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Bioactivity Guided Phytochemical Study of *Clematis hirsuta* Growing in Saudi Arabia

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Abstract – Bioactivity guided phytochemical study of the petroleum ether and butanol extracts of *Clematis hirsuta* resulted in the isolation of 12 compounds. Rat paw edema as a model of acute inflammation was used to evaluate the anti-inflammatory activity of the extracts and the chromatographic fractions. Five known sterols and triterpenes namely: β-amyrin (**1**), lupeol (**2**), β-sitosterol (**3**), oleanolic acid (**4**) and stigmasterol glycoside (**5**) were isolated from the petroleum ether extract. The n-butanol extract afforded two compounds reported for the first time from natural source: (*S*)-(+)-dihydro-5-(hydroxymethyl)-2(3*H*)-furanone (**7**) and (*S*)-(−)-5-hydroxymethyl-2(5*H*)-furanone (**8**). In addition, anemonin (**6**), dihydro-4-hydroxy-5-(hydroxymethyl)-2(3*H*)-furanone (2-deoxy-D-ribono-1,4-lactone) (**9**), biophenol (cimidahurin) (**10**), glucose (**11**) and sucrose (**12**) were also identified. The structures were determined from spectroscopic data including 1D- and 2D-NMR experiments.
Keywords – *Clematis hirsuta*, triterpenes, sterols, anemonin, anti-inflammatory

Introduction

Family Ranunculaceae comprises 59 genera and about 1900 species distributed throughout temperate and cold regions. The genus *Clematis* is represented by 250 species (Evans, 2002). *Clematis* species have been traditionally used for the treatment of inflammatory conditions by the Chinese and indigenous Australians. The roots of *C. terniflora*, *C. japonica* and *C. chinensis* have been used as analgesic, for treatment of rheumatic arthritis and other inflammatory conditions (Xu *et al.*,1996a; 1996b). The ethanolic extracts of *C. pickeringii*, *C. glycinoides* and *C. microphylla* exhibited their anti-inflammatory effect via COX-1, COX-2 and 5-LOX inhibition. Both *C. hirsutissima* and *C. glycinoides* are used as a remedy for headache (Morgan, 1981; Lassak and McCarthy, 1983). *C. papuasica* leaves and stem bark showed a wide spectrum of antibacterial activity (Khan *et al.*, 2001). Antigonhorreal activity was reported for *C. dioica* (Cáceres *et al.*, 1995). The whole plant extract of *C. heracleifolia* was active against recombinant HIV-1 protease (Min *et al.*, 2001). The extracts of the young shoots of *C. vitalba* showed activity against pathogenic yeast and yeast-like microorganisms (Buzzini and Pieroni, 2003). Total extracts of *C. recta* and *C. hirsuta* showed a fungicidal effect (Gruenwald *et al.*, 2000; Cos *et al.*, 2002).

Previous phytochemical investigations of *Clematis* species resulted in the isolation of saponins containing up to 12 sugar units as in clematernosides I and J (Kawata *et al.*, 1998; Kawata *et al.*, 2001; Kizu *et al.*, 1995). Few flavonoids and lignans were also isolated (Kawata *et al.*, 2001; Chen *et al.*, 1993; Yesilada and Küpeli 2006; Dennis and Bierner 1980). Ranunculin, a common glycoside in the Ranunculaceae and its derivatives protoanemonin and anemonin were detected in several *Clematis* species (Southwell and Tucker, 1993; Slavik and Slavikova, 1995). Anemonin has antipyretic and intestinal anti-inflammatory activities by inhibiting the production of nitric oxide, endothelin-1 and intracellular adhesion molecule-1 in rat intestinal microvascular endothelial cells which prevent intestinal microvacular dysfunction (Martin *et al.*, 1988; Duan *et al.*, 2006).

In the present study bioactivity guided phytochemical investigation based on rat paw edema as a model of acute inflammation was conducted to isolate the active principles of the plant.

