NEW GLUCOPYRANOSYLGLYCERYL-*N*-OCTENYL ADIPATE AND BIOACTIVITY OF RETRO AND CONDENSED CHALCONES FROM *TOUSSAINTIA ORIENTALIS*

SS Nyandoro[a*], MHH Nkunya[a], CC Joseph[a], JO Odalo[b] and I Sattler[c]

[a] Chemistry Department, University of Dar es Salaam, P.O. Box 35061, Dar es Salaam, Tanzania
[b]Department of Pure and Applied Sciences, Mombasa Polytechnic University College, P.O.Box 90420-80100, Mombasa, Kenya.
[c]Leibniz Institute for Natural Product Research and Infection Biology, Hans Knöll Institute, Beutenbergstrasse 11a, 07745 Jena, Germany
*E-mail address: samwel@chem.udsm.ac.tz, samnyandoro@yahoo.com

ABSTRACT

*1-(3-β-D-Glucopyranosylglycer-2-yl)-6-N-(oct-2-enyl)-adipate, [orientalin, **1**] was isolated as a new metabolite from the polar leaf extracts of Toussaintia orientalis Verdc (Annonaceae), together with the glycoflavonoids afzelin and quercitrin, and the indolidinoids toussaintines A-C. The reversed chalcones 2-hydroxy-3,4,6-trimethoxychalcone (**2**) and 2-hydroxy-3,4,6-trimethoxydihydrochalcone (**3**), the condensed chalcone (+)-6a,12a-dihydro-6-phenyl-7-styryl-6H,7H-[1]benzopyrano[4,3-][l]benzopyran (**4**), and mixtures of known triterpenoids and steroids were isolated from the less polar extracts of the root and stem bark of the same plant. The structures were established upon detailed analysis of spectroscopic data and other physical parameters. The chalcones exhibited antimicrobial, anti-inflammatory, antiproliferative and cytotoxic activity at varying efficacy levels, the reversed chalcone **2** demonstrating anti-inflammatory potency against COX-2 enzyme that was superior to the standard drug Indomethacin. These results have further indicated the versatility of Annonaceae species in accumulating structurally varied natural products, some of them having unprecedented structures.*

Keywords: *Toussaintia orientalis*; Annonaceae; orientalin, 1-(3-β-D-glucopyranosylglycer-2-yl)-6-*N*-(oct-2-enyl)-adipate; condensed and retrochalcones; anti-inflammatory.

INTRODUCTION

In our previous paper we reported the isolation of unprecedented antibacterial and antifungal *N*-cinnamoyl tetraketide derivatives from *Toussaintia orientalis* Verdc leaves (Samwel *et al*. 2011). The investigations were prompted by the fact that previously we had obtained several antimicrobial, cytotoxic and anti-inflammatory aristolactams, the *pseudo*-nucleoside 1-(2-*C*-methyl-β-D-ribofuranosyl)-uracil and other metabolites from the stem and root barks of that plant species (Odalo *et al*. 2010). That was an unprecedented isolation of a *pseudo*-nucleoside from a plant source, whose structurally similar derivatives are known antiviral marine sponge metabolites (Ichiba *et al*. 1995, Searle and Molinski 1995, Carroll *et al*. 2003, De Clercq 2003) with their clinically used examples including the anti-HIV drug azidothymidine that is dispensed as Retrovir® (Donia and Hamann 2003). Therefore, while carrying out chemical analysis of the leaves it was anticipated that, just as for the stem and root barks, the leaves would also yield the *pseudo*-nucleoside 1-(2-*C*-methyl-β-D-ribofuranosyl)-uracil or related compounds. The presence of such bio-medically potent metabolites in the leaves was considered to have particular significance since the leaves

being regenerative, could be a sustainable source of the compounds.

However, as we reported in Samwel *et al*. (2011), neither the *pseudo*-nucleoside 1-(2-*C*-methyl-β-D-ribofuranosyl)-uracil nor such similar compounds were obtained. Therefore, this prompted us to re-investigate the leaves from a new collection in order to establish unequivocally whether or not the *pseudo*-nucleoside or similar compounds were present in the leaves. Surely, even in the re-investigation neither the *pseudo*-nucleoside nor any similar compounds were obtained from the leaves. Instead we have isolated a new metabolite 1-(3-β-**D**-glucopyranosylglycer-2-yl)-6-*N*-(oct-2-enyl)-adipate (**1**), together with the known glycoflavonoids afzelin and quercitrin (Eldahshan 2011) and the toussaintines A – C (Samwel *et al*. 2011). Furthermore, phytochemical investigations of less polar extracts of the root and stem barks yielded the retro-chalcones **2** and **3**, the condensed chalcone **4** (Nkunya *et al*. 1993, Lien *et al*. 2000), as well as a mixture of polycarpol and 24-methylene-lanosta-7,9(1l)-dien-3β-ol, and that of β–sitosterol and stigmasterol (Jung *et al*. 1990, Hasan *et al*. 1987, Chaurisia and Wichtl 1987, Greca *et al*. 1990), in addition to the aristolactam piperolactam C that we recently reported (Odalo *et al*. 2010) from the same source.

We now report the isolation, structural determination and biosynthetic postulation for the formation of the new metabolite orientalin [1-(3-β-**D**-glucopyranosylglycer-2-yl)-6-*N*-(oct-2-enyl)-adipate, (**1**)]. We further report the isolation, structural determination and biosynthetic considerations, antimicrobial, anti-inflammatory, cytotoxic activity and antioxidant inhibition of the chalcones **2 - 4**.

RESULTS AND DISCUSSION

Repeated chromatography of the dichloromethane and methanol extracts from shade dried leaves gave a yellowish gum, whose structure **1** was established based on analysis of spectroscopic data. The HREIMS showed molecular ion peak at *m/z* 491.3840 ($C_{23}H_{41}NO_{10}$). The presence of free hydroxyl groups was deduced from the appearance of a broad IR band at 3394 cm^{-1}, while the strong absorptions at 1730 and 1650 cm^{-1} appeared in the spectrum and were typical for ester and amide carbonyls, respectively (Silverstein *et al*. 1981). The presence of secondary acyclic amide system was further established by the strong IR band due to nitrogen-hydrogen bond deformation at 1458 cm^{-1} (Kemp 1991).

