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CYTOTOXIC AND ANTIMALARIAL BIFLAVONOIDS ISOLATED FROM THE AERIAL PARTS OF *Ochna Serrulata* (Hochst.) WALP

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ABSTRACT

This paper reports the phytochemistry and biological activities (antimalarial and cytotoxicity) of compounds isolated from aerial parts of Ochna serrulata. Silica gel chromatography followed by repeated chromatotron on the stem bark extract of the plant yielded a new dimeric chalcone (5-deoxyurundeuvine C) along with lophirone C, lophirone A, afzelone B, epicatechin, methyl (2',4'-dihydroxyphenyl)acetate and a mixture of campylospermone A and isocampylospermone A. From the leaves extract, ochnaflavone, 2",3"-dihydroochnaflavone, vitexin-6"-O-acetate, 3'-methoxyvitexin-6"-O-acetate and (2',4'-dihydroxyphenyl)acetic acid were isolated and characterized. A cyanoglucoside was isolated from an extract of flowers and fruits of the plant. The proposed structures of the respective new and known compounds were assigned by using 1D and 2D NMR techniques as well as Mass spectrometry. The cytotoxicity activity of the compounds was tested using sulforhodamine B (SRB) assay in three different cancer cell lines namely, UACC62 (melanoma), TK 10 (renal), and MCF7 (breast). Ochnaflavone and 3'-methoxyvitexin-6"-O-acetate inhibited the growth of UACC62 at TGI (Total Growth Inhibition concentration) 12.91 and 14.11 µM, respectively. The cytotoxic activities of lophirone C were found to be TGI = 35.63, 11.67 and 30.35 µM, against TK, UACC62 and MCF7 cancer cells respectively. Lophirone A exhibited TGI against these cancer cells at 58.96, 26.23 and 40.01 µM, respectively. The rest of the compounds exhibited no significant cytotoxicity against the three cancer cells. Moreover, the compounds were evaluated for antimalarial activity against FCR-3 (chloroquine-resistant Plasmodium falciparum) strains. Ochnaflavone demonstrated the highest activity IC_{50} = 17.25 µM, followed by lophirone A (29.78 µM), 5-deoxyurundeuvine C (31.07 µM), lophirone C (35.31 µM), afzelone B (39.54 µM), 2",3"-dihydroochnaflavone (61.86 µM) and 3'-methoxyvitexin-6"-O-acetate (106.35 µM). These results concur with the traditional use of the genus in the treatment of various ailments.

Keywords: 5-Deoxyurundeuvine C, Biflavonoid, Cytotoxicity, Antimalarial, *Ochna serrulata*

INTRODUCTION

The genus *Ochna* L. consists of trees and shrubs belonging to family Ochnaceae with more than 85 plant species. Most of the plant species in this genus are tropical, distributed from Asia, Mascarene Islands, Madagascar to Africa (Verdicourt 2005). Phytochemical studies have indicated the genus to be a rich source of biflavonoids, (Abdullahi et al. 2014). However, other compounds reported include terpenoids, glycosides, saponins, steroids and fatty acids (Agra et al. 2007). The isolation of ginkgetin from maidenhair tree, *Ginko biloba* L in 1929 marked the beginning of the journey towards the isolation and characterization of biflavonoids. To date different kinds of biflavonoids have been isolated from nature,

the diversity being attributed by the symmetrical or nonsymmetrical units that constitute the skeleton of the molecule. These units might be flavanones, flavones, flavanols, aurones, chalcones and their dihydro derivatives. The manner in which the flavonoid units are linked also contribute to the diversity of these compounds, this might be through either carbon to carbon (-C-C-) or ether (-C-O-C-) bond between two carbons. Furthermore, the link might be between ring A and ring B (AB biflavonoids), two ring A (AA biflavonoids), two ring C (3,3″-CC biflavonoids) and lastly, two B rings (BB biflavonoids). The type of the substituent/ functional groups as well as stereogenic centers in the skeleton further aggravates the diversity (Ferreira et al. 2006).

