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ORIGINAL ARTICLE

Antiplasmodial activity of flavan derivatives from rootbark of *Cassia abbreviata* Oliv.

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Abstract The root bark of *Cassia abbreviata* has been traditionally used by the native population of the coastal region of Kenya to treat malaria. As part of our ongoing investigations into compounds with activity against malaria parasites, we tested the *in vitro* antiplasmodial activity against *Plasmodium falciparum* strain namely; chloroquine-resistant W2 and chloroquine-sensitive D6. The methanolic root extract of the plant was active against the chloroquine-sensitive (IC_{50} = 20.56 μg/ml) and the chloroquine-resistant (IC_{50} = 13.31 μg/ml) strains of *P. falciparum*. Two flavans **1** and **2** were purified, identified and further shown to be antiplasmodial. Compound **2** was more active than compound **1** against both strains of *P. falciparum* with IC_{50} values of 8.12 μg/ml (D6); 8.89 μg/ml (W2) and 26.02 μg/ml (D6); 25.97 μg/ml (W2), respectively. This study partly provides evidence to support the use of *C. abbreviata* as a malaria remedy, as used by the native populations.
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1. Introduction

Cassia abbreviata Oliv. (Fabaceae, Caesalpinioideae), is a small branchy umbrella-shaped deciduous tree 2–25 m tall, with a very distinctive cylindrical fruit (Brenan, 1967; Malan et al., 1996). From ethnomedical information root decoction is used to treat gastrointestinal disorders, malaria, gonorrhea, pneumonia and as a purgative (Chhabra et al., 1987; Gessler et al., 1994; Kokwaro, 1993; Mutasa and Kahn, 1995). The stem bark is used for treating diarrhoea, toothache, abdominal pains and headache (Pelgrave, 1977; Kokwaro, 1993; Orwa et al., 2009). Recently *C. abbreviata* was reported to be used for diabetes treatment in the lower eastern province in Kenya (Keter and Mutiso, 2012), which is experimentally demonstrated by reports of α-glucosidase inhibition and antioxidant activities of its stembark extract (Shai et al., 2010).

A number of compounds including anthraquinones, triterpenoids, alcohols and organic acids have been isolated from the flowers, leaves, root bark and stem bark of *C. abbreviata* (Mutasa and Kahn, 1995). The laxative activity of most *Cassia*

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species is linked to the anthraquinone emodin and its associated glycosides (Sakulpanich and Wandee, 2009). Methanolic extracts of the stem bark antagonized responses to acetylcholine and serotonin in a concentration-dependent manner (Parry and Duri, 1994).

Crude extracts from *C. abbreviata* parts have been screened severally for antiplasmodial activity but no pure active constituent reported (Weenen et al., 1990; Gessler et al., 1994; Connelly et al., 1996; Malan et al., 1996; Muthaura et al., 2007; Rukunga et al., 2009). A 2,4-trans-7,4′-dihydroxymethoxyflavan compound has been isolated and characterized from polar extracts of shredded leaves and twigs of *C. abbreviata*. However, the pharmacological effects were not determined (Dehmlow et al., 1998).

Malan et al. (1996) isolated guibourtinidin dimers from the bark of *C. abbreviata*. Acetone extracts of the bark yielded guibourtinidol-(4β→8)-epicatechin, guibourtinidol-(4α→8)-epiafzelechin, guibourtinidol-(4α→8)-catechin, guibourtinidol-(4α→8)-epicatechin and *ent*-guibourtinidol-(4β→8)-epicatechin (Malan et al., 1996). From the same research work, a novel (2*R*, 3*S*)-guibourrtinidol, representing the first flavan-3-ol with 4′, 7-dihydroxy phenolic substitution pattern, was identified in the heartwood of *C. abbreviata* but no biological activity reported.

Whereas it is clear that crude extracts of different parts of *C. abbreviata* have been screened for antiplasmodial activity, no activity-guided investigation leading to active pure compound has been reported. In this study we investigated the antiplasmodial effects of the methanolic root extract of *C. abbreviata* against two strains of *Plasmodium falciparum* namely; the chloroquine-resistant W2 and chloroquine-sensitive D6 and identified the active compounds.