Both the ^{1}H and ^{13}C NMR spectral features (Table 1), as well as H/H and H/C interactions observed in the COSY, HSQC and HMBC spectra indicated the presence of pyranosyl (Kasai *et al.* 1977; Kasai *et al.* 1979; Breitmaier and Voelter, 1998) and glyceryl units as part of the molecular framework of the isolated compound, the latter unit being linked to the former at the anomeric carbon through an oxygen bridge (HMBC). Thus, the ^{13}C NMR spectrum consisted of resonances characteristic of a pyranosyl unit (δ 105.3, 76.8, 74.9, 72.4, 70.2 and 62.5), the downfield shift observed for the sugar residue anomeric carbon signal (δ_C 105.3) and the corresponding upfield shift of the anomeric proton signal (δ_H 4.01, *d*, 6.9 Hz) being ascribed to the presence of a β–substituted aglycone (Kasai *et al.* 1977). In addition, the ^{1}H and ^{13}C NMR spectra indicated a signal at δ 3.51 and 62.5 respectively, which were assignable to an oxymethylene group. The signals appearing at δ 3.61 and 70.2; and 3.28-3.23, and 76.8, 74.9 and 72.4, respectively were ascribed to four oxymethine units of the pyranosyl skeleton.

Furthermore, the ^{1}H and ^{13}C NMR spectra as well as COSY and HMBC interactions (Fig 2) indicated the glyceryl unit (δ_C 70.8, 68.7 and 64.0) to be linked to the anomeric

carbon through one of its terminal oxymethylene oxygen atoms, and to the rest of the molecule through the oxymethine oxygen, forming an ester functionality and hence accounting for the downfield shift of the glyceryl oxymethine proton signal (δ 5.02) as the result of anisotropy of the ester carbonyl.

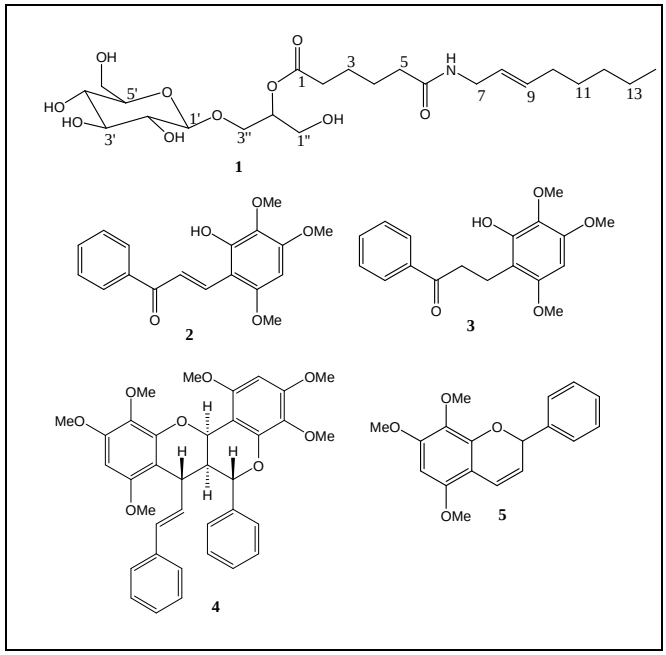

Figure 1: Chemical structures of compounds **1**-**5**

Table 1: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectral data for orientalin (**1**) in CD_3OD".

H/C	δ_H	*J* (Hz)	δ_C	H/C	δ_H	*J* (Hz)	δ_C
1	-	-	175.1	1′	4.01	*d*, 6.9	105.3
2	2.57	*br s*	35.1	2′	3.28-3.23	*m*	76.8
3	1.38	*br s*	26.0	3′	3.28-3.23	*m*	74.1
4	1.38	*br s*	26.0	4′	3.28-3.23	*m*	72.4
5	2.57	*br s*	34.9	5′	3.61	*br s*	70.2
6	-	-	174.8	6′	3.50	*dd*, 12.5, 3.7	62.5
					3.52	*dd*, 12.5, 9.8	
7	2.80	*d*, *J* = 7.8	26.6	1″	4.25	*dd*, 12.1, 7.6	64.0
8	5.12	*br s*	130.9		4.00	*d*, 11.7, 8.2	
9	5.12	*br s*	129.2	2″	5.02	*m*	71.8
10	2.09	*br s*	28.2	3″	3.76	*dd*, 12.1, 4.6	68.7
11	1.10	*br s*	23.8		3.51	*dd*, 12.1, 7.6	
12	1.10	*br s*	23.7				
13	1.06	*br s*	21.6				
14	0.68	*br s*	14.5				

br s = broad signal not well resolved to show multiplicities.

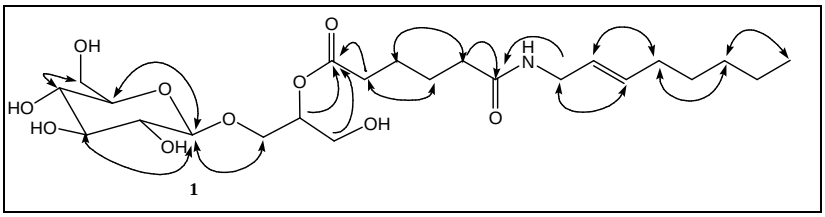

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Figure 2: Important HMBC interactions observed for orientalin (**1**)

