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Flavonoid glycosides from *Olax mannii*: Structure elucidation and effect on the nuclear factor kappa B pathway

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ABSTRACT

Ethnopharmacological relevance: *Olax mannii* Oliv. (Olacaceae) is among the many medicinal plants used in Nigeria for the ethnomedicinal management of both cancer and inflammation. Such plants represent potential sources of innovative therapeutic agents for the treatment of cancer and other malignant disorders. While the majority of medicinal plants exert their anticancer effects by direct cytotoxicity on tumor cells, it is important that other mechanisms through which these plants can exhibit anticancer effects are investigated. Preliminary studies indicated that *Olax mannii* leaves are rich sources of novel flavonoid glycosides. The detailed chemistry as well the mechanisms through which these flavonoid constituents may exert their cancer chemo-preventive and therapeutic effects are, however, not yet investigated.

Aim of the study: The aim of this study is to carry out a detailed chemical investigation of *Olax mannii* leaves and the effects of the isolated constituents on the nuclear factor kappa B (NF-κB) pathway.

Materials and methods: A methanol leaf extract was subjected to various chromatographic separations to achieve isolation of flavonoid glycosides and the structures of the isolated compounds were elucidated by a combination of 1D and 2D NMR and high resolution mass spectrometry. Biological activities were assessed by measurement of cellular viability and proliferation using quantitative IncuCyte videomicroscopy, trypan blue staining and by quantification of the number of metabolically active K562 cells based on quantitation of ATP. The effect of the compounds on the inhibition of the NF-κB pathway as well as toxicity towards peripheral blood mononuclear cells to evaluate differential toxicity was also assayed.

Results: Chemical investigation of the methanol leaf extract of the plant material led to the isolation of three new flavonoid triglycosides, kaempferol 3-*O*-[α-D-apiofuranosyl-(1→2)-α-L-arabinofuranoside]-7-*O*-α-L-rhamnopyranoside (**1**), kaempferol 3-*O*-[*β*-D-glucopyranosyl-(1→2)-*α*-L-arabinofuranoside]-7-*O*-α-L-rhamnopyranoside (**2**), kaempferol 3-*O*-[*β*-D-arabinopyranosyl-(1→4)-*α*-L-rhamnopyranoside]-7-*O*-*α*-L-rhamnopyranoside (**3**), in addition to fourteen known flavonoid glycosides (**4-17**). Of all the tested compounds, only compound **9** (kaempferol 3-O-α-L-rhamnopyranoside) exhibited promising and specific antiproliferative activity on human K562 chronic myelogenous leukemia cells and dose-dependently inhibited NF-κB transactivation.

Conclusion: The presence of this flavonoid glycoside and derivatives may account for the reported efficacy of *Olax mannii* leaf extract in the ethnomedicinal management of cancer and inflammation.

Introduction

Flavonoids are widely known to exhibit a broad spectrum of biological activities such as anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activities (Prasad et al., 2010;

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López-Lázaro, 2009; Orlikova and Diederich, 2012). Many of the biological actions of flavonoids are due to their free radical scavenging, metal ion chelating and antioxidant activities (Seef et al., 2001; Winslow and Krol, 1998). Furthermore, flavonoids suppress inflammation, transformation, proliferation, survival, invasion and angiogenesis, and thus play an important role in inflammatory related diseases, including cancer (Prasad et al., 2010). Evidence further indicates strong association between chronic inflammatory conditions and cancer (Coussens and Werb, 2002; Porta et al., 2009). Several molecular pathways link inflammation to cancer (Porta et al., 2009; Delhalle et al., 2004) including nuclear factor-kappaB (NF-κB) a key transcription factor able to transactivate more than 400 target genes involved in inhibition of apoptosis, activation of cellular transformation, proliferation, invasion, metastasis, chemoresistance, radioresistance, and inflammation (Garg and Aggarwal, 2002). Most of the traditional chemotherapeutic agents induce NF-κB, and subsequently activate cancer cell resistance. Interestingly, flavonoids and other phytochemicals, which inhibit deregulated over-activation of NF-κB, constitute interesting leads for cancer prevention and therapy (Reuter et al., 2009; Schumacher et al., 2010; 2011; Aly et al., 2011; Orlikova et al., 2011).

In the course of our search for new bioactive secondary metabolites from poorly investigated plants, we investigated the Nigerian medicinal plant *Olax mannii* Oliv. (Olacaceae). It is a climbing shrub widely distributed in the tropics especially in Nigeria, Ghana and Sierra Leone (Dalziel, 1956; Hutchison and Dalziel, 1966). Particularly, *O. mannii*, locally known as Tsadar birii in the Northern part of Nigeria, is used by the traditional healers in the treatment of different forms of cancer and inflammatory conditions (Abubaka et al., 2007). In addition, decoction of the leaves and roots of the plant is used to treat fever, yellow fever and snakebite (Burkill 1997). Previous reports have shown that the leaves of related species possess anti-inflammatory and antimicrobial activities (Ajali and Okoye, 2009; Fankam et al., 2011). However, to our knowledge, there is no scientific evidence supporting the ethnomedicinal use of the plant in management of cancer and inflammation. The stimulus that prompted our chemical investigation was the poor record of published natural compounds so far extracted from this medicinal plant (Sule et al., 2005, 2011) as well as our preliminary cytotoxic assay of its crude methanol extract against murine lymphoma L5178Y cells. Moreover, a close observation of the HPLC and the LC–MS data of the crude extract revealed the presence of several peaks, which could be a potential source of novel kaempferol glycosides. Interestingly, kaempferol and its glycosides were reported to reduce prostaglandin E2 synthesis and inhibited cyclooxygenase-2 through their actions on transcription factors NF-κB and peroxisome proliferator-activated receptor γ (Prasad et al., 2010; Munoz-Espada and Watkins, 2006; Xu et al., 2008).

