Polyphenolic Compounds from Flowers of *Hibiscus rosa-sinensis* Linn. and their Inhibitory Effect on Alkaline Phosphatase Enzyme Activity *in vitro*

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Z. Naturforsch. **66 c**, 453–459 (2011); received January 4/July 14, 2011

Graded concentrations (0.1–100 mg/mL reaction mixture) of the methanolic extract of the flowers of *Hibiscus rosa-sinensis* Linn., its water-soluble fraction as well as compounds isolated from this fraction were tested for their inhibitory effect on alkaline phosphatase enzyme activity *in vitro*. Both the methanolic extract and its water-soluble fraction showed significant inhibitory effects on the enzyme activity *in vitro*. On screening the activity of the compounds isolated from the water-soluble fraction, its high inhibitory activity was attributed to the presence of quercetin-7-*O*-galactoside which showed a high potent inhibition of the enzyme activity reaching 100% at 100 mg/mL reaction mixture. Phytochemical investigations of the water-soluble fraction were also carried out and afforded ten polyphenolic compounds including two new natural compounds, namely kaempferol-7-*O*-[6''-*O*-*p*-hydroxybenzoyl-*β*-D-glucosyl-(1→6)-*β*-D-glucopyranoside] and scutellarein-6-*O*-*α*-L-rhamnopyranoside-8-*C*-*β*-D-glucopyranoside). The chemical structure of the isolated compounds was elucidated on the basis of chemical and spectral data.

Key words: Hibiscus rosa-sinensis Linn., Alkaline Phosphatase Inhibition, Novel Flavonoids

Introduction

Contraception, birth control, has always been and will remain an irreplaceable solution for many social and individual problems whereby medicinal plants have been used safely by women of rural communities to prevent conception. Ancient Indian Ayurvedic, Mediterranean, and Mexican literature mention the use of plants for fertility regulation as well as for the production of antifertility drugs; one of the famous local contraceptive agents is *Hibiscus rosa-sinensis* Linn. (Malvaceae). The antifertility activity of the flowers of the Egyptian variety of this species was studied earlier by us, whereby the methanolic extract showed 100% anti-implantation activity associated with luteolysis in the ovaries and complete inhibition of the activity of alkaline phosphatase in the uteri of treated rats at pregnancy day 10 (Hifnawy *et al.*, 2008a). Biologically guided fractionation of the methanolic extract into three fractions (ethyl acetate, *n*-butanol, and water-soluble fractions) revealed that the water-soluble fraction is the biologically most active fraction among the three tested fractions [60% anti-implantation activity with complete inhibition of the alkaline phosphatase activity in the uteri of non-pregnant rats at pregnancy day 10, luteal cell degeneration in the ovaries in addition to embryotoxicity in pregnant rats (Hifnawy *et al.*, 2008b)]. Complete inhibition of implantation is correlated with complete inhibition of the activity of alkaline phosphatase whose role in pregnancy was discussed previously. Hence, our aim here was to test for the first time the direct inhibitory effect of the biologically active methanolic extract and its water-soluble fraction on the activity of the alkaline phosphatase enzyme *in vitro* and to study the relation between the complete inhibition of implantation on the one hand and of the activity of alkaline phosphatase on the other hand. In addition, as a part of our continuing efforts to discover naturally occurring new inhibitory agents, we phytochemically and bi-

ologically investigated the water-soluble fraction of the methanolic extract which led to the identification of ten polyphenolic compounds, namely vitexin, quercetin-7-*O*-galactoside, gallic acid, *p*-hydroxybenzoic acid, neochlorogenic acid, and the aglycones apigenin, quercetin, and kaempferol, together with two new compounds, kaempferol-7-*O*-[6'''-*O*-*p*-hydroxybenzoyl-*β*-D-glucosyl-(1→6)-*β*-D-glucopyranoside] and scutellarein-6-*O*-*α*-L-rhamnopyranoside-8-*C*-*β*-D-glucopyranoside. Of these compounds, quercetin-7-*O*-galactoside was the most potent inhibitor of the enzyme activity.

Material and Methods

General experimental procedures

p-Nitrophenyl phosphate (Fluka, Buchs, Switzerland), calcium phytate (Sigma-Aldrich, St. Louis, MO, USA), Tris-HCl buffer, pH 8.5, Shel-Lab incubator model 1545 (Sheldon Labs, Cornelius, OR, USA), MLW T54 centrifuge (Benchtop, Buch, Germany), Petri dishes, Cecil CE595 double beam digital UV spectrophotometer (Cecil Instruments Ltd, Cambridge, UK) for determination of alkaline phosphatase activity (Gomori, 1955).

