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Antiplasmodial prenylated flavanonols from *Tephrosia subtriflora*

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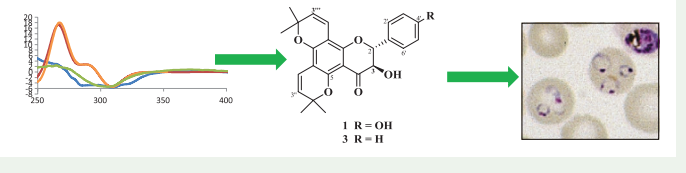
ABSTRACT

The CH_2Cl_2/MeOH (1:1) extract of the aerial parts of *Tephrosia subtriflora* afforded a new flavanonol, named subtriflavanonol (**1**), along with the known flavanone spinoflavanone B, and the known flavanonols MS-II (**2**) and mundulinol. The structures were elucidated by the use of NMR spectroscopy and mass spectrometry. The absolute configuration of the flavanonols was determined based on quantum chemical ECD calculations. In the antiplasmodial assay, compound **2** showed the highest activity against chloroquine-sensitive *Plasmodium falciparum* reference clones (D6 and 3D7), artemisinin-sensitive isolate (F32-TEM) as well as field isolate (KSM 009) with IC_{50} values 1.4–4.6 µM without significant cytotoxicity against Vero and HEp2 cell lines (IC_{50} > 100 µM). The new compound (**1**) showed weak antiplasmodial activity, IC_{50} 12.5–24.2 µM, but also showed selective anticancer activity against HEp2 cell line (CC_{50} 16.9 µM).



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Tephrosia subtriflora; Leguminosae; prenylated flavanonol; subtriflavanonol; antiplasmodial; cytotoxicity

1. Introduction

The genus *Tephrosia* (Leguminosae) is a source of a range of structurally unique polyphenols, such as flavonoids, isoflavonoids and biflavonoids, some of which with antiplasmodial and antioxidant activities (Chen et al. 2014; Plioukas et al. 2016; Nguekeu et al. 2017). In

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continuation of our interest on the genus *Tephrosia* (Atilaw et al. 2017), we have tested the aerial parts of *Tephrosia subtriflora*, a species endemic to the south eastern regions of Kenya (Agnew and Agnew 1994), for antiplasmodial activity. The crude extract was moderately active against chloroquine-sensitive reference clones D6 and 3D7 of *P. falciparum* as well as field isolate KSM 009. Phytochemical investigation of the extract led to the isolation of four compounds, of which, compound **1** is new (Figure 1). The isolation, characterisation, and the antiplasmodial activities of these compounds are reported.

2. Results and discussion

The known flavanone spinoflavanone B (Rao and Prasad 1992) and the known flavanonols MS-II (**2**) (Cao et al. 2004) and mundulinol (Alavez-Solano et al. 2000) were identified by comparison of their spectroscopic data with literature. The new compound (**1**) was isolated as a light yellow amorphous solid. HRMS showed a $[M]^+$ at *m/z* 420.1573, which along with NMR data (Section 3.4., Table S1, Figures S2–S7) was consistent with a molecular formula $C_{25}H_{24}O_6$. The UV (λ_{max} 200 and 267 nm), ^1H and ^{13}C NMR (Section 3.4., Table S1) spectral data suggested a flavanonol skeleton (Figure 1). Thus, the ^1H NMR spectrum showed an AX spin system at δ_H 4.92 and 4.38 (J = 12.1 Hz) characteristic of *trans*-oriented H-2 and H-3 of a flavanonol moiety. A doublet (J = 2.0 Hz) at δ_H 4.05 coupled with H-3 (δ_H 4.38), as shown in the COSY spectrum (Figure S3), was assigned to hydroxy group at C-3 (δ_C 73.1). The ^{13}C NMR spectrum showed signals at δ_C 82.9 (C-2), 73.1 (C-3) and 190.6 (C-4), supporting a flavanonol skeleton. In agreement with this, the HMBC spectrum (Table S1, Figure S5), showed correlations of H-2 with C-1′ and C-4, and H-3 with C-1′ and C-2.