Materials and methods

General experimental procedure

UV spectra were determined on a Dionex instrument with Chromeleon V6.3 as standard software programs. The separation column (125 × 4 mm, length X internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. 1D and 2D NMR spectra were obtained on a AVANCE DMX 600 NMR spectrometers. Mass spectra were recorded using a LCMS HP1100 Agilent Finnigan LCQDecaXP Thermoquest. HRESIMS were obtained with an FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer. Vacuum and pressurized column chromatography was performed with Silica gel 60 M (230–400 mesh ASTM, Macherey-Nagel GmbH & Co.KG, Düren, Germany) and size exclusion chromatography was performed using Sephadex LH-20 (Sigma). TLC was carried out with precoated Silica gel plates (TLC silica gel 60F–254, Merck KGaA, Darmstadt, Germany), and the detection of spots was carried out under UV (254 and 366 nm). Semi-preparative HPLC was carried out with Merck Hitachi L-7100 and L-7400 for pump and UV detector, respectively, using C-18 column (Knauer, Berlin, Germany).

Plant material

The leaves of *Olax mannii* were collected in December, 2010 from Zaria in the northern part of Nigeria. The plant was identified at the Department of Botany Ahmadu Bello University, Zaria, Kaduna State, Nigeria. A voucher specimen has been deposited at the herbarium of the same Institute under the herbarium number 1697. The leaves were air dried for 10 days and pulverized.

Extraction and isolation

Pulverized dried leaves of *O. mannii* (500 g) were extracted with 5 L MeOH for 7 days at room temperature (25 °C) and the extract concentrated *in vacuo* with a rotary evaporator to obtain 79 g of a dark green semisolid. The dried extract was re-dissolved in 50 mL of methanol and the solution made up to 300 mL with water, sonicated for about 10 min and subsequently partitioned successively in *n*-hexane (750 mL X 3), ethyl acetate (750 mL X 3) and *n*-butanol (500 mL X 2). About 10 g of *n*-butanol fraction (BF) was subjected to VLC (silica gel 500 g, sintered funnel 5 L) eluting with 1 L each of dichloromethane:methanol (100:0, 90:10, 80:20, 70:30, 60: 40, 50:50, 40:60, 20:80 and 0:100) resulting in 9 pooled fractions BF1-BF9. Fraction 5 (BF5, 210.6 mg) was chromatographed on Sephadex LH-20 column (2 × 45 cm) using 100% MeOH as solvent to afford several sub-fractions. Based on TLC profile, collected fractions were combined and subjected to semi-preparative HPLC to afford compounds **1** (18.0 mg), **2** (24.0 mg), **3** (8.0 mg), **4** (12 mg), **5** (34.0 mg) and **6** (50.0 mg). The ethyl acetate fraction, EF (1.9 g) was subjected to VLC (silica gel 500 g, sintered funnel 5 L) eluting with 500 mL each of hexane:ethyl acetate (100:0, 60;40, 50:50, 40:60, 30:70, 20:80, 0:100) and ethyl acetate: MeOH (80:20, 60:40, 40:60, 20:80, 0:100) resulting in 12 pooled fractions EF1 to EF12. Promising fractions were further separated on a Sephadex LH-20 column and the resulted fractions were combined based on TLC profile and subjected to semi-preparative HPLC to yield compounds **4** (13.0 mg), **5** (83 mg), **7** (1.7 mg), **8** (8.2 mg), **9** (1.7 mg), **10** (1.3 mg), **11** (3.9 mg), **12** (1.7 mg), **13** (1.0 mg), **14** (1.9 mg), **15** (4.2 mg), **16** (1.7 mg) and **17** (2.5 mg).

Olamannoside A (1)

Yellow solid (18.0 mg). [α]20 D−88.0 (*c* 0.002, MeOH); UV: λ_{max} (PDA)=265, 246 nm. ESIMS (+ve): *m/z*=696.9 $[M+H]^+$, ESIMS (−ve): *m/z*=695.3 $[M-H]^-$. HRESIMS: calcd. for $C_{31}H_{36}O_{18}$ $[M+H]^+$ 697.1974; found 697.1973 $[M+H]^+$. ^1H and ^{13}C NMR (DMSO-d_6), see Table 1.

Olamannoside B (2)

Golden yellow flakes (24.0 mg). [α]20 D−40.29 (*c* 0.002, MeOH); UV: λ_{max} (PDA)=265, 347 nm. ESIMS (+ve): *m/z*=726.9 $[M+H]^+$, ESIMS (−ve): *m/z*=725.1 $[M-H]^-$. HRESIMS: calcd. for $C_{31}H_{38}O_{19}$ $[M+Na]^+$ 749.1905; found 749.1829 $[M+Na]^+$. ^1H and ^{13}C NMR (DMSO-d_6), see Table 1.

Olamannoside C (3)

Yellow powder (8.0 mg). [α]20 D-484.25 (*c* 0.002, MeOH); UV: λ_{max} (PDA)=264, 343 nm. ESIMS (+ve): *m/z*=710.8 [M+H],

Table 1
NMR spectroscopic data of olamannosides A–C (**1**–**3**).