^{1}H (500 MHz) and ^{13}C (125 MHz) NMR: Jeol spectrometer (Kyoto, Japan) in DMSO-d_6; UV: Shimadzu spectrophotometer model UV-240 (Kyoto, Japan); IR: Shimadzu-IR-435 infrared spectrophotometer; CC: Polyamide 6S (Riedel-De Haen, Hannover, Germany) and Sephadex LH-20 (Fluka); PC: Whatman No. 1 and 3MM paper using the following solvent systems: (1) BAW (*n*-BuOH/HOAc/H_2O, 4:1:5); (2) H_2O; and (3) AcOH/H_2O (15:85).

Plant material

Flowers of *Hibiscus rosa-sinensis* Linn. were collected from the field (Experimental and Research Station of the Faculty of Pharmacy, Cairo University, Cairo, Egypt) in spring 2004 and dried in the shade. The plants were authenticated by Dr. Tarek Abdel-Salam, Professor of Botany, Department of Botany, Faculty of Science, Cairo University, Cairo, Egypt, and voucher specimens deposited in the department for future reference.

Extraction, fractionation, and isolation

The shade-dried flowers of the plant (500 g) were coarsely powdered and extracted by successive maceration with petroleum ether (60–80 °C), dichloromethane, and methanol in a Soxhlet extractor at room temperature till exhaustion (5 L each). The extracts were concentrated to dryness under reduced pressure and controlled temperature (40 °C). The respective yields of the petroleum ether (60–80 °C), dichloromethane, and methanol extracts were found to be 4.3 g, 5.1 g, and 56.2 g, respectively.

Forty g of the methanolic crude extract were suspended in 100 mL distilled water and successively extracted in a separating funnel with ethyl acetate and *n*-butanol (3 L each) at room temperature till exhaustion, leaving a residual water-soluble fraction. The three fractions were then dried under reduced pressure at 40 °C; their respective yields were 4.2 g, 3.8 g, and 31.4 g.

The concentrated water-soluble fraction (25 g) of the methanolic extract of *H. rosa-sinensis* flowers was subjected to Sephadex LH-20 column chromatography (500 g, 40 x 1250 mm) and eluted with water followed by different ratios of water/ethanol (1 L each eluent) to give rise to five fractions which were further purified by a series of fractionations on a Sephadex LH-20 column and preparative paper chromatography to afford ten compounds. Fraction I (20% EtOH as eluent, 4.2 g) was resubjected to Sephadex LH-20 column chromatography (150 g, 25 x 500 mm) and eluted stepwise with methanol/H_2O [1:1 (v/v), 1 L] to give compound **1** (150 mg) and the new natural compound **2** (200 mg). Fraction II (40% EtOH as eluent, 5 g) was separated into two compounds by paper chromatography on Whatman 3MM paper using BAW as solvent, which were further purified and crystallized to afford the new natural compounds **3** (150 mg) and **4** (300 mg). Two pure natural compounds of phenolic nature, **5** (150 mg) and **6** (100 mg), were obtained after applying the third fraction (60% EtOH as eluent, 3.35 g) to a Sephadex LH-20 column (100 g, 19 x 250 mm) eluted with ethanol (500 mL each eluent). Fractionation of the material in fraction IV (80% EtOH as eluent, 2 g) by paper chromatography on Whatman 3MM paper with the solvent system BAW led to the separation of two compounds which were further purified and crystallized from aqueous ethanol to give pure crystals of **7** (80 mg) and **8** (50 mg). Finally, the last fraction (absolute EtOH as eluent, 3.2 g) was subjected to a Sephadex LH-20 column (150 g, 25 x 500 mm) eluted with *n*-butanol/water [1:1 (v/v) upper layer, 1 L]

to afford the two flavonoid aglycones **9** (100 mg) and **10** (150 mg).

