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Four New Phenolics and Anti parasitic Secondary Metabolites from *Flacourtia rukam* Zoll. & Mortizi

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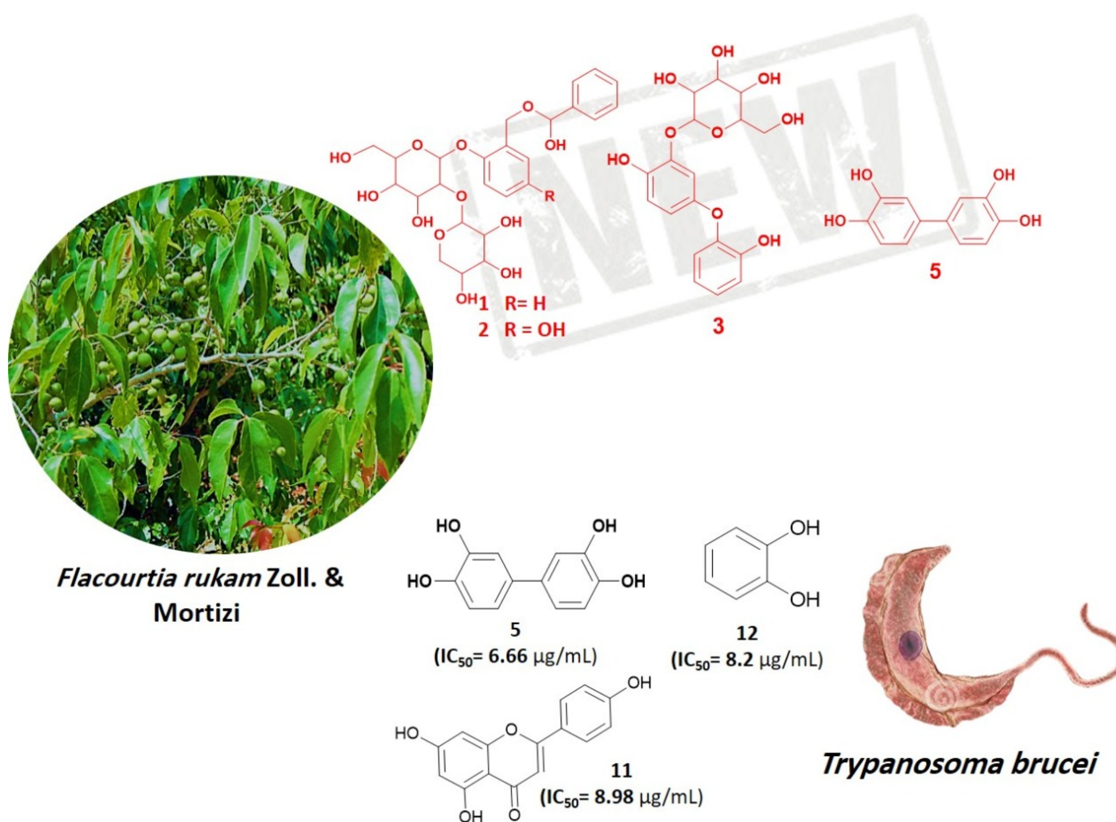
Graphical Abstract



Abstract

Phytochemical investigation of *Flacourtia rukam* Zoll. & Mortizi (*F. rukam*) leaves and bark led to the isolation and characterization of seventeen compounds of which four phenolics were not previously described; 2-[(benzoyloxy)methyl]-phenyl-*O*-*β*-xylosyl-(1→2)-*β*-glucopyranoside (**1**), 2-[(benzoyloxy)methyl]-4-hydroxyphenyl-*O*-*β*-xylosyl-(1→2)-*β*-D-glucopyranoside (**2**), 2-hydroxy-5-(2-hydroxyphenoxy)phenoxy-*β*-glucopyranoside (**3**) and biphenyl-1,1′,2,2′-tetraol (**5**). Interestingly, the later compound is known as a synthetic but this is the first report for its isolation from nature. Chemical structures were established using extensive analysis of spectroscopic data (1D and 2D NMR and HRESIMS). Biphenyl-1,1,2,2′-tetrol (**5**) exhibited a good activity against *Trypanosoma brucei* trypamastigotes with IC_{50}= 6.66 ug/mL. Compounds **2**, **5**, **9**, **10**, **11** and **12** showed a good *in-vitro* anti-inflammatory activity using proteinase inhibitory assay. On the contrary, all tested compounds were inactive as antileishmanial or antimalarial.

Key words

Flacourtia rukam Zoll. & Mortizi, Salicaceae, phenolics, antitrypanosomal, anti-inflammatory.