Nr.	**1**[a,b]		**2**[a,b]		**3**[c,d]	
	δ_C	δ_H (*J*, Hz)	δ_C	δ_H (*J*, Hz)	δ_C	δ_H (*J*, Hz)
2	157.3		158.0		160.2	
3	133.5		134.0		136.4	
4	177.7		178.1		179.9	
5	160.9		160.8		162.8	
6	99.5	6.45, d (2.1)	98.7	6.46, d (2.1)	100.7	6.46 br.s
7	161.7		162.1		163.7	
8	94.6	6.83 d (2.1)	95.0	6.84 d (2.1)	95.8	6.72 br.s
9	156.0		156.3		158.3	
10	105.7		106.0		107.8	
1'	120.5		120.6		122.0	
2'	130.9	8.02 d (8.8)	131.4	8.05 d (8.9)	132.2	7.77 d (8.6)
3'	115.6	6.91 d (8.8)	115.9	6.91 d (8.9)	117.0	6.91 d (8.4)
4'	160.2		161.2		163.2	
5'	115.6	6.91 d (8.8)	115.9	6.91 d (8.9	117.0	6.91 d (8.4)
6'	130.9	8.02 d (8.8)	131.4	8.05 d (8.9)	132.2	7.77 d (8.6)
(3-O)	*Arab*		*Arab*		*Ram*	
1"	106.3	5.80 br.s	106.6	5.93 br.s	103.3	5.39
2"	87.9	4.23 m	89.3	4.44 m	71.9	4.25
3"	75.7	3.86 m	76.1	3.93 m	72.4	3.94 dd (3.0, 9.2)
4"	85.7	3.64 m	86.7	3.48 m	83.4	3.51 d (9.7)
5"	60.3	3.27 m, 3.33 m	60.9	3.31 m (2H)	70.7	3.40 dd (6.1, 9.6)
6"					17.9	0.98 d (6.1)
(1-2)	*Apio*		*Glu*		*Arab*	
1"'	107.9	5.06 d (2.3)	102.1	4.45 d (7.9)	106.7	4.39 (d, 7.4)
2"'	76.1	3.77 br.d (2.3)	73.7	2.98 t (8.1)	73.7	3.56 d (7.5)
3"'	78.8		77.4	3.15[e]	74.8	3.51 d (5.8, 3.6)
4"'	73.6	3.60 d (9.4) 3.89 d (9.5)	77.1	3.28 m	70.2	3.76 br.s
5"'	63.0	3.33 br.s (2 H)	70.2	3.14[e]	67.7	3.51
6"'			61.2	3.52 br.d (11.7) 3.66 br.d (11.7)		3.83 (2.6, 10)
7-*O (Rham)*						
1""	98.4	5.55 br.s	99.8	5.56 br.s	100.0	5.56 br.s
2""	69.9	3.84 m	70.0	3.84 br.d (3.4)	71.86	4.02 br.s
3""	70.3	3.54 m	70.6	3.64 dd (3.4, 9.2)	72.3	3.82 d (9.7)
4""	71.7	3.30 m	72.0	3.30 t (9.2)	73.8	3.49 d (9.7)
5""	70.1	3.43 m	70.5	3.43 m	71.4	3.60 dd (9.4, 6.2)
6""	18.0	1.12 d (6.1)	18.3	1.12 d (6.1)	18.2	1.26 d (6.2)

[a] Measured at 500 (^{1}H) and 125 (^{13}C) MHz.
[b] Spectra measured in DMSO.
[c] Measured at 600 (^{1}H) and 150 (^{13}C) MHz.
[d] Spectra measured in CD_3OD.
[e] overlapped signals.

ESIMS (−ve): *m/z*=709.1 $[M-H]^-$. HRESIMS: calcd. for $C_{32}H_{38}O_{18}$ $[M+Na]^+$ 733.1956; found 733.1849 $[M+Na]^+$.^{1}H and ^{13}C NMR (MeOH-d_4), see Table 1.

Sugar analysis

The absolute configuration of the sugar moieties was assigned by capillary electrophoresis of derivatised hydrolysates according to Noe and Freissmuth (1995).

Cell culture

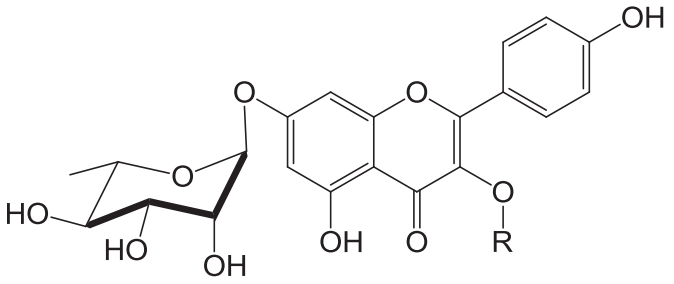
Chronic myeloid leukemia cells (K562) were cultured in RPMI 1640 medium (Lonza, Belgium) supplemented with 10% fetal bovine serum (Lonza), penicillin (100 units/mL) and streptomycin (100 µg/mL) at 37 °C with 5% CO_2. Cells were harvested every 3 days. One day before treatment, the batches of cells were prepared in order to have an exponential growth during the treatment.

Cell viability assessment by trypan blue exclusion assay

Trypan blue is a dye exclusion test used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude trypan blue while dead cells do not. Cells were placed in 24-well plates at 200,000 cells/mL. Different concentrations of compound **9** were mixed in each well while the negative control well was mixed with the same volume of DMSO. At each time point (0 h, 8 h, 24 h, 48 h and 72 h), 20 µL of the cell suspension was mixed with 20 µL of trypan blue. 20 µL of this mixture was applied to a haemocytometer and monitored with a binocular microscope. The unstained cells (viable) were counted in the negative control and treated samples, and the percentage of viability was calculated from three independent tests.

Cell viability assessment by cell titer-Glo assay

This method allows the determination of the number of viable cells based on the quantification of ATP, which indicates cell metabolic activity. Thus luciferase catalysis of luciferin to oxyluciferin and light was used in the presence of Mg^{2+}, ATP and oxygen. Lyophilized enzyme/substrate mixture was reconstituted with the corresponding buffer. An equal volume of reagent and cell suspension (treated or not by compounds for 72 h) was mixed in 96-well plate. Luminescence signal corresponding to the metabolic activity was quantified with a Berthold Orion microplate



1 R α-D-apiofuranosyl (1→2)-α-L-arabinofuranosyl
2 R β Dglucopyranosyl (1→2)-α-L-arabinofuranosyl
3 R α-D-arabinopyranosyl (1→4)-α-L-rhamnopyranosyl

Fig. 1. Chemical structures of the new flavonoid glycosides (1–3).

luminometer and converted to number of viable cells. The percentage of viability was calculated from three independent tests.

Quantitative videomicroscopy

IncuCyte videomicroscopy was used to evaluate the effect of isolated compounds on K562 proliferation. Briefly, cells were grown in 24-well plates pre-coated by Poly-lysine D and incubated for 3 h to allow cell fixation. Cells were treated with the isolated compounds and DMSO alone (2 μL/well) was used as negative control. Results were expressed graphically as percentage of confluence (viable cells) versus time after three independent tests.

