added and the whole kept at 37°C until the appearance of a red coloration. This coloration measured at 540 nm/420 nm indicated the parasite growth.

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