Experimental

General – Melting points were determined on a mettler FP 80 Central Processor supplied with a Mettler FP 81 MBC Cell Apparatus, and were uncorrected. Ultraviolet

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absorption spectra were obtained using a Hewlett-Packard HP-845 UV-Vis spectrometer. Specific rotations were measured on a Perkin-Elmer 241 Mc polarimeter, using a one-decimetre tube. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX-500 instrument at 500 MHz for protons and 125 MHz for carbons using the residual solvent signal as internal standard. Coupling constants (*J*) are in Hz. Standard Bruker pulse programs were used for DEPT, 2D NMR COSY, HMBC and HSQC experiments. EI-MS were obtained using Finnegan MAT 300 mass spectrometer. ES-MS were obtained using Liquid Chromatography/MS Spectrometer (Quattro micro API) equipped with direct prob and a Z-spray electrospray ion source (Micromass®, Quattro micro™, Waters). Silica gel (70-230 mesh, ASTM, Merck) and RP-18 Silica gel F_{254} "Whatmann" were used for column chromatography, Pre-coated silica gel 60 F_{254} plates, 0.25mm thick, Merck, were used for TLC, PTLC and HPTLC.

Plant materials – The aerial parts of *Clematis hirsuta* Guillemin & Perr were collected from "Tanuma" region in March 2000. The plant was identified by the Taxonomist Dr. M. Atiqur Rahman, Medicinal, Aromatic and Poisonous Plants Research Center (MAPPRC), College of Pharmacy, King Saud University. A voucher specimen (#11411) was deposited at the herbarium of the College of Pharmacy, King Saud University.

Extraction and Fractionation – The air dried powdered aerial parts of *C. hirsuta* (2 kg) were exhaustively extracted with 96% EtOH (10 L) by percolation. The solvent was evaporated under reduced pressure using rotary vacuum evaporator leaving 170 g residue. Part of the alcohol extract (155 g) was dissolved in 400 mL of distilled water/alcohol mixture (2 : 1) and successively extracted with petroleum ether (3 × 300 mL), $CHCl_3$ (3 × 300 mL), ethyl acetate (3 × 200 mL) and *n*-butanol (2 × 200 mL). Each extract was separately evaporated to dryness to yield (90, 2.9, 12.5 and 30.5 g, respectively). Both the petroleum ether and *n*-butanol extracts showed pronounced anti-inflammatory activity (protection against edema 50.8 ± 4.5% at a dose of 240 mg/kg and 51.4 ± 4.6 at a dose of 85 mg/kg after 2 hr of carrageenan injection respectively), so they were selected for further phytochemical study.

Chromatography of the petroleum ether fraction – A portion of petroleum ether extract (20 gm) was fractionated on a Sephadex LH20® column (200 g, 80 × 5 cm). Elution started with petroleum ether/ $CHCl_3$ (2 : 1) followed by $CHCl_3$/acetone (4 : 1), $CHCl_3$/acetone (1 : 4), acetone/MeOH (4 : 1), acetone/MeOH (1 : 1) and finally with 100% MeOH. Twenty five fractions, 300 mL each, were collected and similar fractions were pooled to give six main fractions (A-F) that were separately subjected to anti-inflammatory testing. Fractions D (2.57 g, acetone/ MeOH, 4 : 1) and F (2.6 g, MeOH) were the most active (40.1 ± 3.2 and 57.6 ± 2.9% inhibition of edema after 2 hr of carrageenan injection respectively). Part of fraction D (1.5 g) was further separated over silica gel column (100 g, 60 × 2.5 cm). Elution started with petroleum ether/ $CHCl_3$ (9 : 1) and polarity was gradually increased by $CHCl_3$ to give ninety fractions 20 mL each. The fractions were collected and screened by TLC. Crystallization of fractions 15 - 29 (91 mg, 20% $CHCl_3$ in petroleum ether) from MeOH afforded 80 mg of **1** (R_f value 0.47, $CHCl_3$). Fractions 36 - 48 (73 mg, 30% $CHCl_3$ in petroleum ether) were subjected to preparative TLC on silica gel plates and developed with $CHCl_3$ to afford 55 mg of **2** (R_f value 0.33, $CHCl_3$). Fractions 59 - 78 (118 mg, 40% $CHCl_3$ in petroleum ether) on crystallization from MeOH afforded 110 mg of **3** (R_f value 0.45, $CHCl_3$/MeOH, 95 : 5).