*Scutellarein-6-O-α-L-rhamnopyranoside-8-C-β-D-glucopyranoside (***2***):* R_f values x 100: 30 (1), 58 (3), 85 (2). – UV: λ_{max} (MeOH) = 272, 330; (+NaOMe) 282, 327 sh, 398; (+NaOAc) 278, 302 sh, 385; (+NaOAc/H_3BO_3) 275, 322 sh, 340; (+$AlCl_3$) 280, 304 sh, 350, 390 sh; (+$AlCl_3$/HCl) 278, 304 sh, 341, 382 nm sh. – ^{1}H NMR: δ (ppm) = 8.05 (d, J = 8.0 Hz, H-2', 6'), 6.89 (d, J = 8.0 Hz, H-3', 5'), 6.75 (s, H-3), 5.05 (d, J = 2 Hz, H-1"), 4.73 (d, J = 8.5 Hz, H-1'"), 4.12 (dd, J = 5, 2 Hz, H-2" of rhamnose), 3.84 (m, H-2'"), 3.73–3.50 (m, H-6'"a, 6'"b), 3.45 (dd, J = 10, 2 Hz, H-3"), 3.36 (m, H-4'"), 3.30 (m, H-3'"), 3.27 (m, H-5'"), 3.2 (t, J = 10 Hz, H-4"), 3.15 (m, H-5"), 0.85 (d, J = 6 Hz, rhamnose methyl protons), 3.2–3.9 (m, rest of sugar protons). – ^{13}C NMR: δ (ppm) = 164.25 (C-2), 102.58 (C-3), 182.35 (C-4), 158.38 (C-5), 131.4 (C-6), 160.21 (C-7), 104.7 (C-8), 152.91 (C-9), 104.09 (C-10), 121.55 (C-1'), 129.26 (C-2'), 116.20 (C-3'), 162.08 (C-4'), 116.20 (C-5'), 129.26 (C-6'); *6-O-rhamnoside:* 99.8 (C-1"), 70.6 (C-2"), 70.4 (C-3"), 72.2 (C-4"), 68.6 (C-5"), 17.9 (C-6"); *8-C-glucoside:* 74.0 (C-1'"), 71.4 (C-2'"), 78.8 (C-3'"), 70.5 (C-4'"), 81.8 (C-5'"), 60.5 (C-6'").

Kaempferol-7-O-[6'"-O-p-hydroxybenzoyl-β-D-glucosyl-(1→6)-β-D-glucopyranoside] (**3**): R_f values x 100: 33 (1), 14 (3), 10 (2). – UV: λ_{max} (MeOH) = 259, 270, 330 sh, 364; (+NaOMe) 268, 300 sh, 370 sh, 425 (dec.); (+NaOAc) 258, 335 sh, 375; (+NaOAc/H_3BO_3) 258, 330 sh, 372; (+$AlCl_3$) 235, 269, 365, 420; (+$AlCl_3$/HCl) 262, 360, 418 nm. – ^{1}H NMR: δ (ppm) = 8.14 (d, J = 8.5 Hz, H-2', 6'), 6.92 (d, J = 8.5 Hz, H-3', 5'), 6.77 (d, J = 2.0 Hz, H-8), 6.38 (d, J = 2.0 Hz, H-6), 5.20 (d, J = 7.5 Hz, H-1"), 4.92 (d, J = 7.5 Hz, H-1'"), 4.1, 3.92 (br.d, J = 11.6 Hz, H-6"a, 6"b), 3.98, 3.72 (br.d, J = 8.5 Hz, H-6'"a, 6'"b), 3.68 (m, H-5'"), 3.61–3.63 (m, H-4", H-5"), 3.55 (m, H-4'"), 3.54 (dd, J = 9.1, 7.1 Hz, H-3"), 3.52 (d, J = 7.1 Hz, H-2"), 3.51 (d, J = 7.1 Hz, H-2'"), 3.49 (m, H-3'"), 7.71 (d, J = 8.5 Hz, H-2"", 6""), 6.87 (d, J = 8.5 Hz, H-3"", 5""). – ^{13}C NMR: δ (ppm) = 147.64 (C-2), 136.01 (C-3), 177.6 (C-4), 160.35 (C-5), 98.92 (C-6), 162.7 (C-7), 94.42 (C-8), 155.81 (C-9), 104.71 (C-10), 121.55 (C-1'); *moieties:* 99.81 (C-1"), 73.14 (C-2"), 76.3 (C-3"), 69.43 (C-4"), 75.47 (C-5"), 62.9 (C-6"), 103.57 (C-1'"), 73.51 (C-2'"), 76.73 (C-3'"), 70.15 (C-4'"), 77.0 (C-5'"), 63.4 (C-6'"); *p-hydroxybenzoyl moiety:* 124.2 (C-1""), 133.4 (C-2""), 116.08 (C-3""), 164.05 (C-4""), 116.08 (C-5""), 133.4 (C-6""), 169.0 (CO).