NF-κB assay

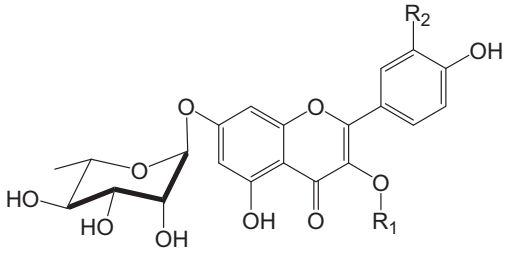
Transient transfections of K562 cells were performed by electroporation using the BioRad Gene Pulser MXcell. Briefly, 500 μL of cell suspension were mixed with 10 μg of firefly luciferase vector, NF-κB pGL4 (Promega, Leiden, The Netherlands) and 10 μg of ph-RG-tk Renilla plasmid (Promega). The mixture was placed in a BioRad electroporation plate and electroporated at 250 V and 650 μF. Transfected cells were cultivated in RPMI 1640 medium with 10% fetal calf serum (FCS) for 24 h. The transfected cells were then harvested and re-suspended in growth medium with 0.1% FCS to reach a final concentration of 10^6 cells/mL and treated with compound **9**. After 2 h of incubation, 20 ηg/mL of TNFα were added in treated wells followed by an additional incubation of 6 h. 75 μL of cell suspension were combined with 75 μL of Dual-Glo™ Luciferase Reagent (Promega) in 96-well plate and incubated for 10 min at 22 °C. Luciferase activity was measured using an Orion microplate luminometer (Berthold detection systems). The same protocol was used to assay *Renilla* activity by adding 75 μL of Dual-Glo™ Stop&Glo Reagent (Promega). The results are expressed as a ratio of Luciferase activity normalized to *Renilla* activity. The IC_{50} value of compound **9** was calculated graphically from three independent tests.

Statistical analysis

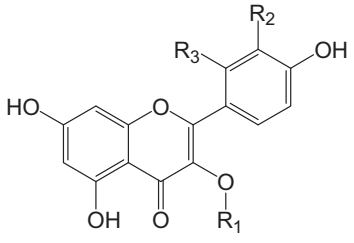
Results from at least three independent experiments were analyzed for statistically significant differences using Graph Pad Prism 6 (Student's *t*-test and One way ANOVA). They were expressed as the mean ± SD. *p*-Values below 0.05 were considered as statistically significant (*), 0.01 more significant (**), 0.001 highly significant (***) or 0.0001 very highly significant (****).

Results and discussion

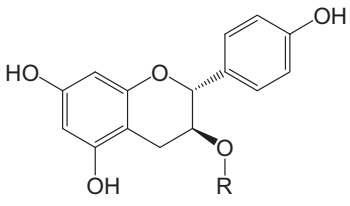
The crude methanol extract (79.0 g) of *Olax mannii* leaves was first dried and then subjected to liquid–liquid fractionation to obtain hexane, ethyl acetate, butanol and water fractions. About 9.71 g of the butanol and 1.9 g of ethyl acetate fractions were separately subjected to vacuum liquid chromatography (VLC) on silica gel followed by column chromatography on Sephadex LH-20. Finally, pure compounds (**1–17**) were obtained by semi-preparative HPLC on reverse phase column. The flavonoid glycosides were obtained as yellow solids and appear purple on TLC under UV light at 366 nm.



4 R_1 = α-L-arabinofuranoside; R_2 = H
5 R_1 = α-L-rhamnopyranosyl; R_2 = H
6 R_1 = β-D-glucopyranosyl (1→4)- α-L-rhamnopyranoside; R_2 =H
7 R_1 = α-L-rhamnopyranosyl; R_2 = OH
8 R_1 = β-D-xylopyranoside; R_2 = H
10 R_1= R_2 = H



9 R_1 = α-L-rhamnopyranoside; R_2 = R_3 = H
12 R_1 = β-D-glucopyranosyl; R_2 = R_3 = H
13 R_1 = β-D-galactopyranoside; R_2 = OH; R_3 =H
14 R_1 = β-D-xylopyranoside; R_2 = OH; R_3 =H
15 R_1 = α-L-rhamnopyranosyl; R_2 = H; R_3 = OH
16 R_1 = β-D-xylopyranoside; R_2 = R_3 =H
17 R_1 = β-D-xylopyranoside; R_2 = H; R_3 =OH



11 R = β-D-glucopyranosyl

Fig. 2. Chemical structures of the known flavonoid glycosides (4–17).