Part of fraction F (1.5 g) was rechromatographed over silica gel column (100 g, 60 × 2.5 cm). Elution started with $CHCl_3$ and polarity was gradually increased with MeOH. Seventy fractions 20 mL each were collected and screened by TLC. Crystallization of fractions 8 - 20 (78 mg, 100% $CHCl_3$) from MeOH afforded 70 mg of **4** (R_f value 0.33, $CHCl_3$/MeOH, 95 : 5). Fractions 30 - 59 (290 mg) were subjected to silica gel column chromatography (25 g, 50 × 1 cm) and elution was started with $CHCl_3$/ MeOH (98 : 2) and polarity was gradually increased with MeOH. Fractions eluted with 4% MeOH in $CHCl_3$ afforded 200 mg of **5** (R_f value 0.31, $CHCl_3$/MeOH, 90 : 10) on crystallization from MeOH.

Chromatography of the *n*-butanol fraction – Part of the butanol extract (27 g) was fractionated on Sephadex LH20® column (300 g, 80 × 5 cm). Elution started with $CHCl_3$/petroleum ether (1 : 4, 500 mL) followed by $CHCl_3$/ petroleum ether (4 : 1, 500 mL), $CHCl_3$/MeOH (4 : 1,1 L), $CHCl_3$/MeOH (1 : 4, 1 L) then 100% MeOH (1.5 L). Fifteen fractions were collected and similar fractions were pooled together after TLC screening to five fractions (I-VI). Fraction V (24. 93 g, 50.4 ± 4.2% protection against edema at a dose of 70 mg/kg after 2 hr of carragenan injection) was chosen for isolation of the active constituents.

Eleven grams of V were chromatographed on a silica gel column (350 g, 120 × 5 cm). Elution was started with $CHCl_3$/MeOH (95 : 5) and polarity was gradually increased with MeOH. Seventy fractions 250 mL each were collected, monitored by TLC and similar fractions

were pooled together to give four fractions. Fraction V-2 (6.82 g) retained the biological activity (49.6 ± 3.1% protection against edema at a dose of 50 mg/kg after 2 hr of carrageenan injection).

Part of fraction V-2 (3.5 g) was further purified over silica gel column (200 g, 80 × 2.5 cm). Elution started with $CHCl_3$/MeOH (99 : 1) and polarity was gradually increased with MeOH. One hundred and ten fractions, 20 mL each, were collected. Fractions 12 - 23 (32 mg), eluted with 2.5% MeOH in $CHCl_3$, were crystallized from MeOH to give 26 mg of **6** (R_f value 0.45, $CHCl_3$/MeOH, 9 : 1).

Fractions 27 - 34 (51 mg) eluted with 5% MeOH in $CHCl_3$ were subjected to PTLC using HPTLC plates and $CHCl_3$/MeOH (98 : 2) as developing system (triple run) to obtain 4 mg of **7** and 3 mg of **8** (R_f value 0.42 and 0.41 respectively, $CHCl_3$/MeOH, 9 : 1).

Fractions 41 - 72 (134 mg) were subjected to repeated column chromatography over silica gel (25 g, 50 × 1 cm) using 5% MeOH in $CHCl_3$ as eluent and the polarity was gradually increased by increasing the MeOH contents. Fractions 8 - 21 eluted with 10% MeOH in $CHCl_3$, afforded **9** (65 mg, R_f value 0.45, $CHCl_3$/MeOH, 8 : 2).

