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C-Flavonoidal Glycosides from *Erythrina caffra* Flowers

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Abstract – A phytochemical investigation of the ethanolic extract of *Erythrina caffra* flowers from an Egyptian origin yielded three *C*-flavonoidal glycosides; 5,7,4'-trihydroxyflavone-8-*C*-*β*-D-glucopyranoside (vitexin) **(1)**, 5,7,4'-trihydroxyflavone-6-*C*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranoside (isovitexin-2''-*β*-D-glucopyranoside**)** **(2)**, 5, 7, 4'-trihydroxyflavone-6, 8-di-*C*-*β*-D-glucopyranoside (vicenin-2) **(3)** and one *O*-flavonoidal glycoside; kaempferol-3-*O*-*β*-D–glucopyranosyl) (1 → 2)-*β*-D-glucopyranoside **(4)**. The structures of the isolated compounds (**1** - **4**) were elucidated using different spectral techniques (UV, 1D and 2D NMR and HRESIMS). This is the first report for the isolation of flavonoidal glycosides from *Erythrina caffra*. The antibacterial, antifungal, antimalarial, and antileishmanial activities of the isolates were evaluated. In addition, the cytotoxic activity of the ethanolic extract and the main fractions were tested using brine shrimp bioassay.
Keywords – *Erythrina caffra, Fabaceae, flavonoids, C-glycosides, O-glycoside, antimicrobial, antiprotozoal, cytotoxicity.*

Introduction

Genus *Erythrina* (Fabaceae) comprises over 110 species of orange or red flowered trees, shrubs and herbaceous plants. It is divided into 5 sub-genera and 26 sections, largely on the basis of morphology, especially the color and the shape of flowers, fruits and inflorescences. It is found throughout the tropical and sub-tropical regions of the world. There are 30 species and subspecies in tropical Africa (Fabian and Germishhuizen, 1997). *Erythrina caffra* is a tree up to 60 feet, indigenous to South Africa and cultivated in Egypt as an ornamental plant (El-Ghittany, 1978). Plants belonging to genus *Erythrina* are well reported as medicinal herbs that have long been used traditionally to treat various diseases, such as infection, cough, malaria, inflammation, bronchitis, asthma, and insomnia (Cui *et al*., 2008). Genus *Erythrina* is very rich in secondary metabolites, two major principle classes are present, including phenolic derivatives (flavonoids, pterocarpans, benzofurans) (Zaatout, 2009; Chukwujekwu *et al*. 2010) and alkaloids (Amer, 1991). In previous studies, we reported the isolation of three novel glycodienoid alkaloids (Amer *et al*., 1991) and seven prenylated flavonoid derivatives from *Erythrina lysistemon* (El-Masry *et al*., 2002). On the other hand, only few reports were published regarding the isolation of *C*-flavonoidal glycosides from genus *Erythrin*a (Nassar *et al*., 2003). Moreover, no phytochemical investigation was conducted to study the flavonoidal glycosides of *Erythrina caffra*.

According to these findings and in continuation to our investigation on genus *Erythrin*a, we attempted to study the flavonoidal content of *Erythrina caffra*. This paper reports the isolation and structural elucidation of three *C*-flavonoidal and one *O*-flavonoidal glycosides from *E. caffra* flowers, as well as their antimicrobial and antiprotzoal activities. The cytotoxicity of the ethanolic extract and the main fractions using brine shrimp bioassay is also reported.

Experimental

General experimental procedures – Melting points were determined on Stuart SMPI melting point apparatus and were uncorrected. UV spectra were determined using a Helios-α UV-Visible Thermo Spectronic, UK supported with Vision 32 software using 1 cm quartz cell. NMR spectra were recorded on Variant Pulse instruments at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR, all data were measured using DMSO-d_6. Mass spectra were measured as HRESIMS using Burker Bioapex FT Mass Spectrometer. CS Chem Draw Ultra version 5.0 program

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was used to calculate the exact molecular weight. Silica gel (230 - 400 mesh) and RP-C18 silica gel, Merck, Darmstadt, W. Germany were used for column chromatography. Precoated TLC silica gel GF-254 plates (0.25 mm thick) and silica gel for TLC GF-254 (10 - 40 μmesh), Merck, Darmstadt were used for TLC and pTLC.

