Isolation, Characterization and Antiplasmodial Activity of Phytochemical Constituents from *Monanthotaxis parvifolia* (Oliv.) Verdc ssp. *kenyensis* Verdc

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Fractionation of the methanol extract of the leaves of *Monanthotaxis parvifolia* (Oliv.) Verdc ssp. *kenyensis* Verdc (Annonaceae) by preparative high performance liquid chromatography yielded seven fractions from which two phytochemical constituents were isolated and identified as quercetin-3-*O-β*-galactopyranoside (hyperoside) and quercetin-3-*O-α*-arabinofuranoside (avicularin) on the basis of spectroscopic data analysis and comparison with published data for the known compounds. This is the first report of the isolation of these compounds from *Monanthotaxis parvifolia*. Hyperoside and avicularin exhibited moderate antiplasmodial activity against chloroquine sensitive (D10) and chloroquine resistant (Dd2) *Plasmodium falciparum* strains (IC_{50} = 10.85 - 38.07 µg/ml). Hyperoside had selectivity index greater than 10 when assayed for cytotoxicity on Chinese hamster ovarian cell line.

INTRODUCTION

Monanthotaxis parvifolia (Oliv.) Verdc ssp. *kenyensis* Verdc (Annonaceae**)** is a rare and sparsely distributed plant species. The plant grows along the riverine and thickets on well drained red soil where the land has never been cleared for cultivation. *M. parvifolia* ssp. *kenyensis* is mainly a climber and grows to a height of 1-6 m. The plant has simple leaves with entire margin which are alternately arranged on the twigs measuring between 10 cm in length and 5 cm in width. The plant has small yellow flowers and the fruits are monocarps that bear 1-3 articles which are ellipsoid in shape on the lower side of the twigs [1].

Decoctions from *Monanthotaxis* species are used traditionally for the treatment of vomiting, headache and fever [2, 3]. Some *Monanthotaxis* species have been studied for their phytochemical constituents but there has been no report on *M. parvifolia* ssp. *kenyensis* [4, 5]. The present study reports the isolation and characterization of phytochemical isolates as well as antiplasmodial activity of this plant.

MATERIALS

Plant material collection and preparation

Aerial parts of *M. parvifolia* ssp. *kenyensis* plant were collected near Thika Town, about 40 km from Nairobi City, Kenya, in May 2006. The plant was authenticated at the National Herbarium, National Museums of Kenya where a voucher specimen (NMK/BOT/CTX/1/2) was deposited. The plant material was dried, ground into fine powder and stored in plastic containers until use.

Malaria parasites

The chloroquine-sensitive (D10) and the chloroquine-resistant (Dd2) *Plasmodium falciparum* malaria parasite strains of Indochina origin were a kind donation from Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia.

Erythrocytes for culture maintenance

Erythrocytes for maintenance of the parasite

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cultures were obtained from the whole blood of consenting O-positive blood group donors who had been screened for antimalarial drugs prior to donating the blood.

Solvents, reagents and materials

General purpose grade solvents such as chloroform, ethyl acetate and methanol (Kobian Ltd, Nairobi, Kenya) were laboratory distilled and used for extraction, fractionation and isolation of the phytochemicals. Analytical grade acetone and methanol (Alpha Chemicals Ltd, Nairobi, Kenya) were used for both analytical and preparative chromatographic work. Filtration to remove particulate matter from crude extracts was performed using Whatmann filter paper No.1 (Whatmann International Ltd, Maidstone, England). Water for the preparation of the standard drugs and crude extracts solution was freshly distilled (Gesedschatt fur Labortechnik GmbH, Burgwedel, Germany) in the laboratory before use. Water used for the culturing procedures was purified by a Millipore Synergy water purification system (Microsep, Tygervalley, South Africa). Ferric chloride and ammonia solutions (Pharmacos Ltd, Essex, England) were used in phytochemical screening.

Analytical grade chloroquine diphosphate was used as the reference drug for the antiplasmodial assay, while *D*-glucose and *D*-sorbitol were used in preparing the respective solutions for washing the blood pellets during synchronization of the malaria parasites (Sigma-Aldrich GmbH & Co., Darmstadt, Germany). Nitro blue tetrazolium (NBT) (Sigma Chemical Company, St. Louis, MO, USA) was used for colour development during the antimalarial assay. Giemsa solution was used to stain malarial parasite's deoxyribonucleic acid (DNA) for microscopy. Malarial parasite cultures were maintained on RPMI-1640 medium sourced from Gibco/BRL Life Technologies (Gaithersburg, MD, USA).