Due to their structural differences, large number of interesting biological activities have been demonstrated by these compounds. These include antimicrobial (Bagla et al. 2014), anti-inflammatory (Park et al. 2006), antiviral (Lee et al. 2006), anticancer (Son et al. 2006), vasorelaxant (Yu et al. 2017) and other activities. It is the fact that the plant has never been phytochemically evaluated before, the ability of biflavonoids to express variety of biological activities, coupled with their low toxicity, that sparked our interest. With this respect, it was considered logical to phytochemically and biologically evaluate the compounds from the aerial parts of *O. serrulata*. The plant that is locally known as *Umbomvane* (Zulu), fynblaarrooihout (Afrikaan) and iliTye (Xhosa), its root decoction is traditionally used for bone disease treatment and *gangrenus proctitis* (Hutchings and Van Staden 1994, Hutchings et al 1996).

MATERIALS AND METHODS

General experimental procedure

Purification of the fractions was achieved by centrifugal chromatography (chromatotron model 7924, Harrison Research), where, circular glass chromatotron plates were used. A layer that is either 2 or 4 mm thick made of preparative silica gel (Merck 7749 with gypsum binding agent) was applied on a glass plate. Silicagel 60F254, (40-63 μm, Merck) was used in column chromatography for purifying the fractions. For the reported NMR spectra recording (^{1}H, ^{1}H -^{1}H COSY, HSQC, HMBC and ^{13}C NMR), Bruker Avance III 400 or Bruker Avance III 500 spectrometers were used. The residual solvent peaks were used for chemical shift referencing (methanol-d4 (^{1}H 3.31 ppm, ^{13}C, 49.1 ppm) and acetone-d6 (^{1}H 2.05 ppm, ^{13}C, 29.9 ppm)). LCT Premier mass spectrometer was used in mass spectral data collection on the time-of-flight (TOF) Waters through electrospray ionization (positive or negative mode). ADP 440+ model polarimeter was used for optical rotations determination; this is the product of Bellingham and Stanley. Separations were monitored by TLC plates (Kieselgel 60 F254, 0.25mm) that was sprayed with anisaldehyde reagent then heated. Anisaldehyde was prepared following: 465 mL methanol in a 1 L volumetric flask were cooled in an ice bath, then addition of 5 mL acetic acid followed by concentrated sulfuric acid (17 mL) and *p*-anisaldehyde (13 mL).

Plant Materials

Aerial parts of *O. serrulata*, obtained from the UKZN Botanical Garden (Sept. 2009) where identification and authentication was done by Allison Young from UKZN Botanical Garden. Voucher specimen with reference number NU, M. Ndoile 01 was deposited at the University of KwaZulu-Natal herbarium.

Extraction and Isolation

Air dried and pulverized 0.92 kg stem bark of *O. serulata* were extracted with methanol (1 L) three times to obtain 9.7 g. Due to the presence of highly polar components, the extract was dissolved in EtOAc:H_2O (8:2)

and extracted three times with 100 mL ethylacetate to yield 7.0 g. Five fractions (Fr. 1-5) were obtained after fractionation of the EtOAC extract in a silica gel column and mixtures of EtOAc:hexanes were used as eluent. Fr. 3 (500 mg) and 4 (1.6 g) were combined and fractionated in a silica gel column eluted with EtOAc:hexanes mixtures yielding 4 fractions (4.1-4.4). Fraction 4.2 (450 mg) was repeatedly purified on a chromatotron with DCM:EtOAc (2:1) eluent to yield 6 mg of **13** and 10 mg of **7**. Repeated chromatotron on fraction 4.3 (600 mg) with DCM:EtOAc (2:1) eluent yielded 10 mg of compound **1** along with **7** (10 mg), **8** (12 mg), **11** (10 mg), **6** (10 mg) and a mixture of **9** and **10** (18 mg).