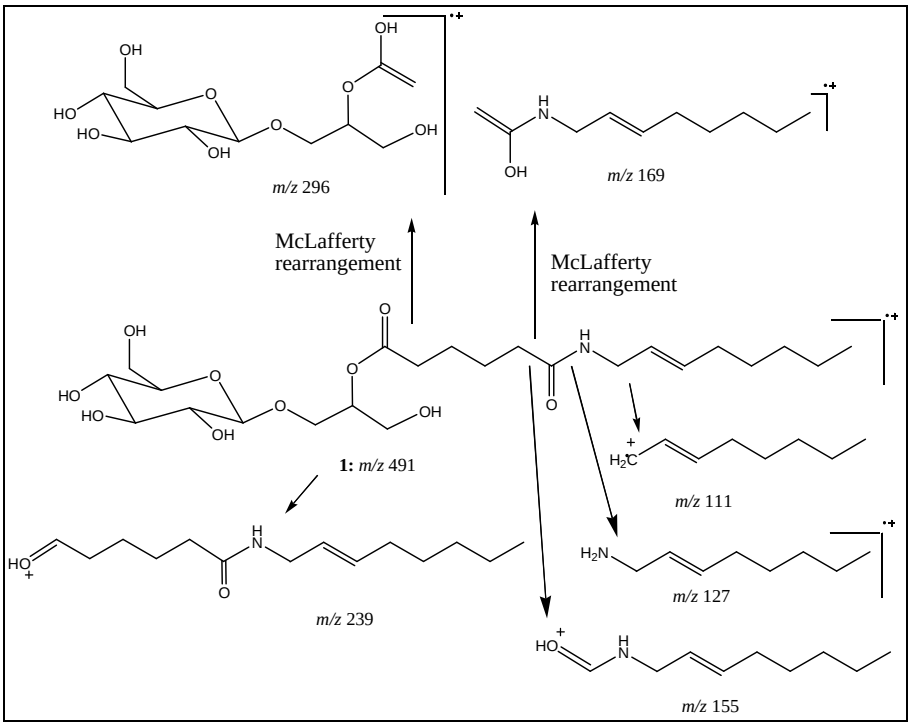
Moreover, both the ^{1}H and ^{13}C NMR spectra (Table 1) as well as the MS fragmentation pattern (Scheme 1) indicated the presence of a 1,6-dioxohexanyl unit (δ_{CO} 175.1 and 174.8), one side of which being linked to C-2 (δ_C 70.8) of the glyceryl group through an oxygen bridge, and on the other side to an oct-2-enyl unit *via* an amino atom (HMBC) forming an amide moiety. The presence of a dioxohexanyl system was further deduced by considering the NMR signals of methylene units (δ_H 2.57, 4H; δ_C 35.1 and 34.9) α to each of the two carbonyl carbons and their corresponding coupling relationships with the "middle" methylene moieties (COSY), and with the carbonyl carbons (HMBC). This was further corroborated by considering the fragmentation pattern observed in the MS that constituted peaks at *m/z* 169 and 296 arising from McLafferty-type cleavage as prompted by each of the two carbonyl groups (Scheme 1).

In addition, the ^{1}H and ^{13}C NMR spectra of **1** also indicated the presence of an isolated *N*-methylene group (δ_H 2.80, 2H, *d*, *J* = 7.8 Hz, and δ_C 26.6) adjacent to one of the two mutually coupling vinylic methine units (δ_H 5.12, δ_C 130.9) of an olefinic moiety as deduced from ^{1}H-^{1}H COSY interaction. From the COSY and HMBC interactions it was evident that the above stated methylene unit as well as the olefinic moiety formed an octenyl portion of the molecule, as further indicated by the appearance of an MS fragmentation peak at *m/z* 111 (Scheme 1). Athoungh some multiplicities did not show the expected splitting patterns hence appeared as broad singlets (*br s*), their coulpling relationship were established from ^{1}H-^{1}H COSY interactions. All the spectral features as hereby discussed established structure **1** for orientalin.

Biosynthetically, orientalin (**1**) could be considered to belong to the series of nitrogenous natural products we recently reported from the leaves of *T. orientalis*, having been derived from a tetraketide parent skeleton (Samwel *et al.* 2011). However, unlike the recently reported nitrogenous compounds, the tetraketide parent skeleton in compound **1** had not subsequently undergone cyclization, but rather it formed a peptide linkage on one end with hexanedioic acid (adipic acid) derived from the oxidation of the respective fatty acid. Thus, the hexanedioic acid could then undergo or would have undergone glycoglycerolation on the other end to form structure **1**.

Although sugar derivatives occur as natural products, and previously also having been isolated from Annonaceae species, this is the first report of the natural occurrence of the glycerylated aminooctenyl adipate sugar derivative (**1**), and the first time to report the occurrence of the glycoflavanoids afzelin and quercitrin from *T. orientalis*. The structures of afzelin and quercitrin were deduced from analysis of their spectral data, which compared well with each other except for the signals appearing as the result of the absence of a C-3′ hydroxyl group in the

former compound, as further confirmed from the literature data (Eldahshan 2011). Isolation of the new metabolite (**1**) have further indicated the versatility of Annonaceae species in accumulating natural products with varied chemical structures, some of the metabolites constituting unprecedented structural frameworks (Leboeuf *et al*. 1982; Nkunya 2005).



Scheme 1: MS fragmentation pattern for orientalin (**1**)

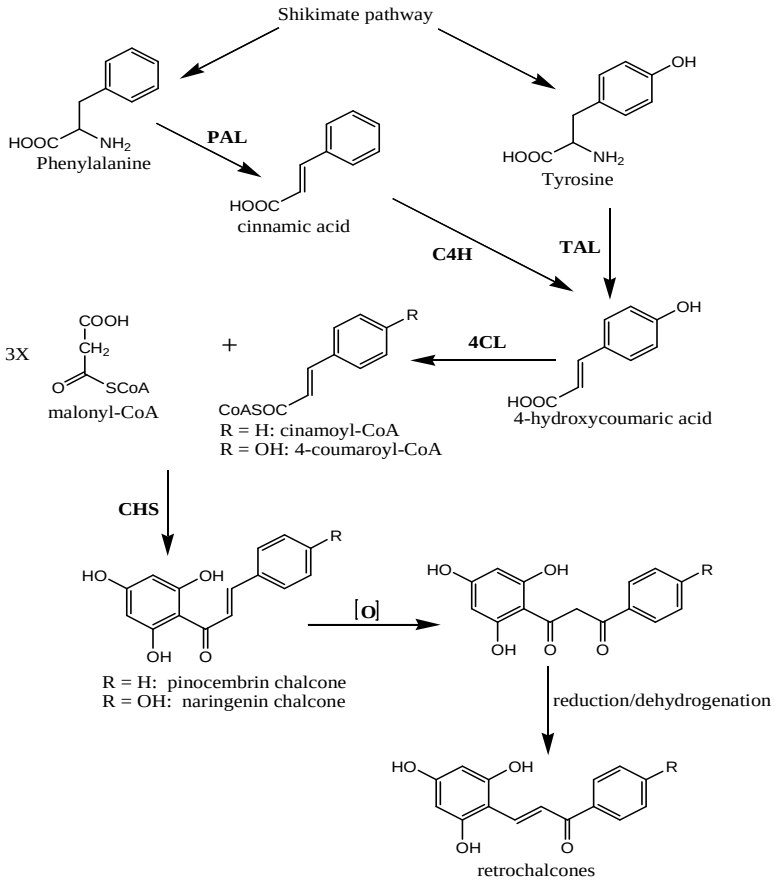
The two retro-chalcones **2** and **3**, and the condensed chalcone dependensin (**4**) were isolated upon repeated chromatography of the pet ether extract of the root bark of *T. orientalis*, with compound **2** being also obtained from the dichloromethane extract of the root barks. The structures of the compounds **2**-**4** were established based on analysis of their spectroscopic data that were comparable to those reported in the literature (Nkunya *et al.* 1993; Lien *et al.* 2000). The co-occurrence of both compounds **2** and **3** is quite interesting from the biosynthetic point of view, although at this stage it is not possible to state the biosynthetic relationship between the two compounds, which would be undoubtedly formed more or less through the same sequence. Generally, chalcones are the first isolable compounds of the flavonoid biosynthesis in plants, but do not necessarily accumulate to any appreciable degree unless the enzyme *chalcone isomerase*, which catalyses the cyclization of chalcone to flavone, is absent (Bohm 1998). Flavonoids are produced from phenylpropanoid formed through the shikimate pathway (Hwang *et al.* 2003). Phenylalanine ammonia lyase (PAL) catalyzes the first committed step of the phenylpropanoid pathway by mediating the conversion of the amino acid phenylalanine to *trans*-cinnamic acid