Fractions 80 - 91 eluted with 20% MeOH in $CHCl_3$ afforded 41 mg of **10** (R_f value 0.44, $CHCl_3$/MeOH, 7 : 3). Fractions 95 - 101, eluted with 25% MeOH in $CHCl_3$ afforded 63 mg of **11** (R_f value 0.26, $CHCl_3$/MeOH, 7 : 3) on crystallization from MeOH. Finally fractions 104 - 108 eluted with 27.5% MeOH in $CHCl_3$ gave 55 mg of **12** (R_f value 0.16, $CHCl_3$/MeOH, 7 : 3) on crystallization from MeOH.

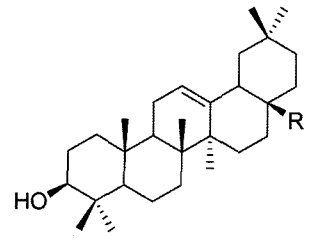
β-Amyrin (1) – $C_{30}H_{50}O$, colorless needle crystals, m.p. 194 - 195 °C ($CHCl_3$). EIMS *m/z* (rel. int. %): 426 $[M]^+$ (21).

Lupeol (2) – $C_{30}H_{50}O$, colorless needle crystals, m.p. 207 - 208 °C ($CHCl_3$). EIMS *m/z* (rel. int. %): 426 $[M]^+$ (14).

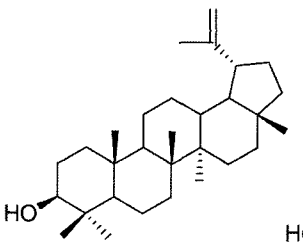
β-Sitosterol (3) – $C_{29}H_{50}O$, colorless powder, m.p. 138 - 140 °C ($CHCl_3$). EIMS *m/z* (rel. int. %): 414 $[M]^+$ (73).

Oleanolic acid (4) – $C_{30}H_{48}O_3$, colorless needle crystals, m.p. 194 - 195 °C (MeOH). ESIMS *m/z* (rel. int. %): 479 $[M + Na]^+$ (100).

Stigmasterol glycoside (5) – $C_{36}H_{58}O_6$, colorless cubic crystals, m.p. 170 - 172 °C (MeOH). ESIMS *m/z* (rel. int. %): 609 $[M + Na]^+$ (100).



1, R= CH_3
4, R= COOH



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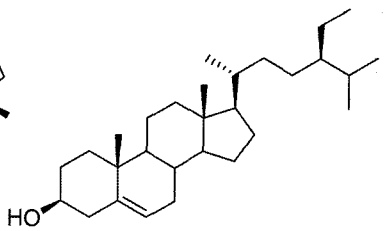

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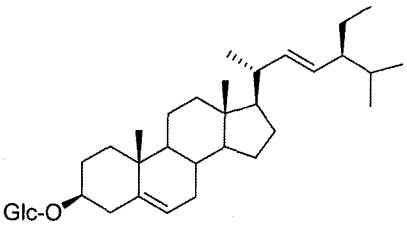

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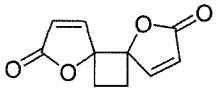

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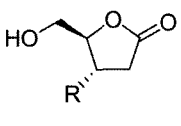
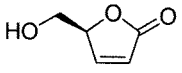


7, R= H
9, R= OH



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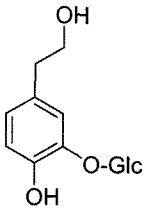

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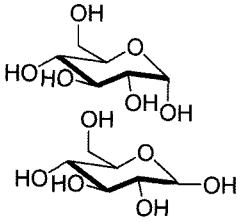

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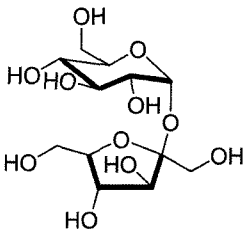

12

Anemonin (6) – $C_{10}H_8O_4$, colorless needle crystals, m.p. 155 - 156 °C (MeOH). ^{1}H- and ^{13}C-NMR: Table 1. ESIMS *m/z* (rel. int. %): 215 $[M+Na]^+$ (100).