1. Introduction

Trypanosomiasis is among the top ten global parasitic diseases.(Garcia 2006) There are two known types; African trypanosomiasis (sleeping sickness, caused by *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense)* and American trypanosomiasis (Chagas disease, caused by *Trypanosoma cruzi*).(Setzer and Setzer 2006) Drugs; as benznidazole and nifurtimox; available for trypanosomiasis are old, complicated to administer and lead to severe adverse reactions which make them currently unacceptable.(Legros et al. 2002) Therefore, it is important to develop new chemotherapeutic approaches against this disease especially from natural source.

Flacourtia is a genus of flowering plants in the willow family; Salicaceae. It was previously a member of family Flacourtiaceae.(Mabberley 2008) *Flacourtia* species were reported to contain different chemical classes such as phenolics (Bourjot et al. 2012, Jayasinghe et al. 2012, Kaou et al. 2010, Ndhlala et al. 2007), lignans (Chai et al. 2009), tannins (Chai, Ren, Xu, Bai, Zhou, Ling, Pu, Li and Tu 2009, Ndhlala, Kasiyamhuru, Mupure, Chitindingu, Benhura and Muchuweti 2007), sterols and triterpenes (Gurav et al. 2013, Saree 1998). These reported compounds possess a broad spectrum of biological activities including antiprotozoal (Bou et al. 2014, Kaou, Mahiou-Leddet, Canlet, Debrauwer, Hutter, Laget, Faure, Azas and Ollivier 2010, Singh et al. 2017) and anti-inflammatory activity(Joshy et al. 2016). Ragasa et al. (2017) reported the isolation of monogalactosyl diacylglycerols, *β*-sitosteryl-*3β*-glucopyranoside-6*β*-*O*-fatty acid esters, *β*-sitosterol, triacylglycerols and chlorophyll A from *Flacourtia rukam* Zoll. & Mortizi fruits(Ragasa et al. 2017). Traditionally, in Malaysia, *Flacourtia rukam* is used in inflamed eyelids, dusted over wounds and used in skin allergies.(Christophe 2006, Lim 2013) Moustapha et al. (2014) compared the antioxidant activity of the methanolic extract of this plant with other Egyptian plants. The results showed that *Flacourtia rukam* exhibited a good antioxidant activity.(Moustafa et al. 2014)

Being a member of the genus *Flacourtia*, it was interesting to do a complete phytochemical study and test the potential antiparasitic and anti-inflammatory effect of *Flacourtia rukam* leaves and bark metabolites.

2. Results and Discussion

A series of extraction, separation, and purification procedures were performed on *F. rukam* leaves and bark which resulted in the isolation and characterization of the four new phenolic compounds (Fig.1); 2-[(benzoyloxy)methyl]-phenyl-*O*-*β*-xylosyl-(1→2)-*β*-glucopyranoside (**1**), 2-[(benzoyloxy)methyl]-4-hydroxyphenyl-*O*-*β*-xylosyl-(1→2)-*β*-glucopyranoside (**2**), 2-hydroxy-5-(2-hydroxyphenoxy)phenoxy-*β*-glucopyranoside (**3**) and biphenyl-1,1′,2,2′-tetraol (**5**). Additionally, thirteen known compounds (Fig.1) were also isolated for the first time from *F. rukam* leaves and bark; 2,3,6-dibenzofurantriol (**4**) (Shi et al. 2008), 6′-caffeoyl-(1-1′) *β*-D-glucopyranosyl-catechol (**6**) (Kumar et al. 2009), rutin (**7**) (Matsubara et al. 1985), isoquercitrin (**8**) (Valentová et al. 2014), dodegranoside B (**9**) (Kumar, Rawat, Rahuja, Srivastava and Maurya 2009), dodegranoside C (**10**) (Kumar, Rawat, Rahuja, Srivastava and Maurya 2009), apigenin (**11**) (Van Loo et al. 1986), catechol (**12**) (Huang et al. 2014), resorcinol (**13**) (Durairaj 2005), *β*-sitosterol-*β*-D-glucoside (**14**) (Bayoumi et al. 2010), phytol (**15**) (Brown 1994), *β*-sitosterol (**16**) (Amin et al. 2017), Friedelin (**17**) (Sousa et al. 2012).