Inhibition assay of alkaline phosphatase enzyme activity in vitro

Sample collection from soil (Gomori, 1955)

Samples were collected from agricultural soil at 2–4 cm depth near the roots of peas, which are rich in phytin and related compounds, in the cities of Kaliobeyah and Zagazig, Egypt. Ten mL of saline solution were added to 1 g of soil sample; the mixture was shaken well and left standing for 20–30 min. The soil suspension was further diluted to 10^{-4}–10^{-6}.

Screening of soil samples for bacteria producing phosphatases (Yoon *et al.*, 1996)

The diluted soil samples were spread on solid ISP medium consisting of (g/L): malt extract (10), yeast extract (4), glucose (4), calcium phytate (5), and agar (20) (initial pH adjusted to 9.0). The plates were incubated at 30 °C for 2 d (Shel-Lab incubator model 1545). The colonies with clear zones around them were considered as potential phosphatase producers.

Isolation and taxonomic characterization of the bacterial strain producing alkaline phosphatase (Yoon *et al.*, 1996)

Among the bacterial phosphatase-producing strains, the isolate AP5 showed the highest alkaline phosphatase activity. The bacteria were motile, Gram-positive, rod-shaped, spore-forming, 0.8–1.2 μm in width and 2–4 μm in length when measured under a transmission electron microscope, suggesting a *Bacillus* strain that was named *Bacillus* sp. AP5.

Cultivation of the bacterial strain producing alkaline phosphatase (Kim *et al.*, 1998)

The bacterial strain producing alkaline phosphatase was cultivated on liquid medium whose composition was: 0.5% peptone, 0.2% glucose, 0.2 mM $CaCl_2$, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 1 mM $MgSO_4$, and 0.004 mM $ZnCl_2$. Na_3PO_4 and $CaCl_2$ were added in the concentration range 0–200 μM and 0–50 mM, respectively, to the basal medium to study the regulation of phosphatase production. The suspension was centrifuged for 5 min at 1,400 x *g* (MLW T54 centrifuge) to sediment the cells, and alkaline phosphatase activity was measured in the supernatant.

Enzyme assay conditions (Kim *et al.*, 1998)

Alkaline phosphatase activity was measured spectrophotometrically by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate (*p*NPP) at 405 nm (Cecil CE595 double beam digital UV spectrophotometer). One mL of reaction assay mixture was composed of 100 μL enzyme solution, 900 μL substrate in 200 mM Tris-HCl buffer (pH 8.5) (Krämer and Green, 2000), 5 mM $CaCl_2$, and 500 μmol *p*NPP. The mixture was incubated at 37 °C for 30 min and the reaction terminated by addition of 50 μL of 4 M NaOH. One unit of phosphatase is the amount, which hydrolyses 1 μmol/mL of substrate per min.

Determination of the effect of the biologically active extracts of Hibiscus rosa-sinensis

H. rosa-sinensis extracts or fractions were added at 0.1, 1, 10, 100 mg/mL to the reaction mixture in six replicates per concentration. Statistical analysis of the results was carried out using Student's unpaired test (Armitage and Berry, 1987). Then the percentage inhibition or activation of the enzyme was calculated relative to a control without inhibitor according to the following equation: inhibition (%) = $(MA_B - MA_T)/MA_B \cdot 100$, where MA_B is the mean absorbance of the blank sample and MA_T is the mean absorbance of the test sample.

Determination of the effect of the compounds isolated from the water-soluble fraction on alkaline phosphatase activity