(*S*)-(+)-Dihydro-5-(hydroxymethyl)-2(3*H*)-furanone (7) – $C_5H_8O_3$, colorless viscous liquid, $[\alpha]_D^{25}$ + 45° (*c* 0.8, $CHCl_3$). ^{1}H- and ^{13}C-NMR: Table 1. ESIMS *m/z* (rel. int. %): 139 $[M+Na]^+$ (100).

(*S*)-(−)-5-Hydroxymethyl-2(5*H*)-furanone (8) – $C_5H_6O_3$, colorless viscous liquid, $[\alpha]_D^{25}$ −122° (*c* 0.7, $CHCl_3$). UV λ_{max} (MeOH) nm: 212. ^{1}H- and ^{13}C-NMR: Table 1. ESIMS *m/z* (rel. int. %): 137 $[M+Na]^+$ (100).

Dihydro-4-hydroxy-5-(hydroxymethyl)-2(3*H*)-furanone (2-Deoxy-D-ribono-1,4-lactone) (9) – $C_5H_8O_4$, semi-solid, $[\alpha]_D^{25}$ + 10° (*c* 1.0, MeOH). ^{1}H- and ^{13}C-NMR: Table 1. ESIMS *m/z* (rel. int. %): 155 $[M+Na]^+$ (100).

Biophenol (Cimidahurin) (10) – $C_{14}H_{20}O_8$, semi-solid. UV λ_{max} (MeOH) nm: 212, 244, 286. ^{1}H-NMR (CD_3OD) δ: 2.73 (2H, t, *J* = 7.0 Hz, H-a), 3.12-3.31 (2H, m, H-4',5'), 3.36-3.39 (2H, m, H-2',3'), 3.60 (2H, t, *J* = 6.7 Hz, H-b), 3.71 (2H, *m*, H-6'), 4.61 (1H, d, *J* = 7.5 Hz, H-1'), 6.64 (1H, d, *J* = 2.0 Hz, H-2), 6.55 (1H, *dd*, *J* = 8.0, 2.0 Hz, H-6), 7.00 (1H, d, *J* = 8.0 Hz, H-5). ^{13}C-NMR (CD_3OD) δ: 39.7 (C-α), 62.5 (C-6'), 64.3 (C-β), 71.4 (C-4'), 74.9 (C-2'), 77.7 (C-3'), 78.3 (C-5'), 104.8 (C-1'), 117.7 (C-2), 119.4 (C-5), 121.4 (C-6), 136.4 (C-1), 145.3 (C-3), 148.4 (C-4). ESIMS *m/z* (rel. int. %): 339 $[M+Na]^+$ (100).

Glucose (11) – $C_6H_{12}O_6$, colorless cubic crystals, m.p. 148 - 149 °C (H_2O). $[\alpha]^{25}$ + 54° (*c* 1.0, H_2O). ESIMS *m/z* (rel. int. %): 203 $[M+Na]^+$ (100). ESIMS *m/z* (rel. int. %): 390 (Silyl derivative, 17).

Sucrose (12) – $C_{12}H_{22}O_{11}$, colorless cubic crystals, 181 - 182 °C (H_2O). $[\alpha]^{25}$ + 65° (*c* 1.2, H_2O). ESIMS *m/z* (rel. int. %): 365 $[M+Na]^+$ (100).