Air dried and pulverized leaves (1.98 kg) of the plant were extracted at room temperature three times with MeOH (2L), to obtain 40 g of extract. This was followed by fractionation on a silica gel column eluted sequencially with DCM, EtOAc and MeOH to obtain 8.2 g, 8.8 g and 20 g of extracts, respectively.

On a short silica gel column, 8.8 g of EtOAc extract were fractionated using increasing polarity of hexanes:EtOAc solvent ratios to yield 5 fractions (L1-5). Fraction L4 (1.0 g) were repeatedly purified on a chromatotron with MeOH:DCM (1:9) to yield **14** (20 mg) and **2** (48 mg). The remainder of L4 combined with L5 were repeatedly purified on a chromatotron eluted with MeOH:DCM (1:9) to yield **2** (16 mg), **3** (18 mg) together with **4** (18 mg) and **5** (13 mg). Air dried and ground fruits and flowers (0.5 kg) were soaked in MeOH to yield 500 mg extract. Upon dissolving the extract in MeOH, off-white fluffy solids were observed, washed with MeOH to afford compound **12** (10 mg).

Biological assays

Cytotoxicity

Isolated compounds were assayed for their cytotoxicity effects following the protocol stated by Wellington et al 2013.

Antimalarial assays

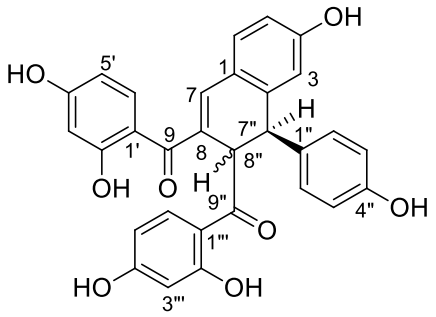
In vitro antimalarial activities of the isolated compounds were evaluated against FCR-3 (chloroquine-resistant *P. falciparum* strain) following the protocol described by Chemaly et al. 2007.

RESULTS AND DISCUSSION

Chromatographic separations on the stem bark and leaves ethylacetate extracts over silica gel column followed by centrifugal thin layer chromatography (chromatotron) yielded one new dimeric chalcone 5-deoxyurundeuvine C (**1**), along with ochnaflavone (**2**) (Kun et al. 1992), 2″,3″-dihydroochnaflavone (**3**) (Likhitwitayawuid et al. 2001), vitexin-6″-*O*-acetate (**4**), 3′-methoxyvitexin-6″-*O*-acetate (**5**), epicatechin (**6**), lophirone A (**7**) (Murakami et al. 1991), lophirone C (**8**) (Pegnyemb et al. 2001), a mixture of isocampylospermone A (**9**) and campylospermone A (**10**), afzelone B (**11**) (Pegnyemb et al. 2003), a noncyanogenic glucoside ((2*Z*)-[(4*R*,5*R*,6*S*)-6-(β-D-glucopyranosyloxy)-4,5-dihydroxycyclohex-2-en-1-ylidene]ethanenitrile) (**12**), methyl (2′,4′-dihydroxyphenyl)acetate (**13**), (2′,4′-dihydroxyphenyl)acetic acid (**14**). Flavones **4** and **5** were previously isolated from leguminoceae and rosaceae families (Brum-Bousquet et al. 1977, Kashnikova et al. 1984), and they are hereby reported to be isolated from family Ochnaceae for the first time. Noncyanogenic glucoside **12** known to family Aquifoliaceae, Ranunculaceae and synthesized by Lefebvre and Drian (2007) is herein, reported for the first time from family Ochnaceae. Compound **13** was first discovered from *Madhuca pasquiery* and later *Ilex aquifolium* (Nahar et al. 2005, Taylor et al. 2011), thus, the first report on