Equipment

A locally assembled hammer mill was used to grind the dried plant material. A rotary vacuum evaporator VV220 with a water bath WB2000® (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) connected to a cooler (Polyscience, Niles, IL, USA), and a diaphragm vacuum pump (KNF Neuberger GmbH, Freiburg, Germany) system was used to reduce the organic solvent extracts to dryness.

Preparative high performance liquid chromatography (HPLC) on reversed-phase column (XBridge™ C_{18} 19 × 250 mm, 5 μm) fitted with a 19 × 10 mm guard cartridge (Waters Corporation, Milford, MA, USA), was used for the fractionation of the phytochemical constituents. A Genevac HT-12 HCl evaporator (Genevac Inc, Stone Ridge, NY, USA) was used to dry the fractions obtained from HPLC for nuclear magnetic resonance (NMR) and mass spectrometry (MS) analyses. The Infrared (IR) spectra were recorded in a FT-IR 8400S spectrophotometer operating in a transmission mode (Shimadzu Europa GmbH, Duisburg, Germany) using 1% potassium bromide (KBr) disks.

The ^{1}H-NMR and ^{13}C-NMR spectra were recorded in deuterated chloroform ($CDCl_3$) and methanol (CD_3OD) at 500 MHz and 125 MHz, respectively, on a Bruker spectrometer (Bruker Daltonik GmbH, Bremen, Germany). A Waters 2424 model photodiode array (PDA) detector (Waters Corporation, Milford, USA) was used to obtain UV spectra of fractions from preparative-HPLC. Mass spectrometry analyses were done using a Waters Synapt G2 Mass spectrometer (Waters Corporation, Milford, MA, USA) coupled to a Mass lynx data analyzer (Waters Corporation, Milford, USA). A cell house 170 incubator (RS Biotech, Irvine, Scotland) was employed in the incubation of parasite cultures and test preparations. The Nikon-model LABOPHOT-2A (Japan) fitted with a 6 V, 30 W halogen lamp microscope at ×100 objective lens was used to monitor parasite growth. All aseptic and microbiological procedures were carried out under bioflow laminar flow equipment (Vermeulen, L.J. BVBA, Westmalle, Belgium).

METHODS

Extraction of plant material

About 800 g of the ground plant material was sequentially extracted for 48 h at 40 °C with petroleum ether (60-80 °C), chloroform and methanol. The solvent extracts were cooled, filtered, reduced to dryness in vacuo and the extract residues stored at 4 °C until use.

Preparation of stock solutions

Culture medium solution was prepared by use of 5.94 g of 4-[2-hydroxyethyl] piperazine-*N*1-[2-ethanesulfonic acid] (HEPES), together with 2 g glucose and 10.4 g RPMI-1640 powder which were transferred into a flat-bottomed flask and dissolved in 1 L of water with the aid of magnetic stirring before making the volume to 1130 ml with water. The medium was adjusted to pH 7.2 using 20% w/v sodium hydroxide. Chloroquine was prepared as a stock solution of 2 mg/ml in Millipore water and stored at -20°C until use. Five percent *D*-sorbitol solution was prepared by adding 5 g *D*-sorbitol into 6.7 ml PBS, the solution made to 100 ml using Millipore water and stored at -20°C until use. The wash medium for human erythrocytes was made up of 10.4 g RPMI-1640 containing L-glutamine, 5.94 g HEPES, 4.0 g D-glucose, 44 mg hypoxanthine, 5% sodium bicarbonate and 4 mg gentamicin dissolved in 900 ml of deionized sterile water.

The complete culture medium was prepared by supplementing the wash medium with 5% Albumax II. The test samples were dissolved in DMSO, sonicated for 20 min and diluted with Millipore water to a stock solution of 2 mg/ml which was stored at -20°C until use. The stock solutions were diluted with the culture medium at the time of the experiment. The complete medium composed of 1:1 ratio of Dulbecco's Modified Eagles Medium (DMEM) (Highveld Biologicals, Lyndhurst, South Africa) and Hams F-12 medium (Sigma, St Louis, MO, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) was used for Chinese hamster ovarian (CHO) cell lines. Emetine hydrochloride was used as the reference drug for the cytotoxicity evaluation.