(Scheme 2). Cinnamate-4-hydroxylase (C4H) introduces a hydroxyl group at C-4 of cinnamic acid in the presence of molecular oxygen to yield *trans*-4-coumaric acid that could also be the product of tyrosine ammnonia lyase (TAL) transformation of tyrosine formed through the shikimate pathway. 4-Coumarate-CoA ligase (4CL) activates *p*-coumaric acid by the addition of coenzyme A to produce a 4-coumarate-CoA ester, which is the preferred substrate of chalcone synthase (CHS). CHS catalyzes the condensation of three molecules of malonyl-CoA with 4-coumaryl-CoA to generate the C_6-C_3-C_6 skeleton found in flavonoids (Scheme 2). During the reaction, a tetraketide intermediate is formed, which then undergoes cyclization to 4,2',4',6'-tetrahydroxychalcone. Retro-chalcones are believed to be formed from the normal chalcones via an intermediate 1,3-dicarbonyl compound which could then undergo reduction of the carbonyl initially at C-l and subsequent dehydration (Saitoh *et al.* 1975). The essential features of this 1,3-carbonyl transposition are illustrated in Scheme 2 for the formation of retro-chalcones.

When first isolated, dependensin was obtained as a racemic mixture together with 5,7,8-trimethoxyflav-3-ene (**5**) and retro-chalcone **2** (Nkunya *et al.* 1993). Dependensin was then conceived to be a product of non-enzymatic acid catalyzed dimerization of **5** while compound **2** was shown to be a decomposition product of **5** (Elyashberg *et al.* 2002). However, dependensin was not detected among the decomposition products of **5** when the latter was exposed to oxygen in the presence of HCl, thereby indicating that despite being obtained in the racemic form dependensin isolated then was a true natural product.

The fact that compound **4** was obtained in an optically active form in the present investigations, could indicate the participation of enzymes either in its biosynthesis or in kinetic resolution of the previously non-enzymetically formed racemic compound. It might also be that there are two enzyme systems in the two plant species, *viz*. *Uvaria dependens* and *T. orientalis* (both belonging to the family Annonaceae) for the synthesis of racemic as well as optically active dependensin. This is a subject that could only be resolved upon analysis of the enzyme systems found in the two plant species.

The occurrence of a natural product both in a racemic and optically active form is unprecedented. While it is generally known that racemic compounds are not products of enzymatic reactions, the above situation might suggest the presence of enzymes that are able to facilitate kinetic resolution of a racemic compound such as dependensin, which would result from non-enzymatic processes to form an optically active compound. However, at this stage such a kinetic resolution process remains a subject for further research.



Scheme 2: Biosynthetic pathway for the formation of retro-chalcones in plants

The chalcones **2-4** isolated from *T. orientalis* were evaluated for their antimicrobial, anti-inflammatory, cytotoxic activity and antioxidant inhibition. The agar diffusion method was used to determine both the antibacterial and antifungal activity of the compounds. Table 2 presents the results as zones of inhibition in the antimicrobial assay. The active compounds showed the activity in the range of 12-16 mm. Generally, in this assay the potent compounds had better activity against fungi than bacteria. The fungal species *P. notatum* (P1) and bacterial species *M. vaccae* (M4) were more susceptible to the test compounds as compared to the other fungi and bacteria assayed. Compound **3** showed a broader spectrum of antimicrobial activity and higher potency (inhibition zone of 16 mm) against *M. vaccae* (M4) than the other chalcones, but had lower efficacy than the standard drug Ciprofloxacin.

Results on the inhibition of purified NAD (P)-linked 3α-hydroxysteroid dehdrogenase as exerted by the isolated compounds at three different concentrations (30, 3 and 0.3 μg/ml), (IC_{50} values and HKI classification of the observed activity) are given in Table 3. Generally, inhibition of NAD (P) linked 3α-hydroxylsteroid dehydrogenase of rat liver cytosol is correlated with anti-inflammatory activity in humans (Penning, 1985), and therefore this test could be

reliably used to evaluate potential anti-inflammatory agents. In that test system, the chalcones **2-4** exhibited varying levels of activity, ranging from inactivity to very good activity. Compound **2** was the most active and fell into Class 3 of the Hans-Knöll Institute (HKI) classification, this indicating a very good anti-inflammatory activity for that compound. The condensed chalcone **4** exhibited good activity, which fell in Class 2 of the above classification, while dihydrochalcone **3** was inactive (Class 0). The loss of activity observed for the dihydrochalone **3** as compared to the chalcone **2** might have been due to the absence of a conjugated carbonyl system in **3**. This would imply the significance of this functionality in the anti-inflammatory activity as it was previously observed for other activities (Rodriguez *et al.* 1997, Ohno *et al.* 1990).