Compound 1: White amorphous solid (MeOH); $[\propto]_D^{25}$+143 (c=0.12, MeOH); UV (MeOH) λ_{max} at 271 nm. The high-resolution electron spray ionization mass spectrometry (HRESIMS) data for compound **1** showed a quasimolecular ion peak at *m/z* 523.1796 $[M+H]^+$ (calcd. for $C_{25}H_{31}O_{12}$, 523.1816) consistent with the molecular formula $C_{25}H_{30}O_{12}$. ^{1}H NMR and ^{13}C NMR data (Table S1) showed resonances characteristic of salicyl alcohol (δ_C 156.9, 126.7, 130.2, 123.3, 130.6, 115.9, 63.5 ppm) (Zhang et al. 2006); attached to benzoyl (δ_C 131.6, 130.6, 129.6, 134.2, 129.6, 130.6, 168.0) and a disaccharide moiety. The identification of the sugar units was confirmed by comparing the ^{13}C NMR chemical shift of sugar part with literature (Du et al. 2004) and by paper chromatographic analysis after acid hydrolysis (Lu and Foo 1997). The disaccharide was confirmed to be glucose (2→1) xylose which is called sambubioside. The attachment site was confirmed by HMBC correlations; $^3J_{C\text{-}H}$ correlation was observed between H-1‴ of xylose (δ_H 4.64, d, J=7.2 Hz) and C-2″ of glucose at δ_c 83.2 ppm (Fig. S7). Coupling constant values (J=7.2 Hz) for both anomeric protons elucidated that glycosidic linkage is of *β*-type. $^3J_{C\text{-}H}$ correlations (Table 1, Fig. 2) of H-7_a at δ_H 5.43 (d, J=12.8 Hz), and H-7_b at δ_H 5.63 (d, J=12.8 Hz) to C-7′; the ester carbonyl at δ_C 168.04 and that of H-1″ of glucose at δ_H 5.07, d, J=7.2 Hz) to C-1 at δ_C

156.9 established the position of the benzoyl and sambubioside moieties on the salicyl alcohol. Compound **1** is a new natural product which is identified as 2-[(benzoyloxy)methyl]-phenyl-*O*-*β*-xylosyl-(1→2)-*β*-glucopyranoside.

Compound 2: White amorphous solid (MeOH); $[\propto]_D^{25}$+131 (*c*=0.13, MeOH); UV (MeOH) λ_{max} at 282 nm. The HRESIMS data for compound **2** showed a quasimolecular ion peak at *m/z* 539.1738 $[M+H]^+$ (calcd. for $C_{25}H_{31}O_{13}$, 539.1765), consistent with the molecular formula $C_{25}H_{30}O_{13}$. ^{1}H NMR, ^{13}C NMR, HSQC and HMBC data (Table S1, Fig. 2) of compound **2** showed a similarity with that of compound **1** except that salicyl alcohol in compound **1** was substituted with gentisyl alcohol (δ_C149.9, 128.2, 116.2, 153.7, 116.4, 118.2, 63.4 ppm) (Lu and Foo 1997). The ^{13}C NMR data for the disaccharide moieties of **1** and **2** were almost identical. This similarity confirmed that compound **2** also contains sambubioside moiety. Compound **2** is isolated from nature for the first time and identified as 2-[(benzoyloxy)methyl]-4-hydroxyphenyl-*O*-*β*-xylosyl-(1→2)-*β*-glucopyranoside.

Compound 3: needle crystals (MeOH); $[\propto]_D^{25}$ -30 (*c*=0.05, MeOH); UV (MeOH) λ_{max} at 271 nm. The HRESIMS data for compound **3** showed a quasimolecular ion peak at *m/z* 381.1168 $[M+H]^+$ (calcd. for $C_{18}H_{21}O_9$, 381.1185) consistent with the molecular formula $C_{18}H_{20}O_9$. The ^{1}H and ^{13}C NMR data (Table S2) exhibited signals for 1,2-disubstituted benzene ring (catechol moiety) [δ_H 6.91 (dd, *J*=1.6, 8 Hz, H-3), 6.96 (m, H-4), 6.82 (m, H-5), and 6.78 (dd, *J*=1.6, 8 Hz, H-6)]; signals for 1,2,4 trisubstituted benzene ring [δ_H 6.87(d, *J*=2.8 Hz, H-6′), 6.78 (d, *J*=8.4 Hz, H-3′), 6.53 (dd, *J*=2.4, 8.4 Hz, H-4′)]; one glucose residue at δ_H 4.73 (d, *J*=7.2 Hz, H-1″), 3.5 (m, H-2″), 3.49 (m, H-3″), 3.46 (dd, *J*=6.8, 3.2 Hz, H-4″), 3.31 (m, H-5″), 3.71 (dd, *J*=4.4, 12 Hz, H_a-6″), 3.78 (dd, *J* = 2.4, 12 Hz, H_b-6″).]. The sugar unit was confirmed as glucose by paper chromatographic analysis after acid hydrolysis (Lu and Foo 1997) and by its carbon chemical shift. Coupling constant value (*J*=7.2 Hz) for anomeric proton confirmed that glycosidic linkage is of *β*-type. The HMBC (Table 2) established the position of glucopyranoside moiety on C-1′; it showed $^3J_{C\text{-}H}$ correlation between δ_H 6.78 (d, *J*=8.4 Hz, H-3′) to C-1′ at δ_C 146.9 ppm, δ_H 6.53 (dd, *J*=2.4, 8.4 Hz, H-4′) to C-2′ at δ_C 143.7 ppm also, it showed $^3J_{C\text{-}H}$ correlation between the anomeric proton of glucose moiety at δ_H 4.73 (d, *J* = 7.2 Hz, H-1″) to C-1′ at δ_C 146.9 ppm. Therefore, compound **3** is a previously undescribed natural product and identified as 2-hydroxy-5-(2-hydroxyphenoxy) phenoxy-*β*-glucopyranoside.