Plant material – Fresh flowers of *E. caffra* were collected during June, 2005 from Alexandria (cultivation garden), Egypt. The plant was botanically authenticated by the late Prof. Nabil El-Hadidi, Department of Botany, Faculty of Science, Cairo University, Egypt and a voucher specimen (EC605) was kept at the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt.

Extraction and isolation – Fresh chopped flowers of *E. caffra* (3.5 kg.) were soaked in ethanol 70% at room temperature. The ethanolic extract was concentrated under reduced pressure at 60 °C to give a viscous residue (150 g). This residue was subjected to hydroalcoholic fractionation using chloroform, ethyl acetate and butanol.

The dry ethyl acetate fraction (2.7 g) was chromatographed over a silica gel column (70 g, *Φ*2.5 cm). Elution was carried out using mixtures of different proportions of (ethyl acetate: methanol: water). Fractions eluted with ethyl acetate: methanol: water (180 : 5 : 4) (490 mg) were purified by crystallization and washing several times with hot methanol to yield 30 mg of compound **1**. Fractions eluted with ethyl acetate: methanol: water (60 : 5 : 4) (50 mg) was subjected to pTLC on fluorescent silica gel plates using ethyl acetate: methanol: water (30 : 5 : 4) as a developing system. The zones were visualized under UV light and using ammonia vapor. The major zone (R_f = 0.38) afforded 19 mg of compound **4**.

The dry butanol fraction (21 g) was subjected to silica gel column (600 g, *Φ*5 cm). Elution was performed using ethyl acetate, with gradual increase of methanol. Two hundred milligrams of the dry fractions eluted with 40% methanol in ethyl acetate were subjected to RP-C18 silica gel column (20 gm, *Φ*1 cm). Elution was performed isocratically using 30% methanol in water. The collected fractions yielded after crystallization from methanol 16 mg of compound **2** and 28 mg of compound **3**.

Compound 1 – m.p: 272 - 273 °C, UV λ_{max}, nm (MeOH): 270, 332; (MeOH + NaOMe) 278, 300 (sh), 352; (MeOH + $AlCl_3$) 277, 305, 349, 385; (MeOH + $AlCl_3$ + HCl) 278, 303, 342, 385; (MeOH + NaOAc) 279, 302 (sh), 388; ^{1}H and ^{13}C NMR: see Tables 1 & 2. HRESIMS (Positive-ion mode) m/z 887.1963 $[2M + Na]^+$ (calculated for 887.2011), (Negative-ion mode) *m/z* 431.1055 $[M-H]^-$ (calculated for 431.0978) and 863.2147 $[2M-H]^-$ (calculated for 863.2035); molecular formula $C_{21}H_{20}O_{10}$.

Compound 2 – m.p: 205 °C, UV λ_{max}, nm (MeOH): 273, 333; (MeOH + NaOMe) 283, 302 (sh), 386; (MeOH + $AlCl_3$) 280, 306, 349, 386; (MeOH + $AlCl_3$ + HCl) 280, 305, 344, 382; (MeOH + NaOAc) 283, 392; ^{1}H and ^{13}C NMR: see Tables 1 & 2. HRESIMS (Positive-ion mode) m/z 595.1596 $[M + H]^+$ (calculated for 595.1663), (Negative-ion mode) m/z 593.1528 $[M-H]^-$ (calculated for 593.1507); molecular formula $C_{27}H_{30}O_{15}$.

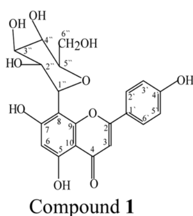
Compound 3 – m.p: > 300 °C, UV λ_{max}, nm (MeOH): 273, 333; (MeOH + NaOMe) 284, 302 (sh), 386; (MeOH + $AlCl_3$) 282, 306, 349, 386; (MeOH + $AlCl_3$ + HCl) 282, 306, 345, 383; (MeOH + NaOAc) 283, 392; ^{1}H and ^{13}C NMR: see Tables 1 & 2. HRESIMS (Positive-ion mode) *m/z* 617.1423 $[M + Na]^+$ (calculated for 617.1482), 1211.2953 $[2M + Na]^+$ (calculated for 1211.3067); molecular formula $C_{27}H_{30}O_{15}$.