Compound **1** was isolated as a yellow solid. It showed UV maxima at λ_{max} 265 and 346 nm, which are typical for flavonoid glycosides. LC-ESIMS of **1** exhibited strong peaks at *m/z* 696.7 $[M+H]^+$ in the positive mode and at *m/z* 695.0 $[M-H]^-$ in the negative mode, which is consistent with the molar mass of 696 g/mol. The molecular formula was established as $C_{31}H_{36}O_{18}$ based on the HR-ESIMS molecular ion peak at *m/z* 697.1973 $[M+H]^+$. ESI-MS/MS showed fragments from subsequent loss of a pentose *m/z* 565 $[M-132]^+$, loss of a deoxyhexose *m/z* 550 $[M-147]^+$, loss of both sugar units *m/z* 432.7 $[M-264]^+$, and finally a fragment at *m/z* 287.2 corresponding to the $[M+H]^+$ ion peak of kaempferol. The latter fragment as well as the UV maxima showed that **1** is a kaempferol derivative with free OH-groups at positions C-5 and C4', while those at C-3 and C-7 are substituted (Nakano et al. 1983). ^1H and ^1H–^1H COSY NMR data showed characteristic aromatic spin systems of rings A and B in kaempferol. For instance, signals of an AA'BB' system at δ_H 8.02 (d, J=8.8 Hz) and 6.91 (d, J=8.8 Hz), each integrated for two protons, were assigned to H-2'/H-6' and H-3'/H-5', respectively. In addition, two *meta* coupled protons at δ_H 6.45 (d, J=2.1 Hz) and 6.83 (d, J=2.1 Hz) were observed and assigned to H-6 and H-8, respectively. ^{13}C NMR spectrum exhibited the presence of three anomeric carbons at δ_C 106.3, 107.9 and 98.4 ppm, and their corresponding protons were detected at δ_H 5.80 (br s), 5.06 (d, J=2.3 Hz) and 5.55 (br s), respectively, in the HMQC spectrum. Careful analysis of 2D NMR data (COSY, HMQC and HMBC) revealed the presence of apiose, rhamnose and arabinose sugar units, which was in agreement with the observed ESI-MS/MS fragments. The sequence of the sugar units and their attachment to the aglycone moiety in **1** were confirmed by ESI-MS/MS, chemical shifts of the anomeric carbons and their protons, and HMBC-correlations of the anomeric protons. The fragment observed at *m/z* 417.9 and the HMBC-correlation of the anomeric proton at δ_H 5.80 ppm to C-3 (δ_C 133.5 ppm) indicated the attachment of the arabinose sugar to C-3 of the kaempferol aglycone. The signal detected at *m/z* 432.7 as well as HMBC correlations of the anomeric proton at δ_H 5.55 ppm and the *meta* coupled protons to C-7 (δ_C 161.7 ppm) indicated the attachment of the rhamnose unit to C-7. Finally, fragments observed at *m/z* 550 and 265, as well as the HMBC correlation of the anomeric proton at δ_H 5.06 ppm to C-2" (δ_C 87.9 ppm) indicated the apiose-(1→2)-arabinose connection. This was confirmed by the characteristic downfield signal of C-2" at δ_c 87.9 ($\Delta\delta_c$ 7 ppm) (Mulinacci et al. 1995). Each sugar unit was completely assigned based on 2D NMR data analysis and comparison with previously reported data. The spin system containing the anomeric proton at δ_H 5.55 ppm corresponded to a rhamnopyranosyl unit. This was corroborated by the characteristic ^{13}C NMR data (Table 1), which are in agreement with those reported for α-L-rhamnopyranoside (Mulinacci et al., 1995; Gohar et al., 2000; Manir et al., 2012). The second pentose was identified as arabinofuranose based on the characteristic ^1H and ^{13}C NMR signals (Table 1), which are in accordance with the literature (Voutquenne et al., 2005; Chan et al., 2008). The observed broad singlet of the anomeric proton at 5.80 ppm is indicative of its equatorial orientation. The furanose nature of the α-

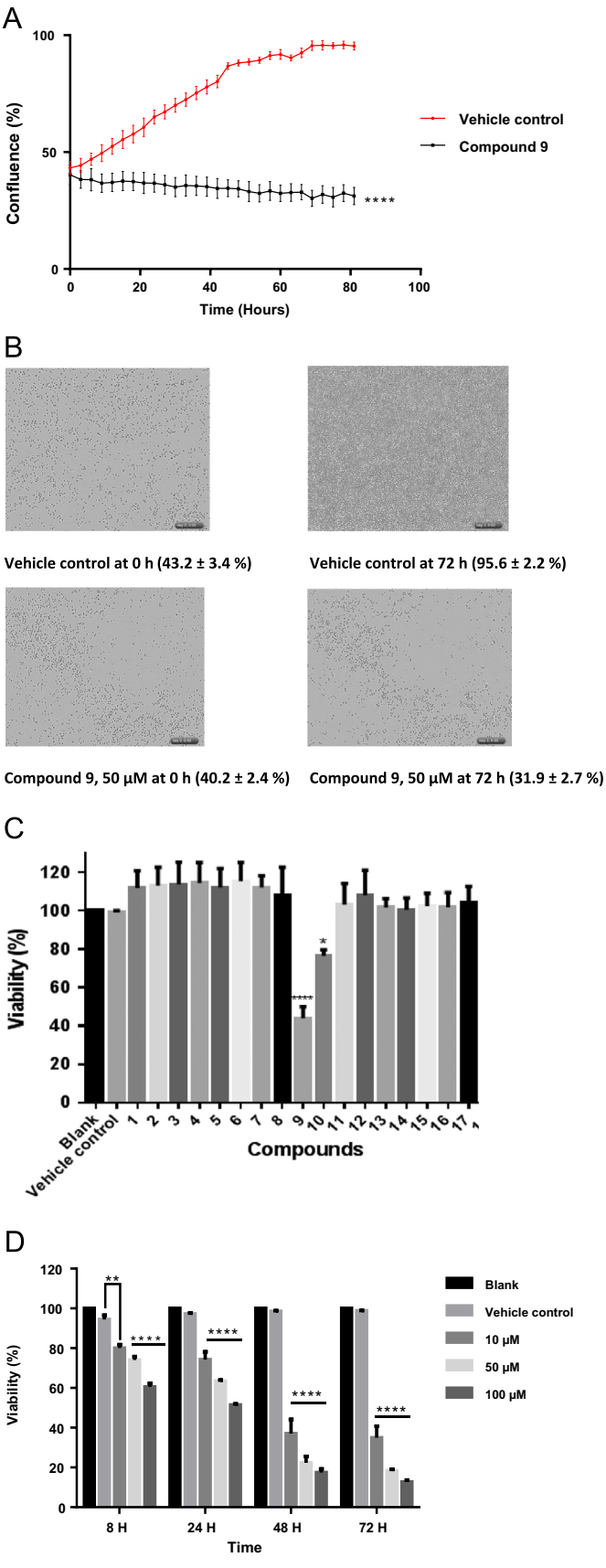


Fig. 3. A. Antiproliferative effect of compound 9 compared to vehicle control by Incucyte. The graph represents the K562 cells growth curve plotting confluence *vs.* time at 3 h intervals. Results are the mean ± SD of three independent tests. Compound **9** affects K562 cell proliferation. B. The effect of compound **9** on K562 cell proliferation was evaluated using an IncucyteTM Live-Cell Imaging System (EssenBioscience). Pictures were extracted from the generated video clip corresponding to the Live cell proliferation analysis of K562 cells at 50 µM compared to the DMSO control. C. Number of metabolically active K562 cells based on quantitation of ATP. Results are shown as mean ± SD of three independent measurements. *p*-Values below 0.05 were considered as statistically significant (*), 0.01 more significant (**), 0.001 highly significant (***) or 0.0001 very highly significant (****). D. Cytotoxic effect of compound **9** on K562 cells using trypan blue exclusion assay. Results are shown as the mean ± SD of three independent tests. *p*-Values below 0.05 were considered as statistically significant (*), 0.01 more significant (**), 0.001 highly significant (***) or 0.0001 very highly significant (****).