Fractionation and characterization of isolated compounds

Preliminary screening of the crude extracts showed the methanol extract from the leaves to have good antiplasmodial activity. The leaf methanol extract was, therefore, scaled up for phytochemical isolation. The methanol extract was prepared into solution for fractionation and purification of compounds at a concentration of 100 mg/ml of the extract in 50% aqueous acetonitrile. The injection volume ranged between 50-1000 μL per run depending on the sample. The mobile phase flow rate was 20 μl/min for all purifications and the column heater was set at 30 °C. FractionLynx® software was used to monitor chromatographic peaks to guide fraction collection Fractions were evaporated to dryness using the Genevac evaporator. Structure elucidation of isolated compounds was performed using spectral data such as ^{1}H- and ^{13}C-NMR spectra as well as EI-MS spectra.

***In vitro* antiplasmodial assay**

Chloroquine-sensitive (CQS) D10 and chloroquine-resistant (CQR) Dd2 strains of *Plasmodium falciparum* parasites were maintained in a continuous *in vitro* culture as per published protocols [5]. The parasites were kept at a 5 % haematocrit with the complete RPMI-1640 medium supplemented with Albumax II. The parasitaemia was maintained below 10 % by the addition of washed, uninfected O$^+$ human red blood cells. The cultures were kept at 37 °C in an incubator with a gas mixture consisting of 93 % N$_2$, 3 % O$_2$ and 4 % CO$_2$ [6]. To maintain the parasites at one phase of growth, usually the ring-phase, they were synchronized by use of 5 % *D*-sorbitol solution [7].

Antiplasmodial activity was determined using the parasite lactate dehydrogenase activity assay [8, 9]. Chloroquine activity was tested at a starting concentration of 100 μg/ml, which was serially diluted two fold in the medium to give

ten concentrations. The samples were tested for activity at a starting concentration of 100 µg/ml, which were serially diluted two-fold in culture medium to give nine concentrations. The IC_{50} was determined from a non-linear dose-response curve fitting analysis using GraphPad Prism version 4. The IC_{50} values are given as a mean value with standard deviations of 2 independent experiments performed in duplicate.

In vitro cytotoxicity assay

Cytotoxicity of host cells is a very important criterion for evaluating the selectivity of the observed pharmacological activity and was therefore included in this study. The method described by Mosmann *et al.* was employed for cytotoxicity assay [10]. This is a quick colourimetric assay method for determining cellular growth and chemosensitivity. The technique makes use of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) bromide salt. The mammalian Chinese Hamster Ovarian (CHO) cell lines were routinely maintained as adherent monolayer cells in 75 cm^3 Falcon culture flasks (Corning Inc, NY, USA) in complete medium which was supplemented with 10 % heat inactivated FBS. The cells were incubated in a 5 % CO_2 and 95 %-air humidified atmosphere at 37°C. The culture medium was changed every three days and the cells sub-cultured [11]. The sample preparations were the same as for antiplasmodial screening. The initial concentration was 100 µg/ml, which was serially diluted in complete medium with 10-fold dilutions to give 4 concentrations, the lowest being 0.1 µg/ml. The formed formazan product was determined using a micro-plate reader at 540 nm. IC_{50} values were determined using non-linear dose–response curve fitting analysis in GraphPad Prism version 4 and are given as a mean value with standard deviations of 2 independent experiments performed in triplicate.

RESULTS AND DISCUSSION

Two compounds were isolated from the methanol extract of the leaves of *M. parvifolia* ssp. *kenyensis*. Spectroscopic analyses led to identification of two structurally related compounds as quercetin-3-*O*-β-galacto-pyranoside (hyperoside) (**1**) and quercetin-3-*O*-α-arabinofuranoside (avicularin) (**2**). The isolated compounds exhibited moderate antiplasmodial activity against both CQS and CQR *P. falciparum* as per literature criteria [12]. Safety of the isolated compounds was compared to their antiplasmodial activity by determining the corresponding selectivity index (SI = ICc_{50}/ICp_{50}). Compound **1** had a selectivity index greater than 10 while compound **2** had a selectivity index less than 10.