The ^1H NMR spectrum further showed signals for an AA′XX′ aromatic spin system resonating at δ_H 7.41 and 6.87, assigned to Ar-$H_{2'/6'}$ and Ar-$H_{3'/5'}$, respectively of ring B. In addition the ^{13}C NMR signals at δ_C 129.4 (C-2′/6′), 115.6 (C-3′/5′) and 156.8 (C-4′) were in agreement with mono-substituted (with OH, δ_H 5.61) ring B. Absence of aromatic protons other than those of ring B indicated that ring A is fully substituted. The presence of two 2,2-dimethylchromene residues were deduced from the characteristic signals for two sets of AX protons at δ_H 6.50 and 5.50{(both, 1H, *d*, J = 10.0 Hz, for H-3″ and H-4″), and 6.58 and 5.56 (both, 1H, *d*, J = 10.0 Hz, for H-3‴ and H-4‴)}, with ^{13}C NMR signals at δ_C 126.9 and 127.0 for C-3″/3‴, respectively and 115.9 and 115.8 for C-4″/4‴, respectively (Table S1). The assignment of the signal at δ_H 6.50 to H-4″ was corroborated by a weak NOESY (Figure S7) cross peak to H-2′, whereas no NOESY correlation could be found between H-2′ and the proton at δ_H 6.58 (H-4‴).

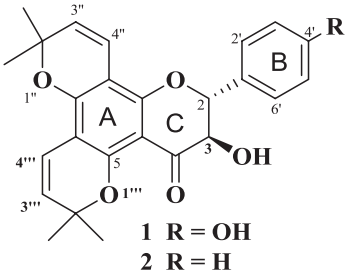

Figure 1. Structures of compounds **1** and **2** isolated from *Tephrosia subtriflora*.

Furthermore, the ^{1}H NMR spectrum showed singlets at δ_H 1.52 (3H), 1.45 (6H) and 1.43 (3H), with the corresponding carbon signals at δ_C 28.4, 28.2 (x 2) and 27.9, assigned to two pairs of *gem*-dimethyl substituents of the two 2,2-dimethylchromene rings (Table S1). The long-range coupling of H-3″ with C-8, and that of H-3‴ with C-6, in the HMBC spectrum (Table S1, Figure S5), supported the placement of the 2,2-dimethylchromene rings at C-5/6 and C-7/8 ring junctions.

The large coupling constant ($J_{2,3}$ = 12.1 Hz) between H-2 and H-3 indicated a *trans*-1,2-diaxial orientation with the phenyl ring B as equatorial (Alavez-Solano et al. 2000; Cao et al. 2004), with two possible configurations; *2R*,*3R* and *2S*,*3S*. Slade et al. (2005) has suggested the $n\rightarrow\pi^*$ transition at 300–340 nm to be used to differentiate the two *trans* enantiomers. However, this band is generaly weak as reported in literature (Alavez-Solano et al. 2000; Cao et al. 2004); this is also the case for compounds **1** and **2** (Figure S10). On the other hand the Cotton effect at *ca*. 305 nm for $\pi\rightarrow\pi^*$ transition is strong and negative in the ECD spectra of these compounds, suggesting *2R*,*3R* absolute configuration as in related flavanonols (Alavez-Solano et al. 2000). In order to ascertain this – first, conformational search of different conformations of the flavononols **1** and **2** were conducted, and the four lowest energy conformations for each compound were identified. These were then used as input structures for quantum chemical calculations. The resulting difference between the global minimum energy structure and the first local minimum next to it was found to be more than 5 kcal/mole in each case. Thus, only the conformations with the lowest energies for compound **1** (Figure 2a) and compound **2** (Figure 2b) were used for ECD calculations. According to Boltzmann weight, the population of other conformations which are higher in energy were negligible. Then ECD quantum chemical calculation for (2*R*,3*R*)-**1** and (2*R*,3*R*)-**2** (Figure S10) were computed. Clearly a strong negative Cotton effect at *ca.* 305 nm for $\pi\rightarrow\pi^*$ transition is associated with 2*R*,3*R* absolute configuration as in other natural flavanonols (Alavez-Solano et al. 2000; Cao et al. 2004). Thus, this transition is more reliable in determining absolute configuration for flavanonols than the Slade et al. (2005) proposal of $n\rightarrow\pi^*$ transition at 300–340 nm. From the on-going, compound **1** was characterised as (*2R*,*3R*)-3,4′-dihydroxydi(2,2-dimethylchromene-5:6,7:8)flavanonol (**1**), to which a trivial name subtriflavanonol