Anti-inflammatory activity – The anti-inflammatory activity was evaluated using rat paw edema as a model for acute inflammation induced in male Wistar rats by 2% aqueous carragenan suspension (Winter *et al.*, 1962) and indomethacin as a positive control. Data were expressed as the mean ± SEM. Significant difference between the control and the treated groups was performed using Student's *t*-test and *P* values. The difference in results was considered significant when $P < 0.001$.

Results and Discussion

Compounds **1-5** were identified as β-amyrin, lupeol, β-sitosterol, oleanolic acid and stigmasterol glycoside by comparing the obtained chemical, physical and spectral, data with the literature (Kolak *et al.*, 2005; Ahmed and Rahman, 1994; Good and Akihisa, 1997). Similarly compound **11** was identified as a mixture of α- and β-D-glucose in their natural ratio in solution form (Pouchert and Behnke, 1992; Agrawal, 1989), while compound **12** was identified as sucrose (Popov *et al.*, 2006).

The ESIMS of compound **6** showed a molecular ion peak at *m/z* 215 $[M+Na]^+$ consistent with the molecular formula $C_{10}H_8O_4$. However, ^{13}C-NMR spectrum (Table 1) showed only five carbon signals sorted by DEPT experiments into 1 × CH_2, 2 × CH and two quaternary carbons, one oxygenated (δ_C 90.3) and one lactonic carbonyl (δ_C 170.7). The ^{1}H NMR spectrum (Table 1) showed three sets of protons (two coupled olefinic doublets at δ_H 6.06 and 7.66 (*J* = 5.5 Hz) and one methylene multiplet signal at δ_H 2.40. The data of **6** pointed out to a dimer with a plane of symmetry passing through cyclobutane ring. The data of **6** were in full agreement with those reported for anemonin isolated from *C. hirsutissima* (Kern and Cardellina, 1983).

The ESIMS $[M+Na]^+$ at *m/z* 139 in combination with the ^{13}C-NMR (Table 1) and DEPT experiments indicated

Table 1. ^{1}H- and ^{13}C-NMR data of compounds **6-9** (δ, *J* in parenthesis in Hz)[a]

No.	**6**[b]		**7**[c]		**8**[c]		**9**[b]	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	---	170.7	-	178.9	-	174.3	-	178.7
2	6.06 (2H, d, 5.5)	121.1	2.58 (2H, ddd, 16.75, 1.0, 2.0)	28.1	7.68 (1H, dd, 5.5,1.5)	155.2	2.93 (1H, dd, 18.0, 7.0) 2.40 (1H, dd, 18.0, 2.0)	39.5
3	7.66 (2H, d, 5.5)	153.1	2.30 (1H, m) 2.11 (1H, m)	22.9	6.21 (1H, dd, 5.5, 2.0)	121.7	4.45 (1H, m)	69.7
4	---	90.3	4.64 (1H, m)	81.4	5.19 (1H, m)	84.9	4.39 (1H, m)	90.1
5	2.40 (4H, m)	23.9	3.79 (1H, dd, 12.5, 3.0) 3.62 (1H, dd, 12.5, 4.5)	63.2	3.90 (1H, dd, 12.5, 3.5) 3.75 (1H, dd, 12.5, 4.5)	61.1	3.72 (1H, dd, 12.5, 3.5) 3.78 (1H, dd, 12.5, 3.5)	62.5

[a]Assigment base on COSY, DEPT, HSQC, HMBC experiments.
[b]Spectra were obtained in $CDCl_3$.
[c]Spectra were obtained in CD_3OD.

that **7** has the molecular formula $C_5H_8O_3$. The five carbons were sorted by DEPT experiments into 3 × CH_2 including one oxymethylene, one oxygenated CH and a lactonic carbonyl. The two degrees of unsaturation indicated that **7** is a cyclic compound. ^{1}H-^{1}H COSY spectrum showed coupling between the CH proton at δ_H 4.64 with both the oxymethylene protons at δ_H 3.62, 3.79 and the methylene at δ_H 2.11 and 2.30. The latter coupled with the third methylene at δ_H 2.58. These data suggested a five membered lactone with a CH_2OH group at C-4. The HMBC data fully supported the above suggestion. Although such structure has not been reported as a natural product, the data of **7** were identical with (*S*)-(+)-dihydro-5-(hydroxymethyl)-2(3H)-furanone available synthetically from Aldrich chemical company (Pouchert and Behnke, 1992).

