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Anti-*toxoplasma gondii* activity of constituents from *Balsamocitrus camerunensis* L (Rutaceae)

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Isolation, characterization and anti-*Toxoplasma gondii* activity of constituents from the CH_2Cl_2/MeOH (1/1) extract of the roots of the cameroonian plant *Balsamocitrus camerunensis* L. were investigated in this study. Four known coumarins derivatives were isolated, namely, marmin (1), imperatorin (2), xanthoxyletin (3), 6,7-Dimethoxycoumarin (4) and an acridone derivative namely 1-hydroxy-3-methoxy-acridone (5). Their structures were established on the basis of their spectroscopic data compared to reported results. Some of the isolated compounds showed noteworthy activity against *Toxoplasma gondii* intracellular parasite in mammals with an inhibition of parasite growth of around 46.44% for compound 4 and 82.12% for compound 3 which was the most active compound.

Key words: *Balsamocitrus camerunensis,* Rutaceae, coumarins, alkaloid, toxoplasmacidial activity.

INTRODUCTION

The discovery and development of anti-*toxoplasma* compounds is an effective way of controlling *Toxoplasma gondii* infections in humans and animals. *Toxoplasma gondii* is an obligate intracellular parasite that is able to infect a wide range of mammalian and avian species. In humans, *Toxoplasma* infections are widespread and can lead to severe disease in individuals with immature or suppressed immune systems. Consequently, *T. gondii* has become one of the major opportunistic infections of the AIDS epidemic (Saeij et al., 2005). Pregnant women without prior exposure to *T. gondii*, but that are infected during the first trimester of pregnancy can pass on the infection to the fetus resulting in abortion or serious neurological and eye pathologies of the baby at birth. (Remington and Klein, 1995)

The family Rutaceae consists of about 1500 species found principally in tropical and temperate regions (Meusel et al., 1978). Rutaceae species are used traditional medicine against elephantiasis, toothache, sexual impotence, gonorrhoea, malaria, dysmenorrhoea and abdominal pain (*Fagara zanthoxyloides)* (Kerharo and Adam, 1971; Ajanohoun et al., 1993; Anokbonggo et

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al., 1990; Watt and Breyer-Brandiwijk, 1962), Epigastric pain, vomiting, diarrhea, abdominal pain, colds, snake bites (*Zanthoxylum ailanthoides*), Aromatic tonic, stomachic and for fever, dyspepsia, cholera, Carminative and stomachic, used as a remedy for toothache, skin diseases, abdominal pain, anorexia, warm infestation, ataxia, Treat poisonous snake bites and also to treat diseases of the digestive (*Zanthoxylum alatum*), Treat fever, stomachache, flatulent colic, toothache and epilepsy (*Zanthoxylum capense*) (Medhi et al., 2013) etc. The chemistry of Rutaceae reveals the isolation of several secondary metabolites such as alkaloids, coumarins, flavonoids, phenylethanoids, lignans, limonoids, terpenoids, tannins and saponins, amongst others (Bouquet and Fouret, 1975; Wansi et al., 2009). Pharmacological studies on this family indicated many biological activities such as antitumor, antimicrobial, antifungal, controlling of cardiovascular and digestive system diseases (Lewis and Elin-Lewis, 1977; Metou et al., 1988; Sofowara, 1985). Previous phytochemical investigations of this genus revealed the presence of coumarins, quinoline alkaloids, free aliphatic acids and steroids, with some of these compounds exhibiting potent antibacterial, fungicidal, and algicidal properties (Tsassi et al., 2010). Plants of genus *Balsamocitrus* (including the decoction of the bark of *B. camerunensis*) are used in traditional African medicine to treat malaria, hypertension, infertility, and influenza (Tsassi et al., 2010; Asase et al., 2005).

As part of our systematic search for new bioactive lead structures from African medicinal plants, *Balsamocitrus camerunensis* L was selected for chemical and biological investigations. *B. camerunensis,* belonging to Rutaceae family, is a small tree of about 5 m high that is a newly discovered species found exclusively in Batouri (Cameroon) and Boukoko (Central African Republic) (Letouzey, 1963). Fouotsa et al. (2013) reported the presence of xanthones and coumarins in the stem bark of *B. Camerunensis*. To the best of our knowledge, there are no reported phytochemical studies on the roots of *B. Cameranensis*. Therefore, the objectives of this study were to isolate naturally occurring compounds from *B. camerunensis* roots and to evaluate their anti-*toxoplasma* activity. We report the isolation and characterisation of five known compounds isolated from the CH_2Cl_2/MeOH (1/1) extract of the roots of the plant *B. camerunensis*, namely marmin 1, imperatorin 2, xanthoxyletin 3, 6,7-Dimethoxycoumarin 4 and 1-hydroxy-3-methoxy-acridone 5, as well as their activity against intracellular *T. gondii* growth and proliferation.