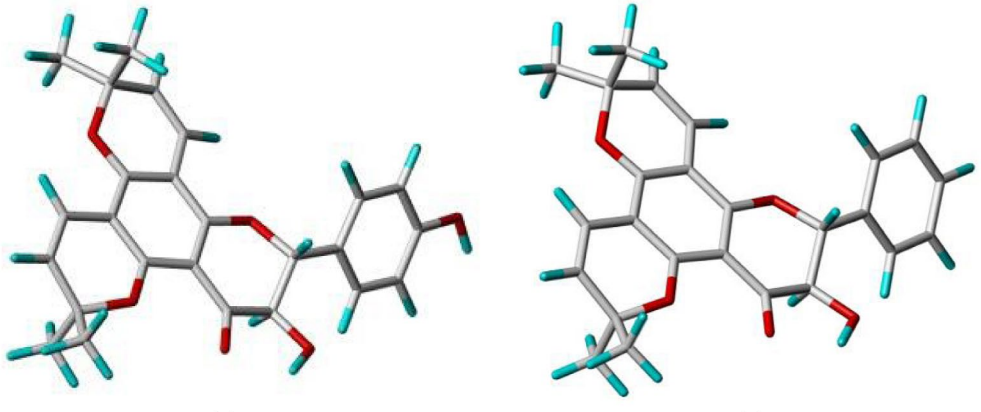

Figure 2. The calculated global energy minimum geometries of (2*R*,3*R*)-**1** (a) and (2*R*,3*R*)-**2** (b).

has been assigned. Similarly the absolute configuration of the related flavononol (**2**) was established as (2*R*,3*R*)-**2**.

The crude CH_2Cl_2/CH_3OH (1:1) extract of the aerial parts of *T. subtriflora* was tested for antiplasmodial activity against chloroquine-sensitive *P. falciparum* reference clones (D6 and 3D7) using a previously established protocol (Smilkstein et al. 2004) with modifications (Cheruiyot et al. 2016; Yeda et al. 2016). The extract showed antiplasmodial activity (Table 1) with IC_{50} value of 4.9–11.4 µg/mL. The pure compounds were also tested for antiplasmodial activity against these strains (Table 1), and compound **2** showed good activity, IC_{50} = 1.7 ± 0.1 µM (against 3D7) and 4.6 ± 1.1 µM (against D6) (Atilaw et al. 2017). The flavanone spinoflavanone B showed moderate activity (IC_{50} = 5.9 ± 0.6 µM against D6, 5.5 ± 1.1 µM against 3D7), while compounds **1** and mundulinol only showed weak activities (Table 1). Compound **2** being the most active was further tested against the artemisinin-sensitive F32-TEM clone, and also showed good activity, IC_{50} = 1.4 ± 0.3 µM. Whereas the activities of compound **2** against the strains 3D7 and F32-TEM were more or less the same, this compound is two-fold less active (IC_{50} = 4.6 ± 1.1 µM) against the D6 strain.

Plasmodium falciparum reference strains and clones undergo a series of short- and long-term continuous culture for distribution by the biological resource centres and research laboratories. Our recent study (Yeda et al. 2016) revealed genotypic and phenotypic stability of *Plasmodium falciparum* field isolates in continuous *in vitro* culture. This observation justifies the inclusion of parasites from natural infection as part of preclinical screening. Thus, the crude extract and the pure compounds were also tested against the field isolate KSM 009. The activities observed against the field isolate are comparable with the activity levels against standard strains (Table 1).

In a cytotoxicity assay, compound **2** did not show significant toxicity (CC_{50} > 100 µM) against both the mammalian African monkey kidney (Vero) and the human larynx carcinoma (HEp2) cell lines (Table 1), indicating that the observed antiplasmodial activity may not be due to general toxicity. However, the new compound (**1**) showed selective cytotoxicity against HEp2 cell line (CC_{50} = 16.9 µM) without significant cytotoxicity against Vero cell line (CC_{50} 102.9 µM).

The antiplasmodial activity of the crude extract could be due to the flavonoids isolated, especially those of the flavanone spinoflavanone B and the flavanonol MS-II (**2**). Other flavonoid subclasses including flavones and chalconoids isolated from the genus *Tephrosia*

Table 1. *In vitro* antiplasmodial activity against D6, 3D7 and KSM 009 strains of *P. falciparum*, and cytotoxicity of crude extract and pure compounds isolated from *Tephrosia subtriflora*.