Compound 5: White amorphous solid (MeOH); $[\propto]_D^{25}$+6 (*c*=0.5, MeOH); UV (MeOH) λ_{max} at 271 nm. The HRESIMS data for compound **5** showed a quasimolecular ion peak at *m/z* 219.0651 $[M+H]^+$ (calcd. for $C_{12}H_{11}O_4$, 219.0657) consistent with the molecular formula $C_{12}H_{10}O_4$. ^{1}H NMR (Table S2) showed ABX spin system [δ_H 6.95 (d, *J* = 2.1 Hz, H-2), 6.84 (dd, *J* = 8.2, 2.1 Hz, H-5), 6.76 (d, *J* = 8.2 Hz, H-6)] whose analysis corresponded to a 1,2,4 substitution. ^{13}C NMR (Table S2) showed only six carbon signals δ_C 146.3 (C-1), 145.2 (C-2), 116.5 (C-3), 134.8 (C-4), 118.9 (C-5), 114.6 (C-6) ppm. Presence of two quaternary carbons at 146.3 (C-1), 145.2 (C-2) suggested the presence of two hydroxyl groups. While appearance of a quaternary *sp*^{2} carbon at 134.8 (C-4) suggested the presence of a dimer attached from this carbon with plane of symmetry in the structure. So, Compound **5** could be identified as biphenyl-1,1,2,2′-tetrol as it was known as a synthetic product (Stevens et al. 2013) but this is the first report for its isolation from nature.