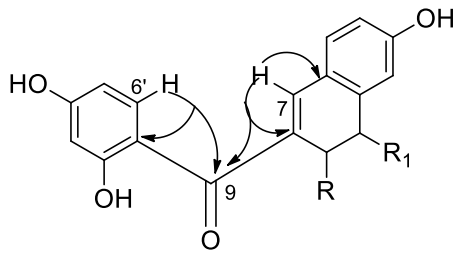
the isolation of compound **13** from Ochnaceae. *Nephila clavata* (spider) toxin has been discovered to contain compound **14** that inhibit L-glutamic acid binding to the brain synaptic membranes, thus, cause paralysis to the prey (Pan-Hou et al. 1987, Kim et al. 1998, Taylor et al. 2011). The first isolation of **14** from plants was from *Ilex aquifolium* seeds, (Nahar et al. 2005) however, in the family ochnaceae, the compound has been isolated for the first time from *Ouratea hexasperma* branches (de Carvalho et al. 2008).

The structures of the above stated secondary metabolites (**1**-**14**) were established by analyses of both NMR spectra (1D and 2D) and mass spectrometry. Known compounds were further confirmed by comparison of the observed and reported spectroscopic data.



1

Compound **1**, a yellow noncrystalline substance, was discovered to possess molecular formula of $C_{30}H_{21}O_8$ that was determined by HRESIMS ([M-H]$^-$, *m/z* 509.1242, calcd 509.1236). The compound displayed NMR spectra that showed signals typical for a chalcone skeleton with trisubstituted alkene (δ_H 7.24) and two vicinal methine protons that were observed to show vicinal spin spin interaction (δ_H 4.99 and 4.34, J = 7.9 Hz), resembling those reported for urundeuvine C with exception on C-5 substituent (Bandeira et al. 2003). Basing on data from ^{1}H NMR, COSY and HMBC cross-peaks, a *p*-substituted and three aromatic systems with protons showing an ABX spin system were identified. H-7 (δ_H 7.24) was linked to C-9 (δ_C 200.0), C-2 (δ_C 133.1), C-8 and C-1 (δ_C 125.3), due to the HMBC correlations observed, thus, allowing the assignment of ring B of the flavonoid moiety (first ABX aromatic system) to both C-7 and C-7″. The HMBC cross-peaks between H-6′ (δ_H 7.68) and C-9 (δ_C 200.0), confirmed the position of ring A (second ABX aromatic system), thus, leading to partial structure **1a** (Fig. 1)



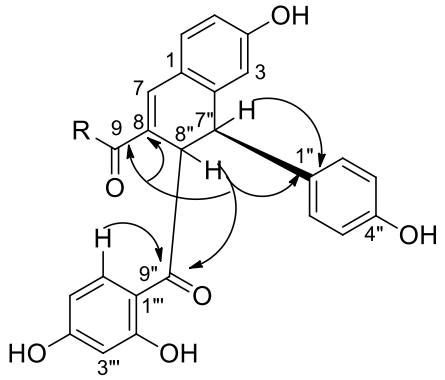
1a

Figure 1: HMBC Cross-Peaks in Partial Structure **1a**

The HMBC cross peaks between H-6″ and the carbonyl C-9″ (δ_C 206.2) that was again correlated to H-8″ (δ_H 4.99), confirmed the position of the third ABX aromatic ring (Ring A′). The position of the *p*-substituted (ring B′) aromatic system indicated by cross-peaks between H-2", 6" (δ_H 7.05) and C-7″ (δ_C 49.6), and H-7″, with C-1″ and C-2″, 6″. The position of C-8″ was further shown by cross peaks of H-8" with both carbonyls C-9″ and C-9, and C-1″ (δ_C 133.1), C-7 (δ_C 141.7), and C-7″. The stated cross-peaks allowed the construction of partial structure **1b** (Fig. 2).

The relative stereochemisty at C-7″ and C-8″ was assigned based on the observed coupling constant (J = 7.9 Hz) for vicinal spin-spin interactions depicting a diaxial orientation between H-8″ and H-7″ (Bandeira et al. 2003). With respect to the mentioned spectroscopic features (Table 1), the new compound **1** was characterized and is thus named 5-deoxyurundeuvine C.