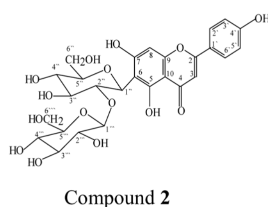
Compound 4 – UV λ_{max}, nm (MeOH): 268, 297 (sh), 346; (MeOH + NaOMe) 273, 302 (sh), 360; (MeOH + $AlCl_3$) 276, 304, 351, 388; (MeOH + $AlCl_3$ + HCl) 276, 302 (sh), 347, 386; (MeOH + NaOAc) 275, 305 (sh), 376; ^{1}H and ^{13}C NMR: see Tables 1 & 2.

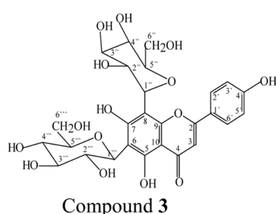
Results and Discussion

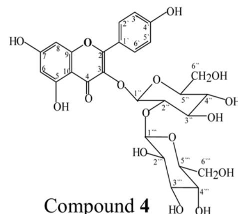
Compound **1** was isolated as yellow crystals. Its UV spectral data in MeOH, $AlCl_3$ and $AlCl_3$/HCl indicated that it had a flavone skeleton with a free hydroxyl group at C-5 and no *ortho*-dihydroxyl groups (Mabry *et al.*, 1970). The bathochromic shifts of Band II with NaOAc (9 nm) and Band I with NaOMe (20 nm) were indicative for the presence of two free hydroxyl groups at C-7 and C-4', respectively. As a result, compound **1** had a 5, 7, 4'-trihydroxyflavone skeleton. HRESIMS (positive-ion and negative-ion modes) of compound **1** were in consistent with the molecular formula $C_{21}H_{20}O_{10}$. ^{1}H NMR spectrum of compound **1** showed resonances for two sets of doublets at δ_H 8.00 and 6.89 assigned to H-2', 6' and H-3',5', respectively. In addition, two singlets at δ_H 6.75 and 6.24 were assigned to H-3 and H-6, respectively. These data indicated that the aglycone moiety was analogous to apigenin except for the absence of one proton in ring A. The presence of 19 carbon signals corresponding to 21 carbon atoms in ^{13}C NMR spectrum illustrated the presence of a flavonoid nucleus with one sugar moiety. The presence of an anomeric proton at δ_H 4.67 attached to carbon at δ_C 73.9 in the HMQC spectrum suggested that compound **1** is *C*-glucoside flavone (Agrawal, 1989; Kumarasamy *et al.*, 2004) The site of the sugar linkage to the aglycone was considered to be at C-8, due to the

presence of a quaternary C-8 signal at δ_C 104.5 and a protonated C-6 signal at δ_C 98.6 (Fernandez *et al*., 1989). Comparing the spectroscopic data of compound **1** with the reported ones (Kumarasamy *et al*., 2004; Zhang and Xu, 2003) confirmed its structure to be 5,7,4'-trihydroxy-flavone-8-*C*-*β*-D-glucopyranoside, commonly known as vitexin. It was previously isolated from *E. crista-galli* (Keh-Feng and Ling-Erl 1997) and *E. indica* (Nassar *et al*., 2003), but this is the first time to be reported and isolated from *E. caffra*.


Compound **1**


Compound **2**


Compound **3**


Compound **4**

Compound **2** was isolated as yellow crystals. Its UV spectral data illustrated the presence of free hydroxyl groups at 5, 7, 4'-positions. The negative-ion mode HRESMS of compound **2** showed a major ion peak at *m/z* 593.1528 $[M-H]^-$ corresponding to the molecular formula $C_{27}H_{30}O_{15}$. NMR spectroscopic data of compound **2** revealed a pattern characteristic for 5, 6, 7, 4'-tetrasub-stituted flavone and indicated the presence of two sugar moieties. The two sugar moieties were confirmed to be attached at C-6 from DEPT spectrum, free C-8 (δ_C 90.4) (Agrawal, 1989) and from the lack of aromatic proton signal for H-6 in ^{1}H NMR spectrum. This was further confirmed from the positive result of Gibb's reagent (King *et al*., 1957). Two anomeric carbons were observed at δ_C 72.2 and 104.2, indicating that one sugar moiety was *C*-glucosyl and the other one was *O*-glucosyl. The large coupling constants of the anomeric protons suggested that both sugars were in the *β*-form (Haribal and Renwick 1998). The inter glycosidic linkage was deduced based on comparing the spectral data with the reported ones (Kumarasamy *et al*., 2004). Final unambiguous assignments and structure elucidation were deduced from 2D NMR (COSY, HMQC and HMBC correlations).