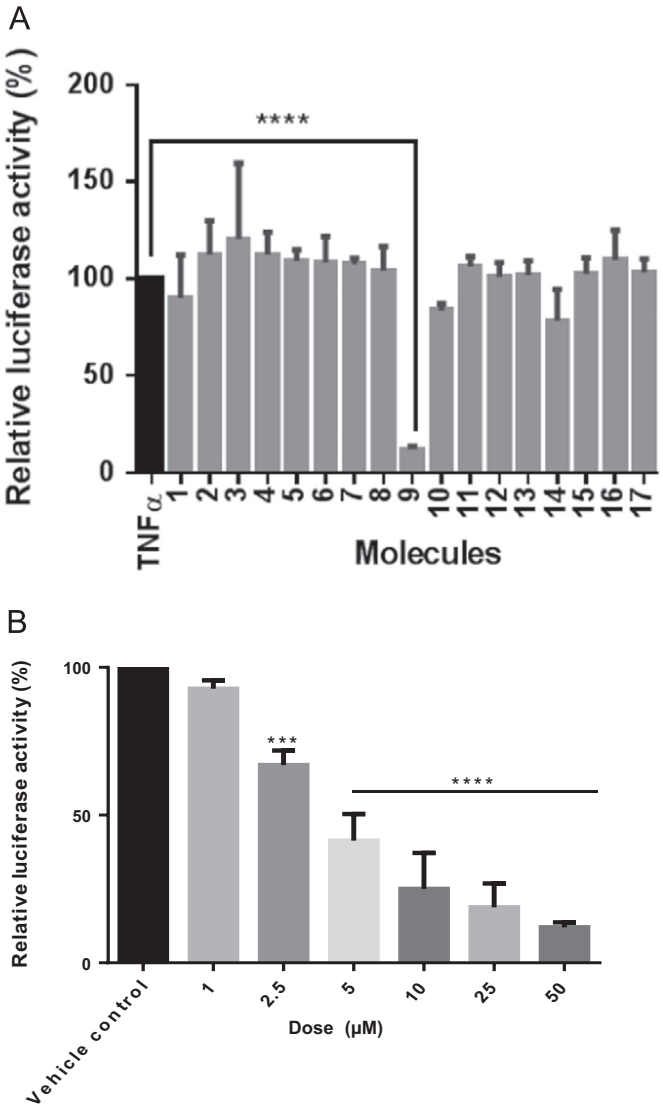


Fig. 4. A Effect of the isolated compounds on NF-κB pathway. Results are shown as the mean ± SD of three individual measurements. *p*-Values below 0.05 were considered as statistically significant (*), 0.01 more significant (**), 0.001 highly significant (***) or 0.0001 very highly significant (****). B Inhibition of TNFα-induced NF-κB activation by compound **9** on K562 cells. Results were presented as mean ± SD of three independent measurements. *p*-Values below 0.05 were considered as statistically significant (*), 0.01 more significant (**), 0.001 highly significant (***) or 0.0001 very highly significant (****).

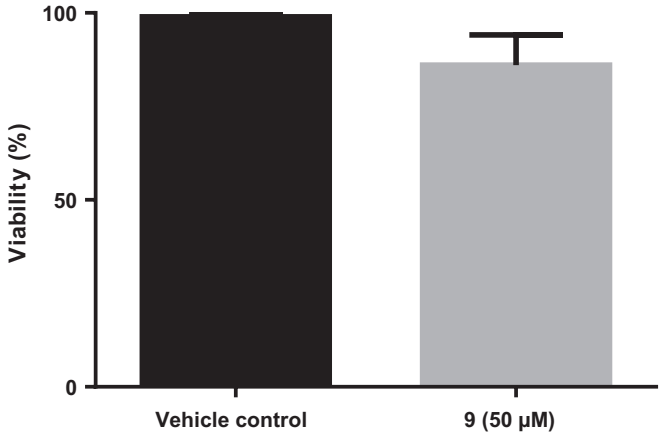


Fig. 5. Cytotoxicity of compound **9** on PBMCs cells after 72 h of exposure. Results are expressed as mean ± SD of three independent tests.

L-arabinosyl unit was further confirmed by the observed downfield signal of C-4" at δ_C 85.7 ($\Delta\delta_c$ 5 ppm) and the upfield signal of C-5" at δ_C 60.3 ($\Delta\delta_c$ 7 ppm) compared with reported data for arabinopyranosides (Abdel-Shafeek et al., 2000; Calis et al., 2001). as well as by the ^3J-HMBC correlation of its anomeric proton to C-4" (δ_C 85.7 ppm). Finally, the apiofuranosyl unit was deduced from the characteristic ^{13}C NMR signals (Table 1), which are in agreement with previously reported data (Ranganathan et al., 1996; Snyder and Serianni, 1987; Rezanka et al., 2009; Materska et al., 2001). The chemical shift of the anomeric proton of the apiofuranosyl unit appearing at δ_H 5.06 ppm is typical for a terminal apiosyl unit (Ranganathan et al., 1996; Snyder and Serianni, 1987; Rezanka et al., 2009; Materska et al., 2001). The D-configuration of the apiofuranosyl unit was confirmed by the chemical shift for CH_2-5"' appearing at δ_H 3.33 ppm, (Ishii, Yanagisawa, 1998) and its α-configuration by the coupling constant of 2.3 Hz of its anomeric proton. The absolute configuration of the sugar moieties of **1** was determined by capillary electrophoresis of derivatised hydrolysates according to Noe and Freissmuth (Karioti et al., 2003). These results confirmed the new structure of **1** as kaempferol 3-*O*-[α-D-apiofuranosyl-(1→2)-*α*-L-arabinofuranosyl]-7-*O*-α-L-rhamnopyranoside, for which the name olamannoside A is proposed.