The percentage inhibitions of cyclooxygenase (COX-1 and COX-2) enzyme of the isolated compounds (which were found to be active in the 3α-HSD assay) at three different concentrations (30, 3 and 0.3 μg/ml) are given in Table 4. Of the tested compounds, the reversed chalcone **2** showed potent anti-inflammatory activity against the COX-2 enzyme exhibiting 88% inhibition at 30 μg/ml concentration as compared to the standard drug Indomethacin which showed 66% inhibition at the same level of concentration. However, the compound was weakly active against COX-1 while the condensed chalcone **4** was weakly active against both COX-1 and COX-2. As previously stated, the difference in activity of the two compounds **2** and **4** could be the result of an α,β-unsaturated carbonyl system in compound **2** as the active site, which is absent in **4** (Rodriguez *et al.* 1997, Ohno *et al.* 1990).

It is of interest to note that the active compounds as hereby reported inhibited the expression of COX-1 and COX-2 enzymes. The enzyme system COX-2 is highly expressed in inflammed tissues and is predominantly associated with development and continuance of inflammation in tissues (Xie *et al.* 1991). The COX pathway, for arachidonic acid metabolism provides many of the necessary inflammatory biochemical mediators (Xie *et al.* 1991), leading to the formation of inflammatory molecules which can exert profound biological effects influencing the process of inflammation. Therefore, the compounds that exhibited potent activity against both enzyme systems could provide templates from which pharmaceutically potent COX inhibitors might be derived. This is therefore an interesting area for future research on the isolated compounds.

When the three chalcones **2-4** were evaluated for their antioxidant inhibition of Xanthin Oxidase (XOD) and Horseradish Peroxidase (HRP) enzymes, the dihydrochalcone **3** exhibited weak inhibitory activity against HRP, but remained inactive against XOD (Table 5). All the other compounds were found to be non-inhibitors against the two enzyme systems at the tested concentrations.

The chalcones **2** and **3** also demonstrated appreciable activity in the antiproliferative and cytotoxic tests, compound **2** being more active than the dihydro- analogue **3**, as both antiproliferative and cytotoxic agents (Table 6). As for the other activities discussed earlier, the observed difference in activity outlined the importance of the conjugated carbonyl system in the activity of these compounds as both antiproliferative and cytotoxic agents. The condensed chalcone **4** exhibited weak activity as both antiproliferative and cytotoxic agent.

Chalcones are reported to possess a broad spectrum of biological activity, among which are cyctotoxicity (Go *et al.* 2005, Elias *et al.* 1995, Beutler *et al.* 1993), antitumour (Dhar 1981, Yamamoto *et al.*

1991, Chang *et al.* 2000, Zi *et al.* 2005), anti-inflammatory (Won *et al.* 2005), antiprotozoal (Frölich *et al.* 2005, Salem and Werbovetz 2005, Chen *et al.* 1994, Chen *et al*. 1997, Liu *et al.* 2001, Zhai *et al.* 1999), and antioxidant (Cioffi *et al.* 2003, Cuendet *et al.* 2000, Mohamad *et al.* 2004) activities. Thus, the biological activity results reported in this paper further indicate the great potential of the chalcones isolated from *T. orientalis* as chemotherapeutic agents, for which additional research could yield even more interesting results.

Table 2: Zones of inhibition (mm) of chalcones (**2-4**) against bacterial and fungal strains given in Table 7

Compounds/Microorganisms	B1	B3	B4	B9	M4	H4	H8	P1
2	0	0	0	0	15	0	0	15
3	12	0	0	0	16	0	11	15
4	0	0	0	0	13	0	0	15
Ciprofloxacin (5 µg/ml)	28	18	23	22	22	--	--	--
Amphotericin (10 µg/ml)	--	--	--	--	--	14	20	18

Table 3: Anti-inflammatory activity of chalcones (**2-4**) against 3α-hydroxysteroid dehydrogenase (% inhibition values)

Compounds	30 µg/ml	3 µg/ml	0.3 µg/ml	IC_{50} (µg/ml)	HKI Class
2	80	51	0	3.06	3
3	34	0	0	---	0
4	74	34	0	8.60	2
Indomethacin	93	27	10	4.59	

Not active (0); active (1); more active (2); highly active (3)

Table 4: Anti-inflammatory activity of compounds **2** and **4** against COX-1 and COX-2

Compounds	Concentration (µg/ml)	% Inhibition of COX-1 and COX-2	
		COX-1	COX-2
2	30	50	88
	3	32	22
	0.3	27	13
4	30	34	13
	3	29	14
	0.3	15	13
Indomethacin	30		66
	3	51	

Table 5: Horseradish Peroxidase inhibition of compound **3**

Compound	Concentration (μg/ml)	% Activity for Positive Control (100%)	Category (HKI)
3	40	29	1
	20	69	
	4	106	
Standard			
NAC	40	2	
	20	2	
	4	51	

Category 1 = Active; 3 = Very high activity

Table 6: Antiproliferative and cytotoxic effects of chalcones (**2-4**)

	Antiproliferative effect (μg/ml)		Cytotoxic effect (μg/ml)
Compounds	**L-929 (GI_{50})**	**K-562 (GI_{50})**	**HeLa (CC_{50})**
2	9.5	11.2	15
3	34.1	41.2	32.1
4	>50	>50	>50

EXPERIMENTAL

General experimental procedures: Column chromatography: Silica gel 60 (Merck 230-400 mesh) and Sephadex® LH-20 (Pharmacia); thin layer chromatography (TLC): Pre-coated plates (Merck, Kieselgel 60 F_{254}, 0.20 mm) and visualization: UV/VIS and anisaldehyde spray reagent/heat at *ca.* 110 ºC; UV (λ_{max}, nm): Perkin Elmer (Lambda 35) spectrophotometer; IR (λ_{max}, cm^{-1}): Perkin Elmer FTIR-100 spectrophotometer; ^{1}H (400 and 600 MHz) and ^{13}C (100 and 150 MHz) NMR: Bruker Avance Topspin Spectrometer and $CDCl_3$ or CD_3OD as solvent, TMS internal standard for ^{1}H NMR and solvent signal for ^{13}C NMR; high-resolution electron ionization mass spectra (HREIMS): GCT Premier TOF mass spectrometer, 70 eV and 180 ºC source temperature; optical rotations: Perkin-Elmer Model 241 and JASCO P-1020 Polarimeters.