Quercetin-3-*O*-β-galactopyranoside (hyperoside) (**1**): UV (MeOH) λ_{max}, (nm): 286, 365; IR (KBr) ν_{max} (cm^{-1}): 3300 (OH), 2900, 1660 (C=O), 1600-1500; ^{1}H-NMR (400 MHz, MeOD), δ (ppm): 8.00 (s, 1H); 7.77 (d, J = 8.4 Hz, 1H); 7.06 (d, J = 8.4 Hz, 1H); 6.59 (s, 1H); 6.40 (s, 1H); 5.30 (d, J = 7.7 Hz, 1H), 4.04-3.98 (m, 2H), 3.85-3.81 (m, 1H), 3.77-3.73 (m, 1H), 3.52-3.47 (m, 2H); ^{13}C-NMR (100 MHz, MeOD), δ (ppm): 179.88 (C-4), 166.55 (C-7), 163.39 (C-5), 159.21 (C-9), 158.82 (C-2), 150.30 (C-4'), 146.18 (C-3'), 136.16 (C-3), 123.34 (C-1'), 123.27 (C-6'), 118.18 (C-5'), 116.47 (C-2'), 105.98 (C-10), 105.83 (C-1"), 100.34 (C-6), 95.15 (C-8), 77.54 (C-5"), 75.48 (C-3"), 73.56 (C-2"), 70.40 (C-4"), 62.34 (C-6"); MS m/z (M+H): 436.09 (M+H), 435.09, 419.09, 391, 303.05 (100 %), 304.05, 287.05, 241.07, 229.14, 217.10, 186.22, 149.01, 124.08.

The ^{1}H-NMR of compound 1 shows five proton signals, three singlets and two doublets. Based on the COSY spectrum the singlet at 8.00, is coupled with the doublet at 7.77. The doublet at 7.77 is in turn coupled with the doublet at 7.06. The coupling constant of 8.4 Hz indicates *ortho* coupling between the protons at 7.77 and 7.06. There is weak *meta* coupling between the protons at 8.00 and 7.77. These three signals are indicative of the presence of a 3', 4'-dihydroxy functional group as in ring B of flavonol which is similar to phenolic aromatic protons in a quercetin B ring. Finally the singlets at 6.40 and 6.59 exhibit weak *meta* coupling. The two signals are consistent with *meta* protons H-6 and H-8 which correspond to those on the A-ring of a quercetin nucleus [13, 15]. The doublet at 5.30

ppm with a coupling constant of 7.7 Hz is characteristic of a highly deshielded anomeric proton. The deshielded aliphatic ^{1}H-NMR signals at δ 4.04 to 3.47 ppm accounting for 6 protons may be attributed to glycosidic protons which resonate downfield relative to other aliphatic protons owing to the presence of multiple C-O bonds. The UV bands observed for compound **1** correspond with those reported in literature for flavonoids [16]. This is further supported by the observation that compound **1** is a yellow amorphous powder which turns ferric chloride green, suggesting that it is phenolic in nature. Search from literature confirms this inference [17]. The IR spectrum with a broad band at 3300 cm^{-1} supports presence of hydroxyl (OH) bonds in the structure while the strong band at 1660 cm^{-1} is characteristic of a carbonyl compound.

The most deshielded signal in the ^{13}C-NMR spectrum at δ 179.88 is due to the carbonyl carbon C-4 in ring C. The aromatic signals between δ 166.55 and 136.15 are due to the deshielded carbon atoms in rings A and C as well as the two hydroxyl-substituted carbons on ring B. The signals appearing below 80 ppm are attributable to the glycosidic carbon atoms. The anomeric carbon is the most deshielded glycosidic carbon atom appearing at 105.83 ppm. The most upfield signal at 62.34 ppm corresponds to the oxymethylene C-6. The ^{13}C-NMR signals of compound **1** corresponded with those reported in the literature for hyperoside [18]. Comparison of mass fragmentation pattern of compound **1** and quercetin indicate that the compound is a derivative of quercetin due to the presence of a base peak at m/z 303. All these data support three aromatic ring systems and presence of a sugar moiety attached to the skeleton and hence the suggested structure. Based on the data compound **1** was identified as hyperoside.