2. Materials and methods

2.1. Plant material

The plant parts (roots) of *C. abbreviata* Oliv. (Fabaceae, Caesalpinioideae) were collected in July 2003 from Taita Taveta District in the coastal region of Kenya. The plant was identified by Dr. S. T. Kariuki (taxonomist) of the Department of Botany, Egerton University. A voucher specimen (KAR 156) of the plant was deposited at herbarium of the Department of Botany, Egerton University. The roots were dried under shade for 2 weeks. The dried roots were further chopped into small pieces and ground to powder then stored at room temperature until extraction.

2.2. Extraction and purification of active compounds

The dried ground plant material (675 g) was defatted with 5 L of petroleum ether (40–60 °C) (Fig. 1). The petroleum ether fraction was not analyzed further and was discarded. The defatted material was further extracted with methanol (5 L) in order to obtain 69 g of crude extract. The crude extract (15 g) was chromatographed on 50 g of silica gel 60 (0.063–0.2 mm) using a glass column with a dimension of 2.5 cm diameter and 10.0 cm long slurry packed volume. The column was eluted with a solvent system gradient eluent of increasing polarity from 100% $CHCl_3$ to 100% MeOH to give 25 fractions. Each of the fractions was spotted on thin layer chromatography (TLC) pates

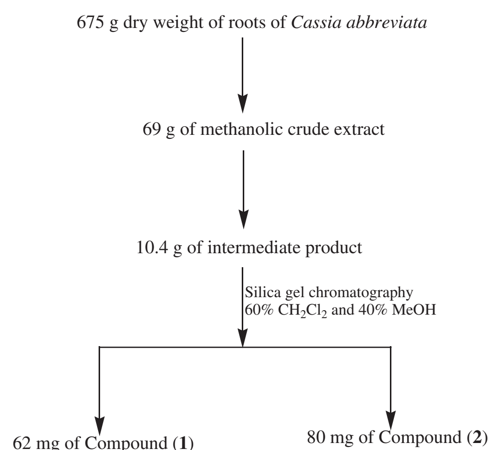

Figure 1 Purification scheme for compounds **1** and **2** from *C. abbreviata*.

(Macherey Nagel, 0.25 mm Dueren). Based on their TLC profile, ten main fractions were pooled from the 25 eluent fractions before being bioassayed against *P. falciparum* D6 and W2 strains. The TLC plates were developed using $CHCl_3$/MeOH (1:1) solvent system that gave good separation. The plates were viewed under UV, developed using anisaldehyde (anisaldehyde/concentrated sulfuric acid (1:1 in 200 ml of methanol) spray reagent and then heated at 100 °C for 15 min. Using the *in vitro* antiplasmodial activity against the D6 and W2 *P. falciparum* strains, the 10 main pooled fractions were tested and the most active fraction was subjected to repeated column chromatography until pure compounds were purified.

2.3. Spectroscopic and spectrometric analysis

NMR spectra (^{1}H and ^{13}C NMR, DEPT-135 and -90) were recorded at 30 °C on a Bruker instrument operating at 300 MHz using standard software packages. The pure compounds were dissolved in deuterated chloroform ($CDCl_3$) for NMR analysis. Chemical shifts (δ) are reported in ppm using the solvent signal as internal standard. Mass spectra were recorded with a LXQ linear ion trap instrument (Finnigan) using electrospray ionization (ESI-MS) in the negative or positive mode. Electron impact mass spectra (EI-MS) were recorded on a Thermoquest-Voyager instrument (equipped with a probe-inlet).

2.3.1. Compound 1: 2,3-Dihydro-5-hydroxy-8-methoxy-2-(4-methoxyphenyl)chromen-4-one

Yellow solid. $C_{17}H_{16}O_5$. ESI-MS, *m/z*: 323 $[M+Na]^+$. ^{1}H and ^{13}C NMR: Table 1. R_f = 0.87 in $CHCl_3$/MeOH (1:1 v/v).

2.3.2. Compound 2: 3,4-Dihydro-2-(4-hydroxyphenyl)-4-methoxy-2H-chromen-7-ol

Brown solid. $C_{16}H_{16}O_4$. ESI-MS, *m/z*: 295 $[M+Na]^+$. ^{1}H and ^{13}C NMR: Table 2. R_f = 0.82 in $CHCl_3$/MeOH (1:1 v/v).