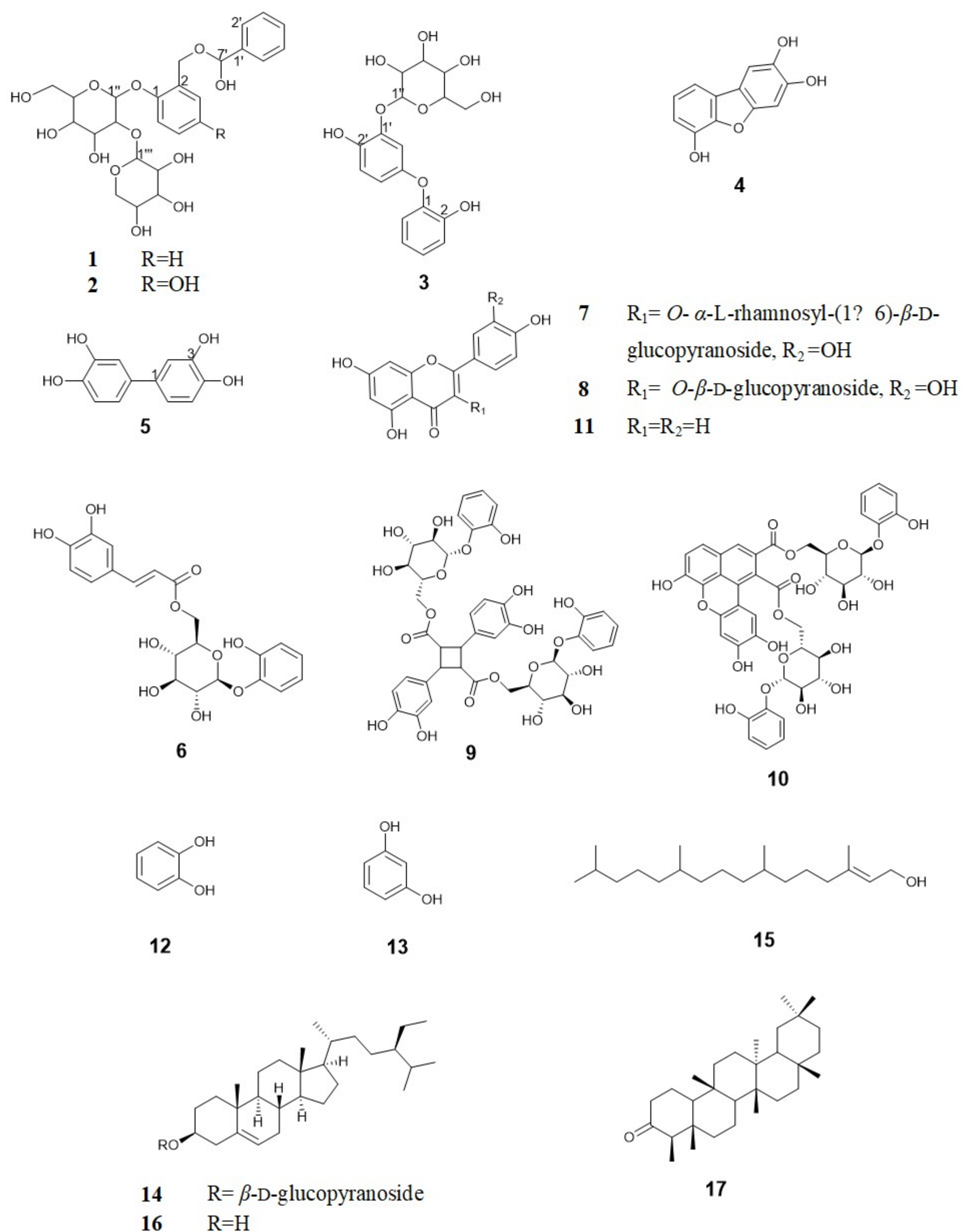


Figure1. Chemical structures of compounds (1-17) isolated from Flacourtia rukam Zoll. & Mortizi leaves and bark

Compound **5** showed a good activity against *Trypanosoma brucei* with IC_{50} = 6.66 μg/mL and IC_{90}=9.68 μg/mL. Compound **11** and **12** showed antitrypanosomal activity with IC_{50}=8.98, 8.2 μg/mL, respectively. Difluoromethyl ornithine (DFMO) was used as a positive control (IC_{50}=3.62 μg/mL and IC_{90}=8.41 μg/mL) (Table 3). On the contrary, all tested compounds were inactive as antileishmanial or antimalarial.

Anti-inflammatory activity of the total ethanolic extracts (leaves and bark) as well as some isolated compounds was conducted by proteinase inhibitory mechanism. Lysosomic neutrophils are a rich source of serine proteinase. Das and Chatterjee (Das and Chatterjee 1995) reported that leukocytes proteinase plays a key role in the development of tissue damage during inflammation as well as a significant role of protection provided by proteinase inhibitors. In the present study, compounds **2**, **5**, **9**, **10**, **11** and **12** showed a good activity with IC_{50}= 44.1, 47.5, 62.6, 79.8, 41.1 and 54.7 ug/mL, respectively compared to the positive control Aspirin® (25.14 ug/mL) as shown in Figure 2. The results confirmed the traditional anti-inflammatory use of *Flacourtia rukam* leaves. (Lim 2013)

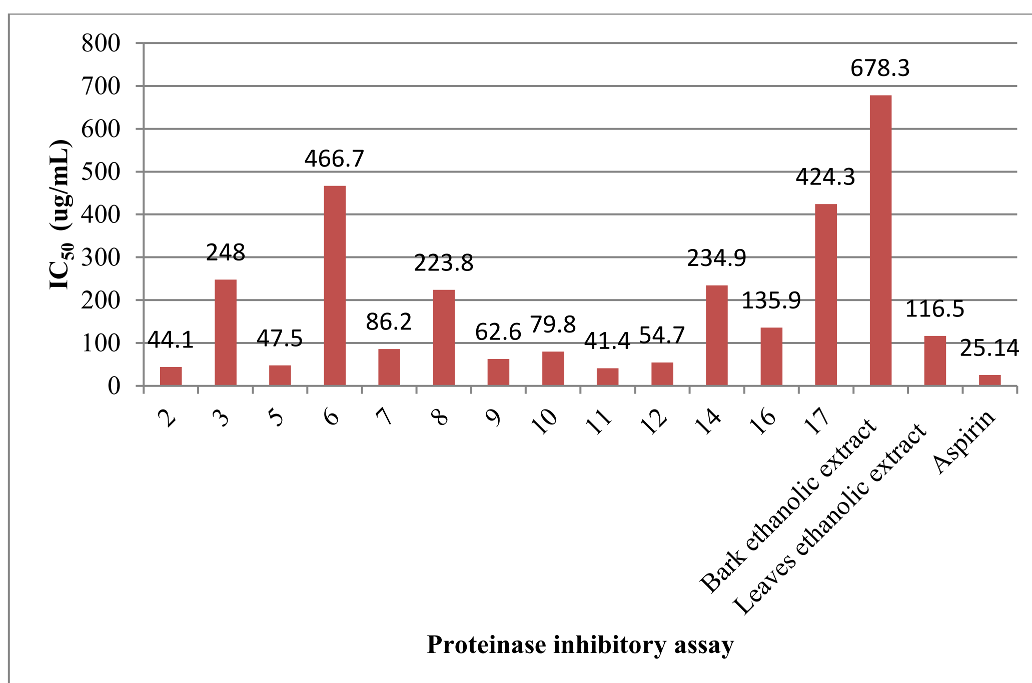


Figure 2. Proteinase inhibitory assay of *Flacourtia rukam* leaves and bark ethanolic extracts and some isolated compounds