Plant materials: The root and stem barks of *T. orientalis* Verdc. were collected in October 2004 while leaves were re-collected in February 2008 from Zaraninge Forest Reserve at the edge of Saadani National Park in Bagamoyo District, Tanzania and the plant's identity was confirmed at the Herbarium of the Department of Botany, University of Dar es Salaam where a voucher specimen is preserved under number FMM 3330.

3.3 Extraction and isolation: The air-dried and powdered leaves (2 Kg) were sequentially extracted using pet ether, CH_2Cl_2 and MeOH at RT (2 x 48 h) and residual solvent removed under *vacuo*. The CH_2Cl_2 extract (38.4 g) on repeated column chromatography, eluting with a gradient of *n*-hexane and EtOAc polarity increase

(silica gel, 25-100% v/v EtOAc/*n*-hexane gradient elution) yielded the previously reported indolidinoids toussaintines A – C (Samwel *et al.* 2011) and orientalin (**1,** 56 mg). The MeOH extract (78.6 g) on repeated column chromatography (silica gel, 25-100% v/v EtOAc/*n*-hexane gradient elution) also gave compound **1** (290 mg), the toussaintines A – C, as well as afzelin and quercitrin (Eldahshan, 2011).

The air dried and powdered root and stem barks (each 1 Kg) were sequentially extracted using pet ether, dichloromethane and methanol at room temperature, each extraction lasting for three days. Removal of residual solvent under reduced pressure furnished the crude extracts (38.2 g). The extracts were subjected to silica gel column chromatography, eluting with a gradient of *n*-hexane and EtOAc, and the fractions were then pooled on the basis of observations from TLC analysis. Repeated chromatography of the pet ether extract of the root bark yielded a mixture of β-sitosterol) and stigmasterol (Chaurisia and Wichtl 1987; Greca *et al.* 1990), a mixture of polycarpol and 24-methylene-lanosta-7,9(1l)-dien-3β-ol, (Jung *et al.* 1990; Hasan *et al.* 1987), 2-hydroxy-3,4,6-trimethoxychalcone **(2)**, 2-hydroxy-3,4,6-trimethoxydihydrochalcone (**3**) and (+)-dipendensin (**4**, $[\alpha]_D$; +16.34^o). The dichloromethane extract of the root bark on repeated chromatography on silica gel and then Sephadex® LH-20 yielded piperolactam C (Odalo *et al.* 2010), **2** and polycarpol, all of which were purified further by recrystallization from EtOAc. The pet ether extract of the stem bark on repeated chromatography yielded compounds **2** and polycarpol, and a mixture of β-sitosterol and stigmasterol, compounds **2** and polycarpol having been recrystallized from MeOH and EtOAc, respectively.

*1-(3-β-D-Glucopyranosylglycer-2-yl)-6-N-(oct-2-enyl)-adipate, [orientalin **1**]*: Light yellow gum; yield, 346 mg; $[\alpha]_D$ -7.9^o (MeOH, *c* 1.38); anisaldehyde – pink then black; IR (film), λ_{max} cm^{-1} 3394, 2927, 2855, 1730, 1650 and 1458; HREIMS, *m/z* (rel. int. %) 491.3840 ([M]$^+$, $C_{23}H_{41}NO_{10}$), 341 (45), 339 (43), 313 (75), 264 (41), 262 (30), 257 (41), 245 (46), 239 (42), 229 (100), 227 (66), 213 (30), 213 (30), 201 (80), 185 (40), 171 (98), 155 (85), 144 (32), 131 (34), 129 (39), 127 (53) 125 (46), 111 (41), 109 (67) and 107 (33); ^{1}H and ^{13}C NMR: Table 1.

Biological Assays

Agar diffusion assay for anti-microbial activity (antibacterial and antifungal activity): The agar diffusion method was used for the determination of antibacterial and antifungal activity of the isolated compounds against the microorganisms listed in Table 7.1. The microorganisms were obtained from the Hans Knolls Institute for Natural Product Research and Infectious Biology (HKI) in Jena, Germany. Approximately 9 mL of Müller-Hinton agar for bacteria and Sabouraud Dextrose Agar for fungi (Oxoid, UK) were poured into petri dishes (9 cm in diameter) and inoculated with the respective test organisms. Wells (4 mm) were punched out of the solid agar using pipette tips, and 1 mL of 50 µg/mL of the test compounds and control antibiotics (Ciprofloxacin, 5µg/mL and Amphotericin, 10 µg/mL) were placed in each well. The petri dishes were then incubated at 30 ^{o}C and 35 ^{o}C for the test bacterial and fungal strains, respectively, for 20 h and the average diameter of the inhibition zone surrounding the wells was measured.

Table 7: Bacterial and fungal strains used to test for efficacy of the isolated chalcones.

Code	Organism Name	Type	HKI Code
BNA	*Bacillus subtilis* ATTC 6633 (IMET) NA	B	B1
SA	*Staphylococcus aureus* (IMET 10760) SG511	B	B3
EC	*Escherichia coli* SG 458	B	B4
PA	*Pseudomonas aeruginosa* K 799/61	B	B9
MV	*Mycobacterium vaccae* IMET 10670	B	M4
SS	*Sporobolomyces salmonicolor* SBUG 549	F	H4
CA	*Candida albicans* BMSY 212	F	H8
PN	*Penicilium notatum*	F	P1

B = Bacterium; F = Fungus

Antiinflammatory activity [3α-hydroxysteroid dehydrogenase (3α-HSD) assay: This assay was carried out following standard procedures as adopted from the literature (Penning 1985). Cell free systems for the determination of antiinflammatory and antiphlogistic activity are critical as it is a complex reaction of living organisms. Nonetheless, *in vitro* assays for the screening of inhibitors of key enzymes like 3α-HSD and prostaglandin H synthase are important tools. The NAD (P)-linked enzyme (3α-HSD) has been purified from rat liver cytosol. This enzyme is known to catalyze the reduction of a variety of 3-ketosteroids, for example 5α-dihydrotestosterone (5α-androstan-17β-*O*-one), 5β-dihydrocortisone (5β-pregnan-17α,21-diol-3,11,20-trione) to the corresponding 3α-hydroxysteroids through the consumption of NADPH and, therefore, plays an important role in cortisone metabolism. The NADPH consumption is determined photometrically as a decrease of extinction at 340 nm. Indomethacin and Ibuprofen are used as reference compounds. A surprising property of the purified enzyme is that it is inhibited by the major classes of non-steroidal and steroidal anti-inflammatory drugs in the rank order of their therapeutic potency. A high correlation exists between the logarithm of the concentration of drug required to produce 50% inhibition of the purified 3α-hydroxysteroid dehydrogenase (log IC_{50} value) and the dose required to produce an anti-inflammatory response in humans. These observations led to the suggestion that the extent of inhibition of 3α-hydroxysteroid dehydrogenase could be used to predict anti-inflammatory drug potency (Penning 1985).