2.4. In vitro antiplasmodial activity assay

The purification of compounds was guided by antiplasmodial testing. The [^{3}H]-hypoxanthine incorporation assay was used to determine the antiplasmodial activity using a semi-automated micro-dilution technique (Desjardins et al., 1979;

Table 1 ^{1}H NMR and ^{13}C NMR data for compound **1**.

No	Experimental		Literature	
	δ_{C-13}	δ_H	δ_{C-13}	δ_H
2	92.6	4.78, dd, J = 12.2 Hz	79.3	5.51, dd, J = 12.2 Hz
3	31.9	2.01, ddd, J = 14.2 Hz, J = 12.3 Hz, J = 3.0 Hz 2.31, dt, dt; J = 14.3 Hz	43.1	3.38, ddd, J = 14.2 Hz, J = 12.3 Hz, J = 3.0 Hz 3.13dt, dt; J = 14.3 Hz
4	192.5	–	196.9	–
5	162.7	–	153.6	–
6	109.3	7.85, d, J = 2.1	108.4	6.26, d, J = 2.4
7	119.9	7.72, d, J = 2.1	120.7	6.67, d, J = 2.4
8	137.0	–	142.4	–
9	143.2	–	148.7	–
10	115.8	–	111.3	–
1′	128.8	–	133.0	–
2′	121.4	7.11, m	128.2	7.08, m
3′	133.6	7.27 m	114.5	6.70, m
4′	149.3	–	159.6	–
5′	124.6	7.72 m	114.5	6.70, m
6′	131.0	7.27 m	128.2	7.08, m
8-OCH_3	55.7	3.45, s	56.2	3.73, s
4′-OCH_3	55.2	3.50, s	55.9	3.73, s
5	–OH	12.09	–OH	12.23

d, doublet; s, singlet and m, multiplet.

Muregi et al., 2003). Two *P. falciparum* malaria parasite clones W2 and D6 were utilized in the susceptibility tests for the crude extracts, the intermediate fractions and purified compounds. The W2 clone is resistant to chloroquine, pyrimethamine, sulfadoxine and quinine, while D6 is resistant to mefloquine but sensitive to chloroquine (Tchuendem et al., 1999; Odhiambo and Odulaja, 2005). These isolates were obtained from our frozen sample library, and maintained in continuous culture to acquire *in vitro* replication robustness depicted by attainment of 3–8% parasitemia within eight-days of culture.

The test samples including extracts, fractions and compounds were dissolved in dimethyl sulfoxide (DMSO) in disposable sterile 50 ml culture test tubes (Fischer Scientific, Pittsburgh, USA) and sonicated for 10 min. Stock solutions were prepared at 50 μg/ml concentration each, by diluting the test sample solutions with complete culture medium with serum (RPMI 1640). Reference drugs, chloroquine (CQ) and mefloquine (MQ) were dissolved in 70% ethanol and starting concentrations of 1000 and 250 ng/ml respectively prepared. These drugs followed by the test samples were put on separate rows of 96-well microculture plates (catalog # 167008; Nunc Inc., Roskilde, Denmark) in first wells only. Twofold dilution across the 11 wells was then done to attain a dose range of CQ (1000–1.953 ng/ml); MQ (250–0.488 ng/ml); test samples (50,000–97.656 ng/ml) with final DMSO concentration < 1% as templates all test extracts and reference standard drugs. Test plates were prepared by transferring 25 μl aliquots of the extract from each well to another sterile plate.

For *in vitro* testing, culture adapted parasites at 3–8% parasitemia were lowered to 1% parasitemia at 1% hematocrit and 200 μl added to pre-dosed drug plates. Negative controls and positive controls (parasitized red blood cells) were incorporated. The plates were incubated for 24 h in 6% CO_2 saturated incubator at 37 °C. Then 25 μl of [^{3}H]-hypoxanthine (0.15 μci) was added and further incubated for 18 h. Harvested cell contents were dried in the oven and thereafter scintillation fluid was put. Drug effect was measured by differential uptake of radiolabeled [^{3}H]-hypoxanthine using an automated liquid scintillation counter (Scintillation and Luminescence counter Top count NXT v2.3) which generated dose dependent scintillation counts per minute (cpm).