3. Experimental

3.1. General experimental procedures

All NMR spectra were obtained on Bruker Avance III 400 MHz with BBFO Smart Probe and Bruker 400 MHz AEON Nitrogen-Free Magnet (Bruker AG, Switzerland)

operating at 400 MHz for proton and 100 MHz for carbon using the residual protons in deuterated solvent as an internal standard. 1D and 2D-NMR spectra (^{1}H, ^{13}C HSQC, HMBC, COSY and NOESY*)* were obtained using standard Bruker pulse programs. All deuterated solvents ($CDCl_3$, CD_3OD, DMSO-d_6 and pyridine-d_5) for NMR measurement were purchased from (Cambridge Isotopes, USA). The optical rotation values were determined using a Jasco P-1020 polarimeter (Easton, MD, USA). HRESIMS data were obtained using a Thermo Scientific LTQ/XL Orbitrap (Waltham, MA, USA). HPLC separations were conducted using an Agilent 1260 Infinity preparative pump (G1361A), Agilent 1260 diode array detector VL (G1315 D), Agilent 1260 Infinity thermostat and column compartment (G1361 A), and Agilent 1260 Infinity preparative autosampler (G2260A). Separation was performed on a ZORBAX SB-C18 preparative column (21.2 × 250 mm i.d, 7 mm particle size) (USA). Column chromatography was performed using silica gel 60 (63–200 µm), polyamide-6 (50–160 µm) and Sephadex LH-20 (Sigma-Aldrich, Germany). *n*-hexane, CH_2Cl_2, EtOAc, MeOH, EtOAc, and *n*-BuOHs solvents were of analytical grade. Pre-coated silica gel 60 TLC plates were purchased from Merck (Darmstadt, Germany). Visualization of the TLC plates was achieved with a UV lamp (λ_{max}254 and 365 nm) and *p*-anisaldehyde′s reagent/acid spray reagent (MeOH/HOAc/*p*-anisaldehyde/sulfuric acid, 85:10:0.5:5) (Waksmundzka-Hajnos et al. 2008).

3.2. Plant material

F. rukam leaves and bark were collected in April 2015 from the botanical garden, Aswan, Egypt. The plant was kindly identified by Dr Abd El-Halim A. Mohammed, Horticultural Research Institute, Department of Flora and Phytotaxonomy Researches, Dokki, Cairo, Egypt. Voucher specimen (BUPD-63) was deposited in Pharmacognosy Department, Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt.

3.3. Extraction and isolation

The air-dried powdered leaves (1.8 kg) and bark (250 g) were exhaustively extracted with 80% ethanol by percolation and the solvent was evaporated under reduced pressure to afford crude extracts (400 g and 30 g respectively).

3.3.1. Fractionation and chromatographic isolation of secondary metabolites from F. rukam leaves.

Three hundred grams of leaves crude extract was suspended in distilled H_2O and partitioned with solvents of different polarities (*n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH saturated with water) to afford (6, 21.8, 28.3 and, 80 g, respectively).

n-Hexane fraction of leaves (FL-NH, 5 g) was used for preparation of the unsaponifiable matter (USM). (El-Kashoury et al. 2013) USM of leaves (2.5 g) was chromatographed on silica gel column. Elution was started with *n*-hexane with 5% increments of EtOAc. Fractions (25 ml, each) were collected and monitored by TLC using hexane-EtOAc (9:1 and 8:2) and *p*-anisaldehyde′s spray reagent. Similar fractions were pooled together to give two compounds; compound **15** (5 mg, eluted with 10% EtOAc in *n*-hexane) and compound **16** (10 mg, eluted with 15% EtOAc in *n*-hexane).

The CH_2Cl_2 fraction of leaves (FL-DC, 20 g) was subjected to VLC fractionation (silica gel) and eluted with 100% CH_2Cl_2 with 10% increments of MeOH to afford two sub-fractions FL-DCI and FL-DCII. FL-DCI (200 mg, eluted with 10% MeOH in CH_2Cl_2) was chromatographed on Sephadex LH-20 column using MeOH as eluent to yield compound **11** (20 mg). FL-DCII (1.2 g, eluted with 15% MeOH in CH_2Cl_2) was rechromatographed on silica gel column (50 g, 2×54 cm) using 100% CH_2Cl_2 with 5% increments of MeOH to afford two sub-fractions (FL-DCIIa, FL-DCIIb). FL-DCIIa (600 mg, eluted with 15% MeOH in CH_2Cl_2) was chromatographed on Sephadex LH-20 column chromatography using MeOH-CH_2Cl_2 (8:2) as eluent to afford compound **12** (270 mg) and compound **13** (4 mg). FL-DCIIb (200 mg, eluted with 15-20% MeOH in CH_2Cl_2) was filtered through Sephadex LH-20 column using MeOH as eluent to obtain compound **14** (20 mg).