	Antiplasmodial activity (IC_{50})			Cytotoxicity (CC_{50})	
Sample	D6	3D7	KSM 009	Vero	HEp2
CH_2Cl_2/CH_3OH (1:1) extract[a]	4.9 ± 0.1	10.5 ± 0.1	11.4 ± 1.3	123.8	135.3
Subtriflavanonol (**1**) [b]	12.5 ± 2.2	24.2 ± 3.5	18.7 ± 1.2	102.5	16.9
MS-II (**2**)[c]	4.6 ± 1.1	1.7 ± 0.1	1.5 ± 0.1	>247.5	>247.5
Spinosaflavanone B	5.9 ± 0.6	5.5 ± 1.1	6.6 ± 1.8	NT	NT
Mundulinol	35.6 ± 0.5	27.8 ± 0.4	22.3 ± 0.6	>248.7	>248.7
Chloroquine	0.0792	0.0140	0.0125	NT	NT
Mefloquine	0.0437	0.0121	0.0185	NT	NT

[a]IC_{50} in µg/mL for crude extract.
[b]IC_{50} in µM for pure compounds and standards.
[c]Compound **2** was also tested against the artemisinin sensitive F32-TEM strain, IC_{50} = 1.4 ± 0.3 µM.
Note: NT = Not Tested.

have also been reported to show antiplasmodial activities (Atilaw et al. 2017). This appears to be the first report on the antiplasmodial activity of flavanonols. It is therefore, worthwhile to test other flavanonols for *in vitro* and *in vivo* antimalarial activities.

3. Experimental

3.1. General experimental procedures

NMR spectra were acquired on Bruker AVANCE III 600 spectrometer using TMS (for ^{1}H) or the residual solvent signal (for ^{13}C) as reference. All 1D and 2D spectra were obtained using standard Bruker software. EI-MS spectra were obtained on a SSq 710 Finnigan MAT mass spectrometer using direct inlet, and 70 eV ionisation voltage. UV spectra were recorded using PerkinElmer Lambda 25, Serial No: 501S14030919 spectrophotometer. ECD spectra were recorded on a J-815 CD-spektrapolarimeter, serial No. A030261168. Optical rotations were measured on a PerkinElmer 341-LC Polarimeter. Column chromatography was run on silica gel 60 (70–230 mesh). TLC and prep TLC were carried out on Merck pre-coated silica gel 60 F_{254} plates (Merck Darmstadt Germany). Gel filtration was done on Sephadex LH-20 (Fluka, Buchs, Switzerland).

3.2. Plant material

The aerial parts of *Tephrosia substriflora* were collected from the natural habitat along Emali–Amboseli road near Emali Township (GPS location: 037° 28′40.18″E and 02° 06′03.13″S) on 30 May 2013. The plant was identified by Mr Patrick C. Mutiso of the University Herbarium, School of Biological Sciences, University of Nairobi, where a voucher specimen (Mutiso-848/May 2013) was deposited.

3.3. Extraction and isolation of compounds from aerial parts of Tephrosia subtriflora

The air-dried and ground aerial parts of *T. subtriflora* (2.4 kg) were extracted with CH_2Cl_2/CH_3OH, 1:1 (4x 1.5 L) by percolation to give 210 g of crude extract. A portion (130 g) of the extract was subjected to CC on silica gel (1.3 kg) eluting with *n*-hexane containing increasing percentages of EtOAc. The fraction eluted with *n*-hexane was separated by CC over Sephadex LH-20 (eluent: CH_2Cl_2/CH_3OH; 1:1) and further purified by PTLC (*n*-hexane/EtOAc; 4:1) to yield spinoflavanone B (10.5 mg). The fractions eluted with 2–5% EtOAc in *n*-hexane were combined and purified by crystallisation (from methanol) to afford MS-II (**2,** 8.0 mg). The mother liquor was further separated by CC chromatography over Sephadex LH-20 (CH_2Cl_2/CH_3OH; 1:1) and PTLC (*n*-hexane/acetone; 4:1) yielding mundulinol (7.5 mg). Crystallisation (from methanol) of the 15% EtOAc in *n*-hexane eluent afforded subtriflavanonol (**1**, 12.2 mg).