The biogenesis of compound **1** originate from 2′,4′,4-trihydroxychalcone, the conjugate addition forms a dimer, then dienone-phenol rearrangement, followed by cyclization to yield **1** (Fig. 3) (Bai et al. 2003).

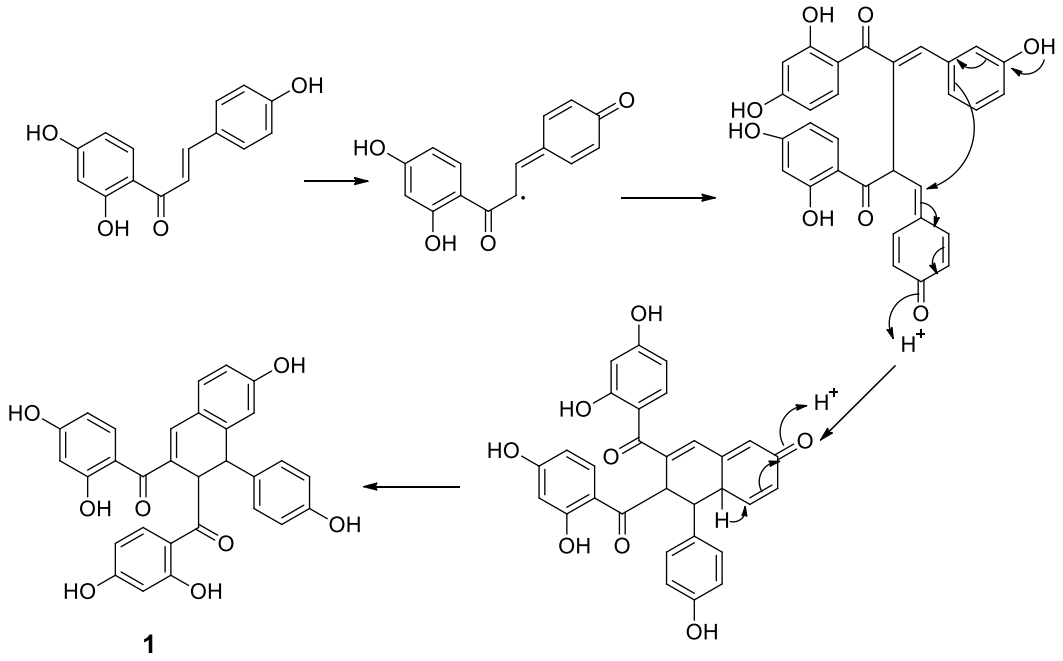


1b

Figure 2: HMBC Correlations in Partial Structure **1b**

Table 1: ^{13}C and ^{1}H NMR Spectroscopic Data for 1 as Compared to Urundeuvine C in CD_3OD

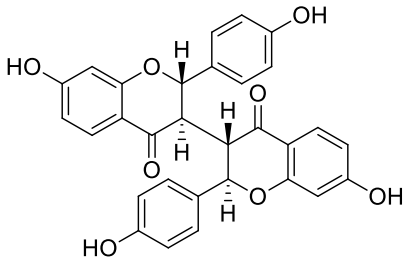
Position	δ_H	*m* (*J* in Hz)	1 δ_C, type	Urundeuvine C δ_C, type
9"		-	206.2, C=O	204.7, C=O
9		-	200.0, C=O	199.2, C=O
4'		-	166.9, C-O	166.9, C-O
4"'		-	166.6, C-O	166.4, C-O
2'		-	166.3, C-O	165.9, C-O
2"'		-	166.2, C-O	165.7, C-O
4"		-	161.4, C-O	157.3, C-O
4		-	157.8, C-O	148.7, C-O
5	6.67	*dd* (2.3, 8.2)	115.4, CH	145.2, C-O
7	7.24	s	141.7, CH	141.6, CH
6'	7.68	*d* (8.8)	135.9, CH	135.7, CH
1"	-	-	133.1, C	134.7, C
6"'	7.72	*d* (9.1)	134.5, CH	134.0, CH
2	-	-	133.1, C	131.4, C
2", 6"	7.05	*d* (8.5)	130.8, CH	130.2, CH
8,1	-	-	125.3, C	124.8, C
6	7.18	*d* (8.2)	132.5, CH	117.31, CH
3	6.27	*d* (2.3)	103.8, CH	116.8, CH
3", 5"	6.67	*d* (8.5)	116.7, CH	116.3, CH
1'	-	-	114.2, C	113.4, C
1"'	-	-	114.0, C	113.1, C
5"'	6.27	*dd* (2.3, 9.1)	109.3, CH	109.0, CH
5'	6.35	*dd* (2.4, 8.8 Hz)	108.9, CH	108.6, CH
3'	6.16	*d* (2.4)	104.2, CH	103.9, CH
3"'	6.35	*d* (2.4)	116.8, CH	103.8, CH
8"	4.99	*d* (7.9)	51.8, CH	51.1, CH
7"	4.34	*d* (7.9)	49.6, CH	48.4, CH