The ESIMS of compound **8** showed $[M + Na]^+$ at 137 *m/z* consistent with the molecular formula $C_5H_6O_3$ with two hydrogens less than **7**. Instead of the two aliphatic methylenes in **7** two olefinic CH were observed in the ^{1}H- and ^{13}C-NMR of **8** (Table 1) (δ_H 6.21 and δ_C 121.7; δ_H 7.68 and δ_C 155.2 ppm). The data of **8** indicated that it is the dehydroderivative of **7**. COSY, HSQC and HMBC experiments were supportive for the suggested structure (*S*)-(−)-5-hydroxymethyl-2(5H)-furanone available synthetically from Aldrich chemical company but new as natural product (Pouchert and Behnke, 1992).

The ^{1}H- and ^{13}C-NMR (Table 1) and ESIMS data of **9** indicated the replacement of C-3 methylene in **7** with a CHOH (δ_H 4.45 *m* and δ_C 69.7). ^{1}H-^{1}H COSY spectrum confirmed the location of the OH at C-3 where the C-2 methylene protons at δ_H 2.40 (dd, J = 18.0, 2.0 Hz) and 2.93 (dd, J = 18.0, 7.0 Hz) correlated with a proton at δ_H 4.45 assigned for C-3. The correlations between the carbon and proton signals were confirmed by HSQC and HMBC experiments. The spectral data of **9** were in good agreement with those reported for the 2-deoxy-D-ribono-1,4-lactone isolated from *Aristolochia arcuata* (Aristolochiaceae) (Francisco *et al.*, 2003).

ESIIMS of **10** showed $[M + Na]^+$ at *m/z* 339 consistent with the molecular formula $C_{14}H_{20}O_8$. The fourteen carbons were clear in the ^{13}C NMR spectrum (experimental) and were sorted by DEPT experiments into 3 × CH_2, 8 × CH and 3 fully substituted carbons. ^{1}H- and ^{13}C-NMR spectra showed an ABX system at δ_H 6.64, d, J = 2.0 Hz, δ_C 117.7; δ_H 7.00, d, J = 8.0 Hz, δ_C 119.4; 6.55, dd, J = 8.0, 2.0 Hz, δ_C 121.4; ethanol moiety at δ_H 2.73, t, J = 7.0 Hz, δ_C 39.7; δ_H 3.60, t, J = 6.7 Hz, δ_C 64.3; in addition to sugar signals assigned for β-D-glucose. HMBC experiment enabled the assignments of the substituent positions: carbon signals at δ_C 136.4, 145.3 and 148.4 were assigned to C-1 bearing the ethanol moiety, oxygenated C-3 and C-4, respectively. The three bond HMBC correlation of the anomeric proton at δ_H 4.61 with C-3 at δ_C 145.3 proved the location of glucose at that position. The data of **10** were in good agreement with the reported data of biophenol (Bianco *et al.*, 1998). In the cited reference, C-6' carbon is missing while C-4' is probably mis-typed at δ_C 104.0.

Compound **5** was tested for anti-inflammatory activity in a dose of 40 mg/kg via intraperitoneal route. Treatment with **5** resulted in 31.0 ± 2.6 and 37.5 ± 1.8% inhibition of edema ($N = 6$, $P < 0.001$), one and two hours after carrageenan injection respectively. Indomethacin at a dose of 10 mg/kg body weight produced 70.6 ± 2.0% inhibition of edema two hours after carrageenan injection.

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