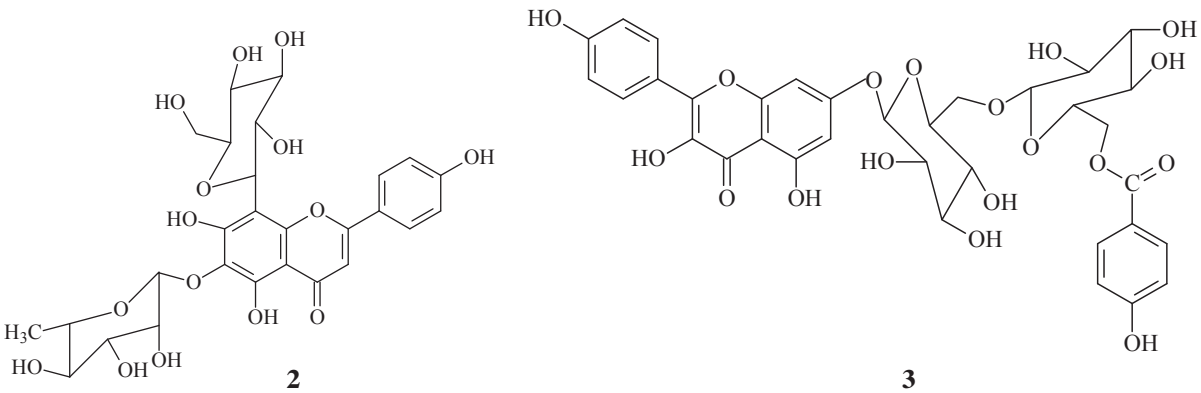
The determination was done as described above.

Results and Discussion

In the course of our screening programme for fertility regulators from natural sources, we found that the water-soluble fraction of the methanolic extract of the flowers of *H. rosa-sinensis* exhibited the highest anti-implantation activity and strongly inhibited the alkaline phosphatase activity *in vitro*. So, this fraction was subjected to phytochemical investigations followed by testing the *in vitro* effect of the isolated compounds on the alkaline phosphatase activity in an attempt to discover the biologically active ingredients of this fraction. Ten polyphenolic compounds, namely vitexin (**1**), quercetin-7-*O*-galactoside (**4**), neochlorogenic acid (**5**), *p*-hydroxybenzoic acid (**6**), gallic acid (**7**), apigenin (**8**), kaempferol (**9**), quercetin (**10**), together with the two new compounds scutellarein-6-*O*-α-L-rhamnopyranoside-8-*C*-β-D-glucopyranoside (**2**) and kaempferol-7-*O*-[6'''-*O*-*p*-hydroxybenzoyl-β-D-glucosyl-(1→6)-β-D-glucopyranoside] (**3**), were isolated from this fraction. The chemical structures of compounds **1**, **4**–**10** were previously reported (Markham and Chari, 1982; Sikorska and Matlawska, 2000; Guvenalp and Demirezer, 2005; Mabry *et al.*, 1970; Smith, 1960; Harborne, 1973). The new natural compounds **2** and **3** gave a positive response to $FeCl_3$ and Mg-HCl tests and showed absorption bands for a glycosidic linkage at 1050 cm^{-1} (glycosidic C-O) in their IR spectra, indicating a flavonoid glycoside.

Compound **2** obtained as yellow prisms (m.p. 185 °C) had a UV spectrum in methanol and in different diagnostic reagents that was characteristic for 6,8-disubstituted flavones (Mabry *et al.*, 1970; Markham, 1982). The sugar rhamnose was obtained on complete acid hydrolysis of **2** giving rise to the intermediate **2a** whose R_f values, colour reactions, and UV spectral data were similar to those of **2**. The intermediate **2a** did not change upon acid treatment but the aglycone scutellarein (Greenham *et al.*, 2003) resulted when **2a** was subjected to periodate oxidation (Hörhammer *et al.*, 1965) in addition to the sugar glucose (paper cochromatography), proving **2a** to be scutellarein-8-*C*-β-D-glucopyranoside and consequently suggesting the substitution of the 8-position of **2** by a *C*-glucosyl moiety.

Final confirmation of the chemical structure of **2** as scutellarein-6-*O*-α-L-rhamnopyranoside-8-*C*-β-D-glucopyranoside (Fig. 1) was achieved by ^{1}H and ^{13}C NMR spectroscopy whereby the identity of the A_2B_2 system of ring B of the aglycone was verified from the two *o*-coupled doublet signals at δ_H 8.05 ppm and 6.89 ppm (each 2H, d, J = 8.0 Hz) assignable to H-2', 6' and H-3', 5', respectively. This identity was supported by two signals at δ_C 129.26 ppm (C-2', 6') and 116.20 ppm (C-3', 5') each for two methin carbon atoms, as well as the singlet signal at δ_H 6.75 ppm assignable for H-3 with its methane carbon signal at δ_C 102.58 ppm (Maitra *et al.*, 1995). The absence of the two *m*-coupled protons of H-6 and H-8 in the ^{1}H NMR spectrum of **2** together with the downfield shift of the C-6 signal in the ^{13}C NMR spectrum at δ_C 131.4 ppm indicated that it is oxygenated (Agrawal, 1989). While, the anomeric proton of the sugar moiety resonating at δ_H 5.05 ppm (d, J = 2 Hz)