The sugar moiety obtained after acid hydrolysis of compound **2** was identified as D-glucose by TLC comparison with reference samples using solvent system chloroform : methanol (6 : 4). Accordingly,compound **2** was identified as 5,7,4'-trihydroxyflavone-6-*C*-*β*-D-gluco-pyranosyl-(1 → 2)-*β*-D-glucopyranoside (Hilsenbeck and Mabry 1990; Cheng *et al*., 2000) known as isovitexin-2"-*β*-D-glucopyranoside. This is the first time to isolate compound **2** from genus *Erythrina*.

Compound **3** was isolated as white needles. Its UV spectral data illustrated the presence of free hydroxyl groups at 5, 7, 4'-positions. ^{1}H NMR spectrum of compound **3** showed one aromatic singlet at δ_H 6.78, assigned to H-3, indicating that the aglycone moiety had substituted C-6 and C-8. The carbon signals of the two sugar moieties in DEPT spectrum, suggested that compound **3** is *C*-diglucoside flavone.

The sites of the sugar linkage to the aglycone were considered to be at C-6 and C-8 positions, since the C-8 signal appeared at δ_C 105.5 and C-6 signal at δ_C 110.0 (Agrawal, 1989) This was further confirmed from the absence of H-6 and H-8 in ^{1}H NMR spectrum. Comparing the spectral data of compound **3** with the reported ones (Agrawal, 1989; Velozo *et al*., 2009) confirmed its structure to be 5,7,4'-trihydroxyflavone-6,8–di–*C*–*β*–D–gluco-pyranoside known as vicenin-2. It was previously isolated from the *E. indica* (Nassar *et al*., 2003) but this is the first report of its isolation from this plant.

Compound **4** was isolated as bright yellow residue. Its UV spectral data suggested a 5, 7, 4'-trihydroxy substituted flavonol skeleton. ^{1}H NMR spectrum of compound **4** indicated a mono- substituted ring B and disubstituted ring A. The absence of the proton singlet of position 3 indicated that compound **4** is 3-substituted kaempferol. Hydrolysis of compound **4** with 2N HCl yielded a flavonol, identical to kaempferol reference sample and a sugar identified by co-chromatography using solvent system chloroform : methanol (6 : 4) as D-glucose. The presence of 25 carbon signals in ^{13}C NMR spectrum indicated the presence of a flavonoidal nucleus and two sugar moieties. The carbon signals of the two sugar moieties in the ^{13}C NMR together with HMQC spectrum suggested that compound **4** is a diglucoside flavonol. The site of the sugar linkage to the aglycone was considered to be at C-3 position, since the hydroxyl group at C-3 is blocked as indicated from the UV spectral data (Mabry *et al*., 1970). The downfield shift of C-3 confirmed the structure to be 3-*O*-kaempferol diglucoside (Agrawal, 1989). The interglycosidic linkage was deduced from the down-field shift of C-2" accompanied by upfield shift of C-1" which inferred the interglycosidic linkage to be glucosyl

Table 1. ^{1}H NMR spectral data, δ_H [mult., J(Hz)] of compounds **1 - 4** (DMSO-d_6)

	1	2	3	4
3	6.75 [1H, s]	6.57 [1H, s]	6.78 [1H, s]	----------
6	6.24 [1H, s]	-----------	-----------	6.08 [IH, s]
8	-----------	6.48 [1H, s]	-----------	6.23 [IH, s]
2', 6'	8.00 [2H, d(8)]	7.67[2H,d(8.4)]	7.94[2H,d(8.4)]	8.04[2H,d(8.4)]
3', 5'	6.89 [2H, d(8.8)]	6.92[2H,d(8.4)]	6.90[2H,d(8.4)]	6.87[2H,d(8.4)]
1''	4.67 [1H, d(8.7)]	4.25[1H,d(6.4)]	4.82[1H,d(9)]	5.21 [1H, d(7)]
2''	3.81 [1H, t(9.2)]	4.09 [1H, br d]		
3''	3.21 [1H]			
4''	3.30 [1H]			
5''	3.20 [1H]	3.15-4.05	3.28-3.85	3.20-3.90
6''	3.52[1H,brd(14)] 3.75[1H,brd(15)]			
1'''	----------	4.82[1H,d(6.6)]	4.74 [1H, d(8)]	4.76 [1H, d(7)]
2'''	----------			
3'''	----------			
4'''	----------			
5'''	----------	3.15-4.05	3.28-3.85	3.20-3.90
6'''	----------			