MATERIALS AND METHODS

Sample collection

The plant *B. camerunensis* L. (Rutaceae) was collected from Dja (Batouri) in the East Province of the Republic of Cameroon, in January 2007 by Mr. Victor NANA of the National Herbarium of Cameroon who identified the plant. A voucher specimen is deposited at the National Herbarium, Yaounde, Cameroon.

Extraction and isolation

The air-dried and powdered roots (1.5 kg) of *B. camerunensis* L. was macerated with CH_2Cl_2/MeOH (1/1) at room temperature for 48 h. Removal of the solvent from the extract under reduced pressure yielded a yellow-brown residue (31.5 g). A part (30 g) of the crude extract was adsorbed on 40 g of silica gel with CH_2Cl_2/MeOH (1/1) as solvent and was submitted for gradient column chromatography on a 200 g silica gel column (type 60; 0.040-0.063 µm, Merck), using *n*-hexane-EtOAc, as the mobile phase and *n*-hexane as the eluent, resulting in the collection of 196 fractions (150 ml for each fraction). The 196 fractions were subsequently pooled into 11 major fractions (A-K) by combining the eluates on the basis of their mobility shift on TLC. Further separation of these fractions was done by repeated column chromatography using silica gel. Fraction A is the combined sub-fractions 1-11 (eluted with *n*-hexane- EtOAc 95:5) and fraction B (sub-fractions 12-26, eluted with *n*-hexane-EtOAc 9:1) was a mixture of fatty acids, hydrocarbons and phytosterols and was discarded. Fraction C (the combined sub-fractions 27-32, eluted with *n*-hexane-EtOAc 85:15) gave xanthoxyletin (3) (27.5 mg). From fraction E (composed of sub-fractions 37-42, eluted with *n*-hexane-EtOAc 85:15), we isolated imperatorin (2) (18.1 mg). Fraction G (composed of sub-fractions 62-71, eluted with *n*-hexane-EtOAc 75:25), was further separated by silica gel column chromatography, eluting with *n*-hexane-EtOAc in order of increasing polarity to yield marmin (1) (45.8 mg). From fraction H (the combined sub-fractions 72-75, eluted with *n*-hexane-EtOAc 75:25 and 70:30), we isolated HEN_4 6,7-Dimethoxycoumarin (4) (75 mg). Further purification of fraction J (the combined sub-fractions 82-86, eluted with *n*-hexane-EtOAc 65:35) gave HEN_5 1-hydroxy-3-methoxy-acridone (5) (97 mg). Fractions D, F, I and K were not further investigated.

Identification of compounds

The melting points were measured using a Büchi apparatus (Büchi melting points B-540) or on a microscope with heating platinum of Reicheirt, and are uncorrected. The optical activities were given at room temperature in methanol on a Perkin-Elmer 341 polarimeter. The α-Rotation of the light-polarized products dissolved in MeOH was measured in a 10 cm tank length. The D line (559 nm) of sodium was used as source of incidental light. The IR spectra were recorded using an infra red spectrophotometer in Fourrier Transformer of Nicolet 400 type on KBr pellet. Positions of the absorption bands were in cm^{-1}. UV spectra were recorded in MeOH solution on a Kontron-Uvikon 932 spectrophotometer. NMR experiments were carried out in various deutereted solvents (MeOH, acetone, $CDCl_3$) on a Varian Inova 500 (499.879 MHz) and a Varian Unity 300 (300.145 MHz) spectrometer for proton at 125.707 and for carbon spectra with TMS as internal standard at 75.087 MHz. Methyl, methylene and methine carbons were distinguished by APT (Attached Proton Test) experiments. Homonuclear ^1H connectivities were determined by using the COSY experiment. One-bond ^1H-^{13}C connectivities were determined with HSQC (Heteronuclear Single Quantum Coherence by 2D-multiple) gradient pulse factor selection. Two- and three-bond ^1H-^{13}C connectivities were determined by HMBC (Heteronuclear Multiple Bond Connectivity by 2D-multiple Quantum) experiment. Chemical shifts were reported in δ (ppm) and coupling constants (J) were measured in Hz. ESI mass spectra were recorded on a Quattro Triple Quadrupole mass spectrometer, with a Finnigan TSQ 7000 with nano-ESI API ion source. EIMS was performed on a Finnigan MAT95 (70 eV). High-resolution mass