The concentration of the test sample that inhibits 50% of the cells (IC_{50} values) was obtained from dose–response curves, using a non-linear dose–response curve fitting analysis via GraphPad Prism v.4.00 software. The concentrations were plotted against scintillation counts per minute (cpm).

2.5. Statistical analysis

Data are expressed as mean ± standard deviation. Statistical analysis was performed using student *t*-test. *P*-values lower that 0.05 were statistically considered as significant.

3. Results and discussion

The methanolic root crude extract of *C. abbreviata* had an *in vitro* IC_{50} value of 13.31 μg/ml against chloroquine-resistant *P. falciparum* W2 strain and 20.56 μg/ml against chloroquine-sensitive *P. falciparum* D6 strain. According to WHO guidelines and previous results from our team (Basco et al., 1994; Pink et al., 2005; Jonville et al., 2008), antiplasmodial activity was classified as follows: highly active at IC_{50} < 5 μg/ml, promising at 5–15 μg/ml, moderate at 15–50 μg/ml and inactive at > 50 μg/ml. The antiplasmodial activity of the methanolic root extract of *C. abbreviata* was therefore classified as promising against W2 and moderate against D6.

Previous antiplasmodial test done for *C. abbreviata* collected and screened in Malawi showed that the plant had potentially high antiplasmodial activity. The organic crude extracts of both leaves and roots of the plant gave the following activities IC_{50} = 10.08 and 2.88 μg/ml, respectively (Connelly et al., 1996). The test for these extracts was done against a multidrug resistant strain of *P. falciparum*, Vietnam–Smith strain

Table 2 ^{1}H NMR and ^{13}C NMR data for compound **2**.

No	Experimental		Literature	
	$\delta_{C\text{-}13}$	δ_H	$\delta_{C\text{-}13}$	δ_H
2	72.6	4.89, dd; J = 12.2 Hz	73.1	5.19, dd; J = 12.2 Hz
3	31.9	2.00, ddd; J = 14.2 Hz, J = 12.3 Hz, J = 3.0 Hz, 2.31, dt; J = 14.3 Hz	34.9	2.02, ddd; J = 14.1 Hz, J = 12.3 Hz, J = 3.0 Hz, 2.31, dt; J = 14.3 Hz
4	82.5	4.25, "t"; J = 4.9 Hz	72.4	4.25, "t"; J = 4.9 Hz
5	137.0	7.72, d J = 8.3 Hz	132.0	7.14, d; J = 8.3 Hz
6	109.3	7.85, d J = 2.3 Hz and 8.4 Hz	107.9	6.44, dd; J = 2.3 and 8.4 Hz
7	162.7	–	155.5	–
8	109.9	6.38, d, J = 2.3 Hz	103.5	6.38, d; J = 2.3 Hz
9	153.2	–	156.3	–
10	115.8	–	113.6	–
1′	128.8	–	133.3	–
2′	121.4	7.11, m	128.0	6.86, m
3′	113.6	7.11, m	115.4	6.86 m
4′	159.3	–	157.0	–
5′	114.6	7.72, m	115.4	7.33, m
6′	131.0	7.27, m	128.0	7.33, m
4-OCH_3	56.7	3.24, s	55.8	3.45, s
4′-OH		12.43		12.23
7-OH		12.94		12.34

d, doublet; s, singlet and m, multiplet.

(VI/S). In that study the activity was suspected to be due to the presence of alkaloids or other polar compounds in the plant parts. However, there was no pure compound reported from this screening.

From this research work, bioassay-guided chromatographic fractionation of the methanolic root crude extract of *C. abbreviata* led to the identification of two structurally related compounds **1** and **2** responsible for the observed antiplasmodial activity (see Fig. 2). Purification was, however, focused on the fraction that showed enrichment of antiplasmodial activity upon fractionation so as to purify the compounds responsible for the observed antiplasmodial activity. The crude extract afforded 10 main fractions, from which the fifth fraction to be eluted had the strongest antiplasmodial activity. This fraction was repeatedly fractionated using silica gel chromatography to afford two compounds **1** and **2**. The proposed chemical structures for the purified compounds were based on the interpretation of the spectra and compared with reported spectroscopic data from the literature.