Table 2: ^{13}C NMR spectral data of compounds **1 - 4** (DMSO-d_6)

	1	2	3	4
2	164.5	167.7	164.0	160.5
3	102.9	101.0	103.0	133.5
4	182.6	184.4	181.1	177.6
5	161.7	162.0	161.8	157.1
6	98.6	107.0	110.0	100.1
7	163.1	162.0	161.8	160.5
8	104.5	90.4	105.5	95.0
9	156.5	155.2	155.1	157.1
10	105.1	103.5	106.2	103.9
1'	122.1	122.5	122.2	121.4
2', 6'	129.5	129.3	129.6	131.0
3', 5'	116.3	116.6	115.8	115.0
4'	160.9	159.0	160.0	157.6
1''	73.9	72.2	73.9	100.2
2''	71.3	82.1	71.3	81.2
3''	79.1	77.9	79.6	76.9
4''	71.0	70.6	69.2	69.5
5''	82.3	81.0	81.4	77.5
6''	61.8	61.5	61.5	61.1
1'''	-------	104.2	74.9	103.5
2'''	-------	73.0	72.5	77.7
3'''	-------	76.0	79.6	77.4
4'''	------	68.5	69.4	69.9
5'''	------	75.0	82.5	74.2
6'''	-----	60.1	61.7	62.9

(1''' → 2'') glucoside. Comparing the spectral data of compound **4** with the reported one, deduced its structure to be kaempferol-3-*O*-*β*-D–gluco-pyranosyl (12)–*β*–D-glucopyranoside commonly known as kaempferol-3-*O*-*β*-D-sophoroside (Xu *et al*., 2005). This is the first time to report and isolate this compound from genus *Erythrina*.

Antifungal, Antibacterial, Antileishmanial, Antimalarial and Cytotoxic activities – The isolated compounds were evaluated *in vitro* for antifungal (*Candida albicans* ATCC 90028, *Candida krusei* ATCC 6258, and *Aspergillus fumigat* ATCC 90906) and antibacterial (*Staphylococcus aureus* ATCC 29213, *Methicillin Resistant Staphylococcus aureus* ATCC 33591, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853 and *Mycobacterium intracellulare* ATCC 23068) activities at conc. 0.8, 4.0 and 20 µg/ml.

These compounds were also tested for antileishmanial (culture of *Leishmania donovani*), antimalarial ((*Plasmodium falciparum* (D6 clone) and *Plasmodium falciparum* (W2 clone)) activities and cytotoxicity against Vero cells (African green monkey kidney fibroblast) at conc. 4.760, 1.587 and 0.5288 µg/ml.

The detailed procedures were previously described (Bharate *et al*., 2007; Ross *et al*., 2008). Unfortunately all the isolated compounds did not show any activity at the tested concentrations.

The brine shrimp lethality bioassay is an effective and rapid method to screen cytotoxic activity. It is a useful bench-top tool (Meyer *et al*., 1982; Anderson *et al*., 1991).

Table 3. Brine shrimp bioassay results (LC_{50} mg/ml)

	Ethanolic extract	Butanol fraction	Ethyl acetate fraction	Chloroform fraction	Fraction rich in compounds **2** and **3**
LC_{50}	0.404	21.177	0.095	0.054	0.105

The natural mortalities of the shrimps were determined in blank artificial sea water and in wells treated with solvent only. Survivors are counted after 24 hours and the percentage of deaths at each dose was recorded. The percent mortalities were corrected for the natural mortalities and plotted against the logarithm of the dose. The data were used to determine the general toxicity and the LC_{50} (mg/ml) of the plant extract and the main fractions for potencies comparison. Caffeine (LC_{50} = 0.306 mg/ml (Meyer *et al*., 1982) purchased from Fluka was used as a positive control and ethanol was used as a solvent and as a negative control in the bioassay test. The total ethanolic extract, butanol, ethyl acetate and chloroform fractions, together with the fraction rich in compounds **2** and **3** were tested in three concentration each (0.1, 1.0 and 10 mg/ml). All of them showed significant cytotoxic activity (LC_{50} <1 mg/ml) (Anderson *et al*., 1991) except the butanol fraction.

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