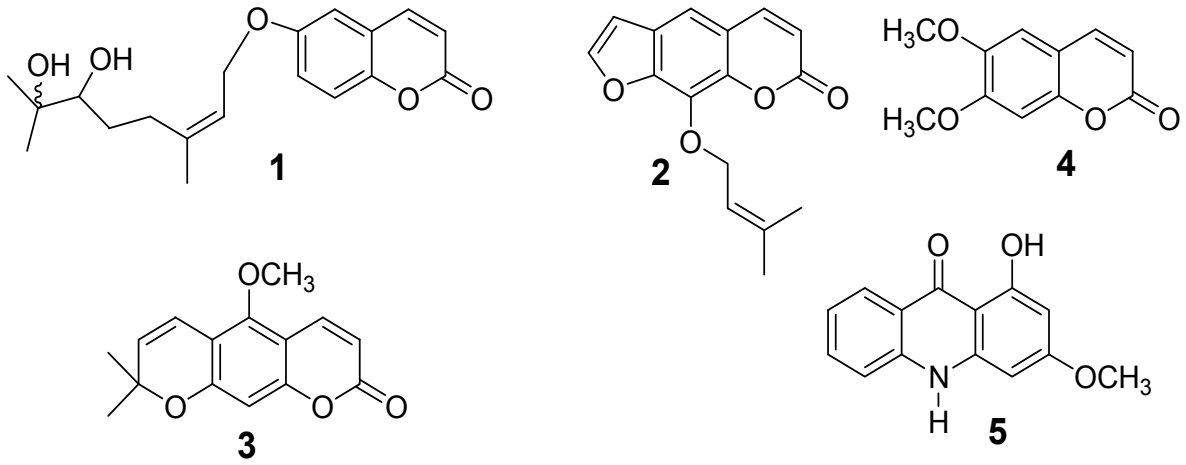

Figure 1. Structures of compounds 1-5.

spectra (HRMS) were recorded by ESI MS on an Apex IV 7 Tesla Fourier-Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA). Column chromatography was carried out on variable diameters of columns by using like stationary phase silica gel with granulometry, 60 Merk between 70-230 mesh and 230-400 mesh. Thin-layer chromatography (TLC) was performed on Polygram SIL G/UV254 (Macherey-Nagel & Co.) and with pre-coated aluminium sheets silica gel 60 F254 TLC. Plates were used to check the purity of compounds. Spots were visualised by UV lamp (254 nm and 365 nm) or by 50% H_2SO_4/H_2O reagent. All reagents used were of analytical grades.

Assays of bio-activity

Cytotoxicity

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

Quantification of Toxoplasma gondii by β-Galactosidase assay

Human foreskin fibroblasts (HFF) cells were infected by tachyzoites of *T. gondii* constitutively expressing *β*-galactosidase. The strain RH of *T. gondii* was used. The molecules to be assayed were added after 1 h of contact between *T. gondii* and HFF cells. *β*-Galactosidase assays were monitored at an absorbance of 540 nm/420 nm, to determine the parasite growth. The experiments were done in triplicate.

HFF cells, grown to full confluency in 96 well plates, were infected with 2×10^4 tachyzoites of genetically modified *T. gondii* constitutively expressing *β*-galactosidase. After one hour incubation of the parasites with HFF cells, the wells were washed with 200 µL of MEM medium, and 200 µL of fresh MEM medium containing dilutions of marmin (1), xanthoxyletin (3), 6,7-Dimethoxycoumarin (4) or 1-hydroxy-3-methoxy-acridone (5) added and incubated for 5 days in the presence of parasites. Two controls were made: uninfected and infected non-treated cells. The plates were centrifuged at 500 *g* for 5 min. The cells were lysed in HEPES 100 mM pH 8, MgSO4 1 mM, Triton X 100 1 %, DTT 5 mM buffer for 1 h at 50°C. Lysis was observed by microscopy. The reaction buffer (phosphate buffer 100 mM pH 7.3, *β* -mercaptoethanol 102 mM, $MgCl_2$ 9 mM) was added in each well, and the plate was incubated for 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 reaction mixture kept at 37°C until the appearance of a red coloration. The absorbance was measured at 540 nm/420 nm to determine parasite growth.

RESULTS

The powdered roots of *B. camerunensis* were extracted with the solvent system CH_2Cl_2/MeOH (1/1). The yellow-brown extract obtained was subjected to repeated column chromatography on silica gel, eluting with hexane followed by ethyl acetate in hexane with increasing polarity as mobile phase to obtain compounds 1-5 (Figure 1). These compounds were in agreement with data reported previously, identifying them as marmin 1 (HEN1), imperatorin 2 (HEN2) (Muller et al., 2004), xanthoxyletin 3 (HEN3), 6,7-Dimethoxycoumarin 4 (HEN4) (Perel'son et al., 1970) and 1-hydroxy-3-methoxy-acridone 5 (HEN5) (Spatafora and Tringaliw, 1997).