Compound **2** was isolated as yellow flakes. It showed UV maxima at λ_{max} 265 and 347 nm. Its LC-ESI-MS exhibited strong peaks at *m/z* 726.9 $[M+H]^+$ in the positive mode and at *m/z* 725.1 $[M-H]^-$ in the negative mode, indicating a molar mass of 726 g/mol. The molecular ion peak observed at *m/z* 749.1829 $[M+Na]^+$ in the HR-ESI-MS designated the molecular formula $C_{32}H_{38}O_{19}$. ESI-MS/MS of **2** showed fragments corresponding to loss of one hexose *m/z* 564.8 $[M-163]^+$, loss of a deoxyhexose *m/z* 580.2 $[M-147]^+$, loss of two sugar units *m/z* 432.8 $[M-295]^+$, and finally the kaempferol aglycone fragment at *m/z* 287.8 ($[M+H]^+$). As in **1** this latter fragment as well as the UV maxima showed that **2** is a kaempferol derivative with free OH-groups at positions C-5 and C4', while those at C-3 and C-7 are substituted.[26] Careful comparison of NMR data as well as ESI-MS/MS data (*m/z* 564.8 $[M-163]^+$) of **2** with those of **1** showed that **2** comprises the kaempferol 3-*O*-α-L-arabinofuranoside]-7-*O*-α-L-rhamnopyranoside subunit. The third sugar unit in **2** was identified as glucose based on its NMR data and comparison with reported data for *β*-D-glucopyranose (Gudej, 2003; Gohar et al., 2000). Furthermore, the chemical shift of the anomeric proton of the glucopyranosyl unit appearing at δ_H 4.45 ppm is typical for a terminal glucosyl unit, and its coupling constant value (7.9 Hz) is consistent with *β*-configuration (Nakano et al., 1983; Mulinacci et al., 1995; Abdel-Ghani et al., 2001). More so, the fragments observed at *m/z* 580.4 and 295, as well as HMBC correlation of the anomeric proton at δ_H 4.45 ppm to C-2" (δ_C 89.3 ppm) supported the glucopyranosyl-(1→2)-arabinofuranoside connection. This connection was further confirmed by the characteristic downfield chemical shift of C-2" of the arabinofuranosyl unit at δ_c 89.3 ($\Delta\delta_c$ 8 ppm) (Chan et al., 2008; Abdel-Shafeek et al., 2000). The absolute configuration of the sugar units of **2** was assigned (Karioti et al., 2003) and the structure was unequivocally completed and identified as kaempferol 3- *O*-[*β*-D-glucopyranosyl (1→2)-*α*-L-arabinofuranoside]-7-*O*-*α*-L-rhamnopyranoside, for which the trivial name olamannoside B is proposed.

Compound **3** was isolated as a yellow powder and showed UV maxima at λ_{max} 264 and 343 nm. The molecular formula was established as $C_{32}H_{38}O_{18}$ based on the observed HR-ESIMS molecular ion peak at *m/z* 733.1849 $[M+Na]^+$. Its ESI-MS/MS showed fragments from loss of a pentose *m/z* 578.8 $[M-132]^+$, loss of a deoxyhexose *m/z* 564.9 $[M-147]^+$, loss of two sugar units *m/z* 432.8 $[M-278]^+$, and the aglycone fragment at *m/z* 286.9 ($[M+H]^+$). The NMR data of **3** were closely related to those of the known kaempferol 3,7-*O*-*α*-L-dirhaminoside (**5**), likewise isolated

during this study, except for the presence of an additional sugar moiety. This was further confirmed by the presence of an additional anomeric carbon at δ_c 106.7 ppm and its corresponding proton at δ_H 4.39 ppm. Confirmation of the kaempferol 3,7-*O*-*α*-L-dirhamnoside substructure of **3** was deduced from the *m/z* 578.8 $[M-132]^+$ MS/MS fragment, 1D and 2D dimensional NMR, as well as comparison with NMR data of **5**. Analysis of the ^1H and ^{13}C NMR data of the additional pentose (Table 1) showed great resemblance to those of xylopyranosyl or arabinopyranosyl units. The possibility of xylopyranose was excluded based on the observed broad singlet at δ_H 3.76 ppm (H-4"') which showed a small coupling with H-5 A"' but no cross coupling with H-5B"' in the COSY spectrum. Thus, the additional sugar was identified as arabinopyranose. The magnitude of the observed coupling constant ($^3J_{H\text{-}1'''\text{-}H\text{-}2'''}$=7.4 Hz) indicated a *trans*-diaxial relation between H-1"' and H-2"', which is consistent with an *β*-configuration of a L-arabinopyranosyl unit (de Almeida et al., 1998; Halabalaki et al., 2011; Yahara et al., 2000). Attachment of the *β*-L-arabinopyranosyl at position 4" was confirmed by the ^3J HMBC correlation of the anomeric proton H-1"' to C-4", as well as by the characteristic downfield chemical shift of C-4" at δ_c 83.4 ($\Delta\delta_c$ 9 ppm). The absolute stereochemistry of the sugar moieties of **3** was assigned and confirmed (Karioti et al., 2003) as described before. Accordingly, **3** was identified as kaempferol 3- *O*-[*β*-L-arabinopyranosyl-(1→4)-*α*-L-rhamnopyranoside]-7- *O*-*α*-L-rhamnopyranoside, for which the trivial name olamannoside C is proposed.