Preparation of cytosol: Adult male Sprague-Dawley rats (150-200 g) were killed by cervical dislocation. The livers were excised and homogenized in 3 volumes of 50 mM Tris-HCl of pH 8.6 containing 250 mM sucrose, 1 mM dithiothreitol and 1 mM EDTA. Homogenates were centrifuged at 100,000 x g for 30 min; the supernatant (cytosol; i.e. source of 3α-hydroxysteroid dehydrogenase) was used for enzyme assays without further processing.

Preparation of purified 3α-hydroxysteroid dehydrogenase: A homogeneous enzyme was prepared according to the method described by Penning (1985). This enzyme had a final specific activity of 3.58 μmol of 5β-dihydrocortisone reduced/min/mg of protein.

***Enzyme assays*:** The reduction of 5β-dihydrocortisone was monitored by measuring the changes in the absorbance of the pyridine nucleotide at 340 nm. Each assay (1.0 ml) contained the following:

Potassium phosphate buffer (pH 6.0, 0.840 ml of 1M), NADPH (20 µL of 9 M), 5β-dihydrocortisone (10 µL of 5 mM), and acetonitrile (30 µL). The reactions were initiated by the addition of enzyme (30-50 µg of cytosolic protein or 0.6 µg of purified enzyme), and optical density change was followed over a period of 5 min. Control incubation experiments by addition of the cytosol in which either 5β-dihydrocortisone or NADPH was absent, indicated that the presence of both substances was required before the cytosol would promote a change in absorbance at 340 nm. The % inhibition of the isolated compounds was generated at three different concentrations (30, 3 and 0.3 µg/ml). Increasing amounts of the isolated compound were added to the standard assay system, and the concentration of the compound required to cause the rate of 5β-dihydrocortisone reduction by 50% (IC_{50}) was computed from the resulting natural logarithm dose-response curves.

Chemiluminescent cyclooxygenase (COX) inhibitor assay: Two isoforms of cyclooxygenase (COX-1 and COX-2) are the rate-limiting enzymes for the biosynthesis of prostaglandins, this being the conversion of arachidonic acid to prostaglandin H_2 molecules that are responsible for the progression of some inflammatory responses (Smith *et al.* 1991; Cao and Prescott, 2002). COX-2 is the inducible isoform expressed in response to pro-inflammatory cytokines, endotoxins and tumor promoters (DuBois *et al.* 1998) and is predominantly associated with inflammation (Pai *et al.* 2001). This inducible COX-2 is believed to be the target enzyme for the anti-inflammatory activity of non-steroidal anti-inflammatory drugs (Xie *et al.* 1991). Therefore, it was of interest to observe if the isolated compounds that had shown anti-inflammatory activity in the 3α-HSD test had effects on these enzymes that would lead to pathogenic changes, if over-expressed. The COX inhibitor assay utilizes the heme-catalyzed hydroperoxidase activity of cyclooxygenases to generate luminescence in the presence of a luminal-type cyclic naphthalene hydrazide and the substrate arachidonic acid. Arachidonate-induced luminescence has been shown to be an index of the real-time catalytic activity and demonstrated the turnover inactivation of the enzyme (Forghani *et al.* 1998). The chemiluminescent COX assay induces both COX-1 and COX-2 enzymes in order to screen isoenzyme-specific inhibitors.

Preparation of reagents: All the reagents used were commercially obtained from Cayman Chemical Company in the form of a Chemiluminescent COX (ovine) Inhibitor Screening Assay kit. The assay buffer (0.1 M Tris-HCl, pH 8) was prepared by diluting 3 ml of commercially available assay buffer concentrate with 27 ml of HPLC grade water and stored at 4 ^{o}C. The heme solution was prepared by diluting 58 µl of the commercial heme with 942 µl of the assay buffer (dilute). The COX-1 and COX-2 enzymes, which were stored at -80 ^{o}C were each prepared for the assay by diluting 30 l of enzyme with 570 µl of assay buffer (dilute) and stored on ice. Arachiodonic acid, which was used as the substrate was stored at -80 ^{o}C. The supplied substrate (100 lµ) was diluted by 10µ0 µl of 0.1 M KOH and the resulting solution diluted with 9.8 ml of assay buffer (dilute) to achieve a final concentration of 116 µM. The chemiluminescent substrate, which contained a naphthalene hydrazide solution, was used as supplied.

Assay procedures: The assay was performed in triplicate at 25 ^{o}C and the test samples were dissolved in DMSO. Heme (10 µl), assay buffer (10 µl), and solvent (10 µl) were added to three wells, which served as background wells. Three other wells which were designated as 100% initial activity wells were each constituted with heme (10 µl), enzyme (either COX-1 or COX-2, (10

μl)), and solvent (10 μl). Three other wells, which served as the inhibitor wells were constituted with heme (10 μl), enzyme (either COX-1 or COX-2 (10 μl)), and test sample (10 μl). The assay buffer (200 μl) was added to all the wells. The plate was then inserted into the Luminometer and the chemiluminiscent subsrate (10 μl) dispensed from the Luminometer and arachidonic acid (50 μl) was immediately dispensed to all the wells and reading taken for 10 seconds per well. The percent inhibition of the enzymes was determined from the average Relative Luminescent Units (RLU) values as follows:

$$\% \text{ Inhibtion} = 100 - \left(\frac{\text{RLU inhibitor sample-RLU background}}{\text{RLU initial activity-RLU background}} \text{X } 100\right)$$

Antioxidant inhibition activity: The antioxidant tests were carried out by employing Xanthine-Oxidase (XOD) and Horseradish peroxidase (HRP) assay systems. XOD is an important oxidative enzyme, which is involved in normal biological processes and also in pathological conditions. XOD catalyses the oxidation of hypoxanthine, and xanthine to uric acid which plays a crucial role in causing gout (Kong *et al.* 2000), whereas, the generation of superoxide radicals (ROS, reactive oxygen species) are involved in many pathological processes such as inflammation, atherosclerosis, cancer and aging (Halliwell *et al.* 1992). Therefore, it was of interest to observe if the isolated compounds could affect this enzyme that leads to pathogenic changes, if over-expressed.