The compounds reported in this study marmin (1), xanthoxyletin (3), 6,7-Dimethoxycoumarin (4) and 1-hydroxy-3-methoxy-acridone (5) were evaluated on embryonic fibroblastic MRC5 cells for cytotoxicity using the sulforhodamine B assay (Fricker and Buckley, 1996) which gave an IC_{50} of 10 µg/mL.

We obtained an inhibition of parasite growth of around 5% for compound 1 (HEN1), 82.12% for compound 3 (HEN3), 46.44% for compound 4 (HEN4) and 22.03% for compound 5 (HEN5) (Figure 2).

DISCUSSION

Clinical treatment of toxoplasmosis in HIV-infected or AIDS patients has mostly been with atovaquone, a hydroxy-1,4-naphthoquinone, which is a structural analog

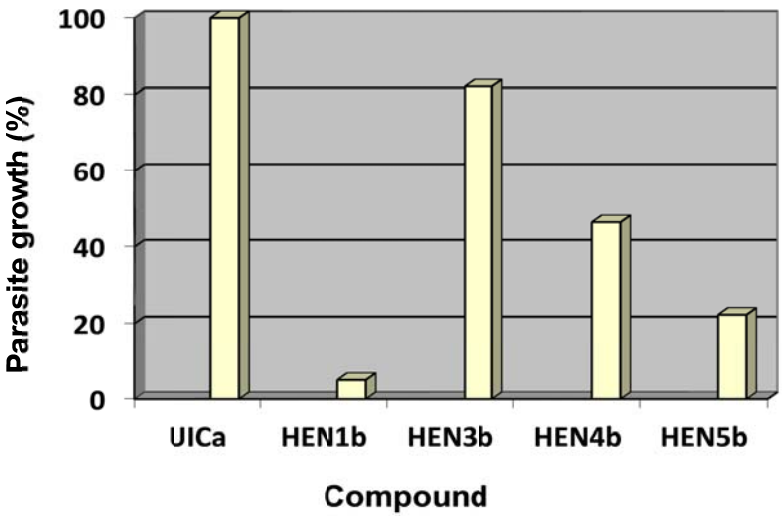

Figure 2. *Toxoplasma gondii* growth in presence of the different tested products. Parasites were incubated for 5 days in the presences of the products (in percentage). [a](UIC) Untreated infected cells. [b](HEN1) marmin, (HEN3) xanthoxyletin, (HEN4) 6,7-Dimethoxycoumarin and (HEN5) 1-hydroxy-3-methoxy-acridone.

of ubiquinone. *T. gondii* infection in immune-competent individuals often results in latent chronic infection, with parasite cyst formation in brain tissue. When the immune system of infected individuals wanes, such as in AIDS patients, the parasite is reacted leading to acute infection and toxoplasmosis clinical manifestation (Luft and Remington, 1992). CNS toxoplasmosis affects 7% of AIDS patients (Jones et al., 1999). Medications for immune-competent 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 (Kovacs and Masur, 2000), some patients are intolerant to regimens. Thus there is urgent need to develop more effective and tolerable drugs against *T. gondii* infection. Therefore, the current study was conducted to develop a new anti-*Toxoplasma* agent from our local medicinal plant *B. camerunensis*.

The anti-*T. gondii* activity of *B. camerunensis* has not been reported before. In this study, we found that 6,7-Dimethoxycoumarin(HEN4) significantly inhibited *T. gondii* growth with an inhibition of parasite growth of around 46.44% (Figure 2). The most potent anti–*T. gondii* activity was found with xanthoxyletin (HEN3), which showed an inhibition of parasite growth of around 82.12% (Figure 2), indicating a good anti–*T. gondii* activity.

Similar results were also reported by Vardamides et al. (2008). They used two compounds isolated from the stem bark of *Turraeanthus africanus* to evaluate the anti-*T. gondii* activity on embryonic fibroblastic MRC5 cells for cytotoxicity using the same methods (sulforhodamine B assay which gave an IC_{50} of 10 µg/mL). The results show that Turraenthin (bezoic acid derivative) inhibited *T. gondii* growth of around 55% while Sesamin (lignane) inhibited *T. gondii* growth of around 20%.

The search for anti-*T. gondii* agent from Cameroonian medicinal plants has led to the finding that compounds isolated from *B. camerunensis*, in particular HEN3, showed potent anti-*T. gondii* activity. These results, first reported in this work, have allowed us to propose that *B. camerunensis* are likely the sources of new compounds that could be used to treat *T. gondii* infections. Further studies will be necessary to determine the toxicity of these active compounds and to purify the other fractions.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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