Avicularin (quercetin-3-*O*-β-arabinofuranoside, **2**): UV (MeOH) λ_{max}, nm: 258, 360; IR (KBr) ν_{max} cm^{-1}: 3400 (OH), 2395, 1650-1600; ^{1}H-NMR (400 MHz, MeOD) δ (ppm): 7.55 (d, *J* = 1.9 Hz, 1H), 7.50 (dd, *J* = 8.4, 1.9 Hz, 1H), 6.92 (d, *J* = 8.4 Hz, 1H), 6.40 (d, *J* = 1.4 Hz, 1H), 6.22 (d, *J* = 1.4 Hz, 1H), 5.49 (s, 1H), 4.35 (d, *J* = 2.1 Hz, 1H), 3.97 – 3.87 (m, 2H), 3.57 – 3.49 (m, 2H); ^{13}C-NMR (101 MHz, MeOD) δ, ppm: 178.83 (C-4), 164.92 (C-7), 161.58 (C-5), 157.99 (C-9), 156.87 (C-2), 148.60 (C-4'), 144.72 (C-3'), 133.89 (C-3), 121.73 (C-1'), 123.29 (C-6'), 115.59 (C-2'), 115.00 (C-5'), 108.17 (C-1"), 104.72 (C-10), 98.51 (C-6), 93.40 (C-8), 86.63 (C-4"), 81.99 (C-2"), 77.33 (C-3"), 61.17 (C-5").

The ^{1}H-NMR spectrum of **2** shows the presence of five proton signals in the aromatic region with a substitution pattern similar to that described for compound **1**. However, the signals for compound 2 were well resolved. The signal at 7.55 ppm was a doublet coupling with the signal at 7.50 ppm with a small coupling constant of 1.9 Hz thus indicating *meta* coupling. The proton at 7.50 ppm exhibits *ortho* coupling (*J* = 8.4 Hz) with the proton at 6.92 ppm. As discussed above, this pattern is found in ring B of flavonol. The protons at 6.40 and 6.22 ppm have a small coupling constant (*J* = 1.4 Hz) which suggests *meta* coupling as seen in ring A of flavonol. The singlet at 5.49 can be attributed to the deshielded ric proton. The three signals at 4.35 to 3.49 ppm accounting for five protons are attributable to glycosidic protons. The six glycosidic protons point to the presence of a pentose rather than a hexose glycone. The ^{13}C-NMR data were generally in agreement to those of a 15 carbon basic skeleton of quercetin. Apart from the deshielded anomeric carbon (108.17 ppm), there are four glycosidic carbon atoms at 86.63 (C-4"), 81.99 (C-2"), 77.33 (C-3") and 61.17 (C-5") thus confirming the presence of a pentose glycone moiety. The 13C-NMR data agrees with that reported in the literature for avicularin [19]. The MS spectrum of compound **2** also had a prominent base peak at m/z 303, which is characteristic of a quercetin structure. Based on the data compound **2** was identified as avicularin.
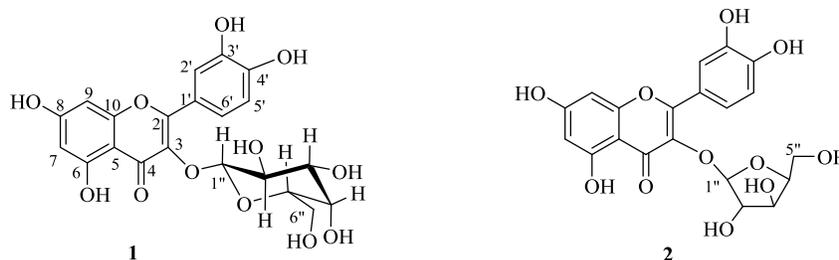
Figure 1. Chemical structures of hyperoside (1) and avicularin (2).

Although similar quercetin derivatives have previously been isolated from *Japecanga* leaves [13], *Bauhinia longifolia* [14], *Saxifraga stolonifera* (L) Meeb [15] and *Bistorta manshuriensis* [19] this is the first account reporting their isolation from *Monanthotaxis parvifolia* ssp. *kenyensis*, *in vitro* antiplasmodial activity and cytotoxicity on mammalian cells.

CONCLUSION

This study has provided the first report on the isolation of two quercetin glycosides, quercetin-3-*O*-β-galactopyranoside (hyperoside) and quercetin-3-*O*-α-arabinofuranoside (avicularin) from the leaves of *M. parvifolia* ssp. *kenyensis*. Biological assays showed that hyperoside and avicularin were active against both CQS and CQR plasmodial strains with IC_{50} < 50 µg/ml while hyperoside exhibited selective toxicity against mammalian cells with a SI > 10. The results lend credence to the use of *Monanthotaxis parvifolia* ssp. *kenyensis* Verdc in traditional medicine for the treatment of malaria related symptoms.

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