2 **3**

Fig. 1. Chemical structures of scutellarein-6-*O*-*α*-L-rhamnopyranoside-8-*C*-*β*-D-glucopyranoside (**2**) and kaempferol-7-*O*-[6'''-*O*-*p*-hydroxybenzoyl-*β*-D-glucosyl-(1→6)-*β*-D-glucopyranoside] (**3**).

was characteristic for those reported for 6-*O*-rhamnose. On the other hand, the resonances of C-8 at δ_C 104.7 ppm with the C-anomeric signal of glucose at δ_H 4.73 ppm (d, J = 8.5 Hz) indicated C-glucosylation of the 8-position. These data inferred the presence of a rhamnose moiety at 6-*O*-position and a glucose one at 8-*C*-position of the aglycone apigenin (Markham, 1982; Hörhammer *et al.*, 1965).

R_f values, colour reactions, and UV spectral analysis of compound **3** (dark yellow crystals, m.p. 199 °C) in methanol and in the presence of diagnostic reagents suggested compound **3** to be a flavonol (band I and II at 364 and 259 nm, respectively) with the 7-position substituted (Markham, 1982). Acid hydrolysis of **3** yielded kaempferol as the aglycone moiety, together with glucose as the sugar moiety and a *p*-hydroxybenzoyl moiety identified by paper co-chromatography with authentic samples which consequently indicated that **3** is kaempferol acylated at its 7-position by these two moieties. While hydrogen peroxide oxidation of **3** gave rise to the disaccharide gentiobiose [glucose (1→6) glucose], which was identified through paper co-chromatography with authentic samples, consequently suggesting **3** to be a kaempferol-7-acylated gentiobioside.

Further investigations of compound **3** were achieved through ^{1}H NMR spectroscopy which gave data similar to those of 7-*O*-substituted kaempferol (Harborne, 1993) where the doublet signals of H-6 and H-8 appeared at δ_H 6.38 ppm and 6.77 ppm, respectively, *i.e.* they were shifted downfield to those of kaempferol. The presence of two doublet anomeric hexose proton resonances at δ_H 5.20 ppm (H-1") and 4.92 ppm (H-1''') with J = 7.5 Hz ensured the presence of a gentiobiose moiety. The presence of a *p*-hydroxybenzoyl moiety as well was ensured by the characteristic proton signals which resonated at δ_H 7.71 ppm (d, J = 8.5 Hz, H-2'''' and 6'''') and 6.87 ppm (d, J = 8.5 Hz, H-3'''' and 5''''). These data confirmed that **3** is kaempferol-7-*O*-(*p*-hydroxybenzoyl gentiobioside). Final confirmation of the chemical structure of **3** was achieved by ^{13}C NMR spectroscopy whereby the two *β*-anomeric carbon signals resonated at δ_C 99.81 ppm and 103.57 ppm similar to those of two glucopyranoside moieties confirming their linkage to be (1→6) from the downfield shift of the C-6" of one of the glucose moieties at δ_C 62.9 ppm. Also, the aromatic carbon signals at δ_C 133.4 ppm (C-2'''' and 6''''), and 116.08 ppm (C-3'''' and 5'''') and the carbonyl group signal at δ_C 169.0 ppm were indicative of a *p*-hydroxybenzoyl moiety (Agrawal, 1989), which was assigned to be at the terminal glucosyl C-6''' as its signal appeared downfield at δ_C 63.4 ppm. Consequently, compound **3** (Fig. 1) was elucidated as kaempferol-7-*O*-[6'''-*O*-*p*-hydroxybenzoyl-*β*-D-glucosyl-(1→6)-*β*-D-glucopyranoside].

The *in vitro* inhibition of alkaline phosphatase is closely related to its *in vivo* inhibition in reproductive organs just as in the case of beryllium: alkaline phosphatase (from both kidney and *E. coli*) was shown to be inhibited by low concentrations of beryllium sulfate (Thomas and Aldridge, 1966). In another study, administration of beryllium nitrate caused significant inhibition of the activity of the enzyme in female reproductive organs (ovary, uterus, cervix, and vagina), with maximum

inhibition observed 10 days after exposure (Mathur *et al.*, 1989). Our previous studies (Hifnawy *et al.*, 2008a, b) showed that such mechanism was correlated with the inhibition of implantation, *i.e.* contraception. In the present study the biologically active methanolic extract of *H. rosa-sinensis* flowers as well as its water-soluble fraction were found to completely inhibit the enzyme *in vitro* over a wide range of tested concentrations (Table I).