Lucegenin derived chemiluminescence (LDCL) was used to measure inhibition of XOD as described by Li *et al.* (1998). ROS reduces lucigenin by producing lucigenin cation radical which reacts with the peroxidase radical to yield an unstable dioxetane intermediate. The lucigenin dioxetane decomposes to produce two molecules of *N*-methylacridone, one of which is in an electronically excited state, which upon relaxation to the ground state emits a photon. The production of ROS can then be monitored through sensitive measurement of the photon emission. Allopurinol, which inhibits XOD activity, was used as a standard drug in this assay. XOD catalyzes the degradation of azothioprin, an immunosuppressant substance that is used after organ transplant. Allopurinol is used for co-medication in order to slow degradation of azothioprin.

The assay mixtures for enzymatic system contained XOD (4 μg/ml) and xanthine (0.5 mM) in air-saturated PBS (1 ml) containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and dark-adapted lucigenin (5 μL, 5 μM). The lucigenin-derived chemiluminescence was monitored with a Luminometer at 37 ^{o}C and light emission was recorded after every min for 20 min, and was then expressed as mean arbitrary light units/min. The blank reaction, that is, a control contained all components except the enzyme. The concentrations of the test samples were 0.16, 0.8 and 4 μg/ml.

N-acetylcysteine (NAC) was used as a standard scavenger. Thiol-containing molecules possess antioxidant properties that are of interest in the pharmacological inactivation of reactive oxygen species (ROS), particularly in the treatment of chronic inflammatory diseases. The *in vitro* antioxidant activity of a new agent is examined and compared with NAC. This assay was used to assess the antioxidant potential of the isolated compounds.

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and Horseradish peroxidase (HRP, E.C. 1.11.1.7, Grade II) were obtained from Boehringer Mannheim (Germany). Hydrogen peroxide (30%) was obtained from Merck (Milan, Italy) while Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, in pure form, >98%), was obtained from Fluka (Milan, Italy). All other reagents and compounds were of analytical-grade. All solutions were prepared with pyrogen-free reagent-grade water, using a Milli-Q system (Millipore, Milan, Italy). A Luminoscan Ascent luminometer (Labsystems, Helsinki, Finland) was used to perform the automated assays. The data were recorded using a computer and the Ascent software for kinetics measurements. The 96-wells blank microplates employed in the automated assay were from Thermo Labsystems, Helsinki, Finland.

The reagent solutions used in the assay were prepared, stored and employed according to published literature for the chemiluminescent (CL) antioxidants assay (Girotti *et al*. 2002). The calibration curve (1–10 μM Trolox) was measured once a week and any time when the stable reagents (luminol and HRP solutions) were newly prepared. Otherwise, at each experimental session only one standard concentration was measured, that compared with the calibration curve, was used to calculate a "correction factor". When this value was lower than 0.8 or higher than 1.2 a new calibration curve was prepared. In each well of the microplate were manually injected 10 μl of the sample or standard solution and 20 μl of HRP solution. The reaction started when 200 μl of CLM were injected automatically in each well. Signal intensities (RLU) and count numbers were automatically recorded.

Antiproliferative and cytotoxicity assay: Antiproliferative and cytotoxic activity of the compounds was determined as described in the literature (Dahse *et al.* 2001). The compounds were assayed on cell lines K-562 (human chronic myeloid leukaemia) and L-929 (mouse fibroblast) for their antiproliferative effects (GI_{50}, concentration which inhibited cell growth by 50%), and against HeLa cells for their cytotoxicity effects (GC_{50} = concentration at which cells are destroyed by 50%; used partially in referring to the lysis of cells). The cells were incubated at 10 different concentrations of each of the target compounds. Cells of established suspended cell lines K-562 (DSM ACC 10) and adherent Huvec (L-929) were cultured in the RPMI medium (Dahse *et al.* 2001). The adherent cells of L-929 were harvested at the logarithmic growth phase after trypsinization, using 0.25% trypsin in PBS containing 0.02% EDTA (Biochrom KG L2163). For each experiment with K-562, L-929, and HeLa cells approximately 10,000 cells were seeded with 0.1 mL RPMI 1640 (GIPCO BRL 21875-034) containing 25 μg/ml Gentamicin sulfate (BioWhittaker 17-528Z but without HEPES) per well of the 96-well microplates (K-562: NUNC 163320, L-929, HeLa: NUNC 167008). For the cytotoxicity assay, the HeLa cells were pre-inoculated for 48 h without the test substances. The dilution of the compounds was carefully made on the monolayers of HeLa cells after the pre-incubation time.

Cells of L-929, K-562 and HeLa in the presence of the respective test compounds were incubated for 72 h at 37^{o}C in a humidified atmosphere and 5% CO_2. Suspension cultures of K-562 in microplates were analyzed by an electronic cell analyzer system (CASY 1, SCHÄRFE, Reutlingen, Germany) using an aperture of 150 μm. The contents of each well (0.2 ml) in the microplate were diluted 1:50 with CAYSTON (SCHÄRFE). Every count/ml was automatically calculated from the

arithmetic mean of three successive counts of 0.4 ml each. From the dose response curves the GI_{50} values were calculated using the computer program CASYSTAT. The GI_{50} value was defined as the 50% intersection line of the concentration-response curve, determined by the cell count/ml as compared to the control. The essential parameters for the estimation of growth inhibition and for changes in the diameter distribution curve were expressed as diagrams. The monolayer of the adherent Huvec (L-929) and HeLa cells were fixed by glutaraldehyde and stained with a solution of methylene blue. After washing gently, the stain was eluted by 0.2 ml of 0.33 M HCl into the wells. The optical densities were measured at 630 nm in a microplate reader.

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