Compound **1**, named 2,3-dihydro-5-hydroxy-8-methoxy-2-(4-methoxyphenyl)chromen-4-one, was purified as a yellow solid (62 mg) and was readily soluble in chloroform, dichloromethane and methanol. The ^{1}H NMR, ^{13}C NMR and DEPT spectra indicated a typical flavanoid basic structure that is functionalised at positions C-4, C-5, C-8 and C-4′ (see Fig. 2). The ^{1}H NMR spectrum showed a characteristic resonance signal at δ_H 12.09, corresponding to a phenol OH group at position C-5 (refer Table 1).

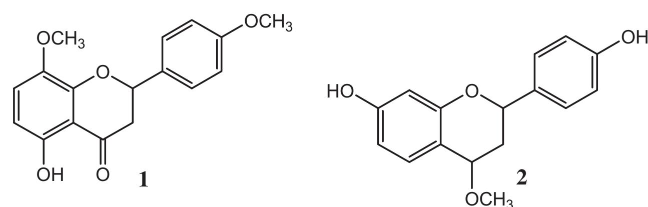

Figure 2 Chemical structures for purified compounds **1** and **2**.

Compound **2** (80 mg) was also purified from the methanolic root extract as brown solid that was highly soluble in chloroform and methanol. The structure of compound **2** was elucidated and was identified to be 2,4-trans-7,4′-hydroxy-4-methoxyflavan by common name and 3,4-dihydro-2-(4-hydroxyphenyl)-4-methoxy-2H-chromen-7-ol by IUPAC naming system. Compound **2** was previously purified from shredded leaves and twigs of *C. abbreviata*. The ^{1}H -NMR and ^{13}C NMR spectral data generated experimentally are summarized in Table 2 and the corresponding literature values given (Dehmlow et al., 1998). The bioassay-guided fractionation process yielded two compounds; (**1**) (62 mg) and (**2**) (80 mg) with the latter having a more improved antiplasmodial activity (D6 IC_{50} value of 8.1 ± 0.8 μg/ml and W2 IC_{50} value of 8.9 ± 2.1 μg/ml) relative to the intermediate fractions and the crude extract. The chemical structures of compounds **1** and **2** were deduced based on the NMR spectra and EI-MS values when compared to literature values.

There are no previous reports of compounds **1** and **2** being investigated for antiplasmodial activity though their structures

Table 3 *In vitro* antiplasmodial activity values for chloroquine, mefloquine, quinine and compounds **1** and **2**.

Compound	Antiplasmodial D6 (IC_{50} μg/ml) ± S.D.	Antiplasmodial W2 (IC_{50} μg/ml) ± S.D.
Chloroquine	0.026	0.029
Mefloquine	0.030	0.044
Quinine	0.93	0.106
Crude extract	13.31 ± 4.1	20.56 ± 3.2
1	26.02 ± 1.0	25.97 ± 1.0
2	8.12 ± 0.8	8.89 ± 2.1
Control	0	0

S.D., standard deviation.

reveal that they could be biologically active. The antiplasmodial activities for compounds **1** and **2** are summarized in Table 3. Compound **1** showed a moderate activity, with IC_{50} values of 26.02 μg/ml (D6), and 25.97 μg/ml (W2) while compound **2** was more potent with IC_{50} values of 8.12 μg/ml (D6), 8.89 μg/ml (W2) good antiplasmodial activity as per the criterion. The results can be discussed based on a criterion described by Pink et al. (2005) in which for antiparasitic drug discovery, a compound can be considered a hit if it is active *in vitro* against whole protozoa with an IC_{50} of $\leqslant$1 μg/ml. Based on the criterion, both compounds **1** and **2** cannot be considered hits but active enough to account for the reported antimalarial activity reported by traditional herbal practice.

4. Conclusion

The identification of antiplasmodial flavans from *C. abbreviata* suggests that they may play a role in the medicinal properties of the plant, but their potential for the development of antimalarial drugs is limited due to their level of activity and the chemical structures of these classes of compounds.

Conflict of interest statement

The authors confirm that there is no conflict of interest. The funding agencies had no role in the review framework, concepts, interpretation of literature and the final conclusions.

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