On the other hand, regarding the compounds isolated from the water-soluble fraction, even though some compounds caused enzyme activation (Table I), the overall effect of the extract and the fraction was inhibitory on the enzyme, indicating a predominance of the compounds that possess inhibitory activity on the enzyme, namely gallic acid, *p*-hydroxybenzoic acid, and quercetin-7-*O*-galactoside. The first two compounds have carboxylic groups in addition to OH groups;

Table I. Effect of the addition of different concentrations of the methanolic extract, of the water-soluble fraction, and of isolated compounds of *Hibiscus rosa-sinensis* to the reaction mixture on the alkaline phosphatase activity *in vitro*.

Drug	Tested concentration [mg/mL reaction mixture]	Mean absorbance ± σ measured at 405 nm	Inhibition or activation[a] (%)
Control	-	0.242 ± 0.002	-
Methanolic extract of *Hibiscus rosa-sinensis*	0.1	0.000 ± 0.000*	−100
	1		
	10		
	100		
Water-soluble fraction of the methanolic extract of *Hibiscus rosa-sinensis*	0.1	0.000 ± 0.000*	−100
	1		
	10		
	100		
Apigenin	10	0.453 ± 0.002*	+87.2
Gallic acid	0.1	0.171 ± 0.008*,+	−29.3
	1	0.098 ± 0.007*,+	−59.5
	10	0.079 ± 0.006*,+	−67.4
	100	0.014 ± 0.003*	−94.2
p-Hydroxybenzoic acid	0.1	0.210 ± 0.004*,+	−13.2
	1	0.195 ± 0.004*,+	−19.4
	10	0.179 ± 0.002*,+	−26.0
	100	0.137 ± 0.003 *	−43.4
Kaempferol	10	0.347 ± 0.018*	+43.4
Kaempferol-7-*O*-(6'''-*p*-hydroxy-benzoyl gentiobioside)	10	0.449 ± 0.012*	+85.5
Neochlorogenic acid	10	0.325 ± 0.009*	+34.3
Quercetin	10	0.349 ± 0.010*	+44.2
Quercetin-7-*O*-galactoside	0.1	0.099 ± 0.014*,+	−59.1
	1	0.03 ± 0.002*,+	−87.6
	10	0.009 ± 0.003*,+	−96.3
	100	0.000 ± 0.000*	−100
Scutellarein-6-*O*-rhamnoside-8-*C*-glucoside	10	0.310 ± 0.006*	+28.1
Vitexin	10	0.322 ± 0.009*	+33.1

[a] +, activation; −, inhibition.
* Significantly different from control (blank) at $p \leq 0.05$.
\+ Significantly different from the mean absorbance caused by the addition of higher concentrations of the same compound to the reaction mixture at $p \leq 0.05$.

therefore inhibition could be attributed to an alteration in the pH value of the reaction mixture. Although quercetin-7-*O*-galactoside is not as acidic as the two other compounds, it showed potent inhibition of the enzyme activity, reaching 100% at the 100 mg/mL concentration level and, unlike the other two compounds, slightly decreasing (to only 96.3%) upon 10-fold dilution (Table I). From the above data, the compound quercetin-7-*O*-galactoside was the major active ingredient in the fraction; this was also supported by its high yield in the chemical study.

This is the first report highlighting the inhibitory activity of quercetin-7-*O*-galactoside on alkaline phosphatase enzyme activity. As there are only a few studies concerning the influence of flavonoids on alkaline phosphatase activity (Iio *et al.*, 1980; Iio, 1985), it follows that quercetin-7-*O*-galactoside (**4**) may be useful as a lead compound for the development of agents inhibitory on the activity of the alkaline phosphatase enzyme, a mechanism found to be closely related to contraception. In addition, two novel compounds have been identified; scutellarein-6-*O*-*α*-L-rhamnopyranoside-8-*C*-*β*-D-glucopyranoside (**2**) and kaempferol-7-*O*-[6'''-*O*-*p*-hydroxybenzoyl-*β*-D-glucosyl-(1→6)-*β*-D-glucopyranoside] (**3**), which exhibited a significant activation of the enzyme.

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