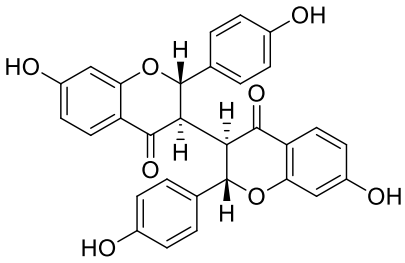
1

Figure 3: Proposed Biogenesis of Compound 1

The outer bark of *O. integerrima* yielded isocampylospermone A (**9**) and campylospermone A (**10**). The compounds were named as biflavanone 1 and biflavanone 2, where, the latter was later published as campylospermone A from *Campylospermum mannii* stem bark (Ichino et al. 2006, Elo Manga et al. 2009). As assigned by Ichino et al. (2006), the relative configuration (C-3-C-3″) was based on optical rotation, where, **10** showed a significant specific rotation (+83.2 in MeOH) thus, chiral, whereas **9** indicated a very small specific rotation (+6) and thus, meso isomer. Following analogy of the reported chamaejasmin where its meso isomer was named as isochamaejasmin, we hereby propose the name isocampylospermone A be assigned to compound **9**.



9



10

Biological activities

The cytotoxic activities of the compounds were evaluated by assessing their growth inhibition activity in a 3-cell line panel consisting of UACC62 (melanoma), TK 10 (renal), and MCF7 (breast). The discussed

results herein, are summarized in Table 2, therefore, for each compound the following parameters were assessed: 50% growth inhibitory effect, signifying the growth inhibitory power of the compound (GI_{50}), the drug concentration that result in total growth inhibition, signifying the cytostatic effect of the compound (TGI), lethal concentration of the compound killing 50% of the cells, signifying the cytotoxic effect of the compound (LC_{50}).

Table 2: Cytotoxic Activities of the Isolated Compounds

Compound	Parameter	UACC62 (μM)	TK10 (μM)	MCF7 (μM)
8	GI_{50}	-	18.39	-
	TGI	11.67	35.63	30.35
	LC_{50}	18.25	54.27	54.19
7	GI_{50}	16.78	36.14	23.19
	TGI	26.23	58.96	40.01
	LC_{50}	35.59	131.29	62.25
2	GI_{50}	-	14.16	13.27
	TGI	12.91	-	-
	LC_{50}	25.16	-	-
Etoposide	GI_{50}	3.77	16.80	5.98
	TGI	74.06	65.48	-
	LC_{50}	-	-	-

Note: Fixed concentration (19.84 μM) of **5** was used and %GI TK10 (8.02), UACC62 (-41.88) and MCF7 (7.6).