3.4. Subtriflavanonol (1)

Yellow amorphous solid. UV (MeOH) λ_{max}: 200 and 267 nm; $[\alpha]_D^{20} = -41.7$ (c 0.001, CD_2Cl_2); ECD (CH_2Cl_2) λ nm ($\Delta\varepsilon$; $M^{-1}\,cm^{-1}$): $(+5.55)_{246}$, $(0)_{273}$, $(-5.66)_{305}$, $(0)_{353}$, $(+0.29)_{368}$; ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.92 (1H, *d, J* = 12.1 Hz, H-2), 4.38 (1H, *dd, J* = 12.1, 2.0 Hz, H-3), 7.41 (2H, *m,* H- 2′/6′), 6.87 (2H, *m*, H-3′/5′), 5.50 (1H, *d, J* = 10.0 Hz, H-3″), 6.58 (1H, *d, J* = 10.0 Hz, H-4″),

5.56 (1H, *d, J* = 10.0 Hz, H-3‴), 6.50 (1H, *d, J* = 10.1 Hz, H-4‴), 1.52 (3H, *s*), 1.45 (6H, *s*), 1.43 (3H *s*) (for Me_{2-2}″ and Me_{2-2}‴), 4.05 (1H, *d, J* = 2.0 Hz, 3-OH), 5.61 (1H, *br* s, 4′-OH); ^{13}C NMR (150 MHz, $CDCl_3$): *δ* 82.9 (C-2), 73.1 (C-3), 190.6 (C-4), 102.6[a] (C-4a), 155.9 (C-5), 104.8 (C-6), 155.4 (C-7), 102.7[a] (C-8), 157.5 (C-8a), 129.1 (C-1′), 129.4 (C-2′/6′), 115.6 (C-3′/5′), 156.8 (C-4′), 78.5 (C-2″), 126.9 (C-3″), 115.9 (C-4″); EIMS *m/z* (rel. int.): 420 $[M]^+$ (13), 285 (16), 269 (18), 121 (15), 107 (100), 105 (25), 79 (28), 77 (38), 65 (19), 55 (24), 53 (31), 43 (58); HRMS *m/z* 420.1573 for $C_{25}H_{24}O_6$ (calculated for 420.1573).

3.5. In vitro *antiplasmodial activity assay*

Antiplasmodial activities of crude extract and pure compounds were tested against chloroquine-sensitive *P. falciparum* reference clones (D6 and 3D7), artemisinin-sensitive clone (F32-TEM) as well as field isolate KSM 009 (the current circulating strain of *P. falciparum* in East African region) using non-radioactive assay technique following established protocols (Smilkstein et al. 2004) with modifications (Cheruiyot et al. 2016; Yeda et al. 2016).

3.6. *Cytotoxicity assay*

The cytotoxicity of crude extract and pure compounds isolated from *T. subtriflora* against the mammalian cell lines, African monkey kidney (Vero), and human larynx carcinoma (HEp2) cell lines was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as described in literature (Prayong et al. 2008). The logarithmic graph of cytotoxicity versus sample concentration was used to calculate the CC_{50} values (Prayong et al. 2008).

3.7. *Theoretical calculation*

Different conformations and configurations of the studied compounds were optimised at the B3LYP/6-311G(d,p) (Lee et al. 1988; Becke 1993) level of theory without any restrictions. The ECD were computed using the Time-Dependent DFT (TDDFT) (Bauernschmitt and Ahlrichs 1996; Autschbach et al. 2002) algorithm in the programme package GAUSSIAN 09 (Frisch et al. 2009)(5). The Becke3–Lee–Yang–Parr functional and the 6-31G*(d) basis set was applied. About 15 singlet and 15 triplet states were solved (keyword TD (NStates = 15, 50–50). All GAUSSIAN results were analysed and the spectra display using the SpecDis 1.62 (Bruhn et al. 2014). The molecules were displayed using SYBYL -X 2.1.1 (2013).

4. Conclusion

A new flavanonol along with three known flavonoids were isolated from CH_2Cl_2/CH_3OH (1:1) aerial parts extract of *T. subtriflora*. The Cotton effect at *ca.* 305 nm for $\pi \rightarrow \pi^*$ transition in ECD spectra is useful in determining the absolute configuration of flavanonols. One of the compounds, MS-II (**2**) showed good antiplasmodial activity (Nogueira and Lopes 2011) against four strains of *P. falciparum*, including KSM 009 – one of the currently circulating isolates of *P. falciparum* in the East African region, with no significant cytotoxicity. The new compound (**1**) showed antiplasmodial activity and also showed selective cytotoxicity against Hep2 cell line.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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