The EtOAc fraction (FL-EA; 28 g) was fractionated on polyamide using H_2O-MeOH gradient (in 10% increments) to obtain two main sub-fractions; FL-EA-I and FL-EA-II. FL-EA-I (10 g, eluted with H_2O-MeOH; 9:1) was chromatographed on silica gel column (100 g, 2.5×42 cm) using mixtures of CH_2Cl_2-MeOH gradient (in 10% increments) to obtain two sub-fractions. FL-EA-Ia and FL-EA-Ib.

FL-EA-Ia (1 g, eluted with 25-30% MeOH in CH_2Cl_2) was subjected to silica gel column chromatography using mixtures of CH_2Cl_2-MeOH as eluent in 5% increments to afford compound **1** (4 mg, eluted with 25-30% MeOH in CH_2Cl_2) and compound **2** (15 mg, eluted with 30% MeOH in CH_2Cl_2). FL-EA-Ib (360 mg, eluted

with 30% MeOH in CH_2Cl_2) was purified by RP-HPLC using H_2O (A)-MeOH (B) each containing 0.03% HCOOH in a gradient mode: A/B 70/30–30/70; 25 min- 30/70-00/100; 5min, a flow rate of 5 mL/min to afford compound **3** (t_R= 27 min, 12 mg). FL-EA-II (3.2 g, eluted with 30-50% MeOH in H_2O) was rechromatographed on silica gel column (150 g, 2.7 x 51 cm) using mixtures of CH_2Cl_2-MeOH as eluent in 10% increments to obtain two sub-fractions; FL-EA-IIa and FL-EA-IIb. FL-EA-IIa (600 mg, eluted with 10% MeOH in CH_2Cl_2) was chromatographed on silica gel column using mixtures of CH_2Cl_2-MeOH as eluent in 5% increments of MeOH to afford two sub-fractions, each of which was separately purified on Sephadex LH-20 column using MeOH as eluent to obtain compound **4** (4 mg) and compound **5** (10 mg,). FL-EA-IIb (360 mg, eluted with 10% MeOH in CH_2Cl_2) was rechromatographed on silica gel column using mixtures of CH_2Cl_2-MeOH as eluent in 5% increments of MeOH followed by Sephadex LH-20 column chromatography using MeOH as eluent to obtain compound **6** (20 mg).

n-BuOH fraction of the plant leaves (FL-NB; 40 g) was fractionated on polyamide using H_2O-MeOH gradient to obtain three main fractions (FL-NB-I, FL-NB-II and FL-NB-III); FL-NB-I (700 mg, eluted by 20-30% H_2O in MeOH) was purified on Sephadex LH-20 column chromatography using MeOH as eluent to obtain compound **7** (20 mg). FL-NB-II (4.5 g, eluted with 50-60% H_2O in MeOH) was chromatographed on silica gel column using mixtures of CH_2Cl_2-MeOH as eluent in 10% increments to obtain two sub-fractions; FL-NB-IIa (30 mg, eluted with 25% MeOH in CH_2Cl_2) and FL-NB-IIb (40 mg, eluted with 25% MeOH in CH_2Cl_2). Each fraction was filtered through Sephadex LH-20 column chromatography using 20% H_2O in MeOH as eluent to obtain compound **8** (12 mg) and compound **9** (15 mg). FL-NB-III (500 mg, eluted by 80% MeOH in H_2O) was chromatographed several times on Sephadex LH-20 column chromatography using MeOH as eluent to obtain compound **10** (40 mg).

3.3.2. Fractionation and chromatographic isolation of secondary metabolites from F. rukam bark.

The crude alcohol extract of the stem bark (25 g) was suspended in distilled H_2O and partitioned with solvents of different polarities (*n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH) to afford different fractions (2.5, 1, 2.5 and 11 g, respectively).

n-Hexane fraction of the stem bark (FB-NH, 2 g) was used for the preparation of the USM. Fractionation of the USM of bark (0.5 g) led to the isolation of two compounds; compound **17** (25 mg, eluted with 5% EtOAc in *n*-hexane).and compound **16** (5 mg, eluted with 15% EtOAc in *n*-hexane).

TLC investigation of the EtOAc (FB-EA) and *n*-BuOH (FB-NB) fractions with different solvent systems showed similar spots so that both fractions were combined. Portion of the combined polar fractions (2.5 g) was chromatographed several times on Sephadex LH-20 column using MeOH as eluent to afford compound **6** (7 mg).

*3.3.3. Acid hydrolysis of compounds **1-3**.*

Each compound (about 2 mg) was dissolved in 1 ml of 2 N HCl and the solution was then heated on a boiling water bath for 1 h. The hydrolyzed products were separately compared with standard sugar samples on Whatman No.3 chromatographic paper developed with *n*-BuOH-CH_3COOH-H_2O (6:4:1 v/v). The sugar spots were visualized by spraying with aniline hydrogen phthalate and heating in an oven at 110°C for 10 min. (Lu and Foo 1997)

*3.3.4. Compound **1***

White amorphous solid (MeOH); $[\propto]_D^{25}$+143 (*c* =0.12, MeOH); UV (MeOH) λ_{max} 271 nm;; ^1H and ^{13}C NMR (100 MHz, CD_3OD) spectral data are given in Table S1; HRESIMS *m/z* 523.1796 $[M+H]^+$ (calcd. for $C_{25}H_{31}O_{12}$, 523.1816).