All compounds demonstrated a concentration-dependent cytotoxicity against the three mentioned cancer cell lines. Compound **8** expressed cytotoxic activity across the three cancer cell lines that corresponds well with the reported anticancer activity (Ajiboye, et al. 2014). The comparison of compounds **7** and **8** indicates that, the latter has expressed twice as much cytotoxic activity, this might be due to the unusual structure of **7** that might have altered its binding capacity. The observed activity of these two compounds correlated well with the reported tumor promotion inhibition (Murakami, et al. 1991, Ajiboye, et al. 2014). The growth inhibition properties of **2**, a renowned biflavone with diverse biological activities was rather unusual. Interestingly, the compound totally inhibited the growth of melanoma cancer cells only, thus, more scientific studies need to be conducted to establish the selectivity behaviour expressed. The same trend is observed for C-glucosylated flavone, 3′-methoxyvitexin-6″-*O*-acetate, where, growth inhibition of melanoma cancer cells was observed to be highly effective at 19.84 μM. All compounds demonstrated higher cytotoxicity against UACC62 (melanoma) cancer cell lines than the rest, this might explain the susceptibility of the respective cancer cell lines to biflavonoids. A topoisomerase inhibitor, etoposide was used as a standard, expressing almost the same level of cytotoxicity across the three cancer cell lines. It is worth mentioning the very low cytotoxicity activities demonstrated by antioxidants **13** (2.55 x 10–3 $mg.mL^{-1}$), and **14** (1.5 x 10-3 $mg.mL^{-1}$), this observation implies that these compounds could be deployed in the reported activity

(antioxidant) (Nahar et al. 2005; Taylor et al. 2011) without causing harm to other cells.

Antimalarial activities

As indicated in Table 3, the chloroquine-resistant *Plasmodium falciparum* strains (FCR-3) were used to evaluate the growth inhibitory activities of the isolated compounds.

Table 3: Antimalarial Activities of the Isolated Compounds

Compound	IC_{50} (µM)	Compound	IC_{50} (µM)
2	17.25	**11**	39.54
7	29.78	**3**	61.86
1	31.07	Mixture of **9** and **10**	64.31
8	35.31	**5**	106.35

The antimalarial activity of compound **9** and **10** was previously reported by Ichino et al. (2006), where, the compounds showed activity at 0.16 and 10.19 µM (**9** and **10**, respectively) and at 0.51 and 8.82 µM (**9** and **10**, respectively) against multidrug resistant strains and multidrug sensitive strains respectively (Ichino et al. 2006). However, the observed activity did not reflect the reported, this might be attributed by the fact that individual compounds were used in the reported activity, whereas mixture of the two compounds was used in the observed activity. Regarding Structure Activity Relationship (SAR), it is interesting to note that the dihydro-derivative of **2** (2″,3″ dihydroochnaflavone) expressed a decreased antimalarial activity as well as cytotoxicity as compared to **2**, this might be due to breakage of conjugation, thus altering of binding activity.

The observed biological activities of compound **1** did not correlate with those reported for its derivatives isolated from *Myracrodruon urundeuva* Fr. All. (*Astronium urundeuva* Engl.). These compounds have shown neuroprotective effect by reducing oxidative stress and apoptotic injury (Helio et al. 2009), analgesic effects and anti-inflammatory activity (Viana et al. 2003), thus, this calls for derivatization of the compound **1** to enhence its biological potency.

Compound description summary

*5-Deoxyurundeuvine C (**1**)*: yellow non-crystalline solid substance; $[\alpha]^{23}_{D}$= -5.2 (c 0.2, MeOH); HRESIMS (negative ionization mode), $[M-H]^-$, m/z 509.1242 (calculated for $C_{30}H_{21}O_8$ 509.1236).^{1}H and ^{13}C NMR data see Table 1.

CONCLUSION

Overall, a total of fourteen compounds were isolated and characterized, of these eight were biflavonoids, indicating the plant species to be a rich source of this class of compounds. The biological activity of the compounds ranged from moderate to low cytotoxicity and antimalarial activities. This correlates well with the traditional use of the plant for treatment of various ailments including bone diseases and *gangrenus proctitis* in Zulu traditional medicine system.

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