The structures of the fourteen known flavonoid glycosides were identified based on their ^1H NMR, ^{13}C NMR, and mass spectrometric data as well as comparison with published data as kaempferol 3-*O*-α-L-arabinofuranosyl-7-*O*-α-L-rhamnopyranoside (**4**) (Nakano et al., 1983; Gohar and Maatooq, 2003), kaempferol 3,7-*O*-α-L-dirhamnopyranoside (kaempferitin) (**5**) (Yahara et al., 2000), kaempferol 3-*O*-[*β*-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside]-7-*O*-α-L-rhamnopyranoside (**6**) (Shao et al., 2003), quercetin 3,7-*O*-α-L-dirhamnopyranoside (**7**) (Fang et al., 2005). kaempferol 7-*O*-α-L-rhamnopyranoside (**8**),[27] kaempferol 3-*O*-α-L-rhamnopyranoside (**9**) (Halabalaki et al., 2011), kaempferol-3-*O*-*β*-D-xylopyranosyl-7-*O*-*α*-L-rhamnopyranoside (**10**) (Nakano et al., 1983; Gohar and Maatooq, 2003), (+)-afzelechin 3-*O*-*β*-D-glucopyranoside (**11**) (Park et al., 2006), kaempferol 3-*O*-*β*-D-glucopyranoside (**12**) (Mulinacci et al., 1995), quercetin-3-*O*-*β*-galactopyranoside (**13**) (Gudej, 2003), quercetin 3-*O*-*β*-D-xylopyranoside (**14**) (Halabalaki et al., 2011), morin 3-*O*-*α*-L-rhamnopyranoside (**15**) (Halabalaki et al., 2011), kaempferol 3-*O*-*β*-D-xylopyranoside (**16**) (Halabalaki et al., 2011), morin 3-*O*-*β*-D-xylopyranoside (**17**) (Halabalaki et al., 2011). Fig. 1 and 2

All isolated compounds were screened for their cytotoxic activity against human K562 leukemia cells using quantitative IncuCyte videomicroscopy. Among the tested compounds, only compound **9** exhibited a significant antiproliferative effect on K562 cells at 50 μM (Fig. 3A, results not shown for inactive compounds). A marked reduction in cell proliferation was observed for compound **9** when compared to DMSO after 72 h of treatment. Results obtained by IncuCyte were corroborated by quantification of the number of metabolically active K562 cells based on quantitation of ATP (Fig. 3B). Again only compound **9** produced a significant inhibition of K562 cellular metabolism at a concentration of 50 μM after 72 h of treatment. Finally, the cytotoxicity of compound **9** on K562 cells was also assessed by trypan blue test (Fig. 3C). Results showed that compound **9** was non-toxic to K562 cells at 10 and 50 μM after 8 h of exposure but became significantly toxic after 24 h and the toxicity was dose dependent with an IC_{50} of 15.2 μM (Fig. 3D). Furthermore, NF-κB assays were performed for all isolated compounds (Fig. 4A). Compound **9** showed significant, dose-dependent inhibition of NF-κB (Fig. 4B) with an IC_{50} of 4.1 μM. Of specific interest is the fact that compound **9** was non-toxic toward peripheral blood mononuclear cells at 50 μM after 48 h of exposure (Fig. 5) thus underlining the excellent differential toxicity of this compound.

NF-κB is a family of closely related protein dimers, which can be activated by free radicals, inflammatory stimuli, cytokines, carcinogens, tumor promoters, endotoxins, γ-radiations, ultraviolet (UV) light and X-rays (Delhalle et al., 2004). Activation of NF-κB mediates a number of diseases including cancer, atherosclerosis, myocardial infarction, diabetes, allergy, arthritis etc. Agents that suppress NF-κB could potentially prevent or delay the onset of or treat NF-κB-linked diseases. One of the probable mechanisms of action by which dietary flavonoids exercise anti-tumor properties is through the suppression of NF-κB signaling. Kaempferol has been shown in previous reports (Prasad et al., 2010; Xu et al., 2008) to suppress NF-κB signaling. Surprisingly, of all kaempferol glycosides we tested for NF-κB inhibitory activity, only compound **9** (kaempferol 3-*O*-*α*-L-rhamnopyranoside) exhibited significant inhibition of NF-κB with IC_{50} value of 4.1 μM.

Previous studies indicated that flavonoids generally possess low bioavailability when administered to humans owing to their limited absorption and rapid elimination (Hollman et al., 1997). There are also reports that glycosidic moieties present in flavonoids enhance or reduce absorption depending on the particular aglycone (Felgines et al., 2000). Studies also showed that different flavonoids effectively inhibit NF-κB and other inflammatory responses, in spite of having differential bioavailability (Prasad et al., 2010). It is therefore likely that *in vitro* and *in vivo* effective concentrations are not the same for different flavonoids and/or their glycosides (Prasad et al., 2010) and different flavonoids and their glycosides may also possess different mechanisms of action *in vitro* and *in vivo*. Compound 9 (kaempferol 3-*O*-*α*-L-rhamnopyranoside), however, which exhibited significant inhibition of NF-κB and also showed selective toxicity on K562 cells, offers great promise in cancer chemoprevention and therapy.

Conclusions

Our investigation of the ethyl acetate and butanol fractions of the methanol leaf extract of *Olax mannii* led to the isolation of 17 flavonoid glycosides of which three are new compounds. Compound **9** (kaempferol 3-*O*-*α*-L-rhamnopyranoside) exhibited significant and selective cytotoxicity and inhibition of NF-κB. Kaempferol 3-O-*α*-L-rhamnopyranoside is a common basic unit of the two most abundant flavonoid glycosides (Kaempferitrin and its derivative) isolated from *O. mannii*. We postulate a possible breakdown of these major compounds *in vivo* to the more active kaempferol 3-O-*α*-L-rhamnopyranoside to account for the reported efficacy of *O. mannii* leaf extract in the ethnomedicinal management of cancer and inflammation (Abubaka et al., 2007).

Conflict of interest

The authors declare that there are no conflict of interest

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