*3.3.5. Compound **2***

White amorphous solid (MeOH); $[\propto]_D^{25}$+131 (*c* =0.13, MeOH); UV (MeOH) λ_{max} 282 nm; ^1H and ^{13}C NMR (100 MHz, CD_3OD) spectral data are given in Table S1; HRESIMS *m/z* 539.1738 $[M+H]^+$ (calcd. for $C_{25}H_{31}O_{13}$, 539.1765).

*3.3.6. Compound **3***

Needle crystals (MeOH); $[\propto]_D^{25}$ -30 (*c* =0.05, MeOH); UV (MeOH) λ_{max} 271 nm; ^1H and ^{13}C NMR(100 MHz, CD_3OD) spectral data are given in Table S2; HRESIMS *m/z* 381.1168 $[M+H]^+$ (calcd. for $C_{18}H_{21}O_9$, 381.1185).

*3.3.6. Compound **5***

White amorphous solid (MeOH); $[\propto]_D^{25}$+6 (c =0.5, MeOH); UV (MeOH) λ_{max} 271 nm; ^{1}H and ^{13}C NMR (100 MHz, CD_3OD) spectral data are given in Table S2; HRESIMS *m/z* 219.0651 $[M+H]^+$ (calcd. for $C_{12}H_{11}O_4$, 219.0657)

3.4. Biological activities

3.4.1 In-vitro antimalarial activity

The *in-vitro* antimalarial activity was determined for the isolated compounds except **1**, **9**, **10**, **13** and **17** (due to lack of quantity) against D6 (chloroquine sensitive) and W2 (chloroquine-resistant) strains of *P. falciparum,* which were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (WRAIR), Washington, DC. The assay is based on the determination of plasmodial lactate dehydrogenase (LDH) activity. (Jain et al. 2005, Mohamed et al. 2016) The IC_{50} values were obtained from the dose-response curves. Artemisinin and chloroquine were included as positive drug controls, and dimethyl sulfoxide was included as a vehicle control.

3.4.2. In-vitro antileishmanial and antitrypanosomal activities

Also, the same compounds were tested for the different activities namely, *Leishmania donovani* promastigote assay, *L. donovani* axenic amastigote assay*, L. donovani* THP1 macrophage amastigote assay*, Trypanosoma brucei* trypamastigotes assay and THP1 toxicity assay. The Alamar Blue assays as described earlier were used.(Manda et al. 2014, Rahman et al. 2011) IC_{50} and IC_{90} values were computed from the dose response curves using XLFit®.

3.4.3. In-vitro proteinase inhibitory assay

The total ethanolic extracts (leaves and bark) as well as all isolated compounds (except **1**, **4**, **13** and **15**; due to lack of quantity) were tested for *in-vitro* anti-inflammatory assay using proteinase inhibitory assay (Trypsin inhibition) according to Guo et al. (2015).(Guo et al. 2015)

Briefly, 0.06 mg trypsin (dissolved in 1 mL of 20 mM Tris-HCl buffer, pH 7.4) was mixed together with samples to a final concentration of 5, 10, 20, 50, 100, 250, 500

and 1000 µg/mL. After incubation for 5 min at 37°C, 1 mL of 0.8% azocasein (Sigma) in 20 mM $NaHCO_3$ (pH 8.1) was added to the reaction mixture at 37°C for 20 min. The reaction was stopped by adding 2 mL of 10% (w/v) trichloroacetic acid solution. The assay mixture was centrifuged at 12,000 *g* for 10 min, then equal volumes of 1.0 M NaOH and the supernatants were mixed, and absorbance was measured at 440 nm against the buffer as a blank. All determinations were carried out in triplicate manner and values are expressed as the mean±SD. The results were expressed as percentage inhibition, Aspirin was included as a standard. Percentage of inhibition and IC_{50} were calculated. Inhibitory activity (%)=(1 − As/Ac) ×100, where, As is the absorbance in the presence of test substance and Ac is the absorbance of control.

4. Conclusion

Seventeen compounds were isolated from *Flacourtia rukam* leaves and bark, four of them were new phenolics in addition to thirteen known compounds including one triterpene, two sterols, one diterpene and other nine known phenolic compounds. Biphenyl-1,1′,2,2′-tetrol exhibited a good antitrypanosomal activity and could be a good candidate as a potential antitrypanosomal drug. Moreover, *Flacourtia rukam* is a rich source of phenolics to which the anti-inflammatory effect in traditional use can be attributed.

Conflict of interest statement

Authors declare that there is no conflict of interest

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