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ORIGINAL ARTICLE

Structure elucidation and vasodilator activity of methoxy flavonols from *Calycotome villosa* subsp. *intermedia*

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Abstract Two flavonols identified as 3,5,7,4′-tetrahydroxy-3′-methoxyflavone (**1**) and 3,5,7,4′-tetrahydroxy-8-methoxyflavone (**2**) were isolated from the seeds of *Calycotome villosa* subsp. *intermedia*. The structure elucidation of the isolated compounds was performed by the spectroscopic methods (UV, IR, ^{1}H NMR, ^{13}C NMR and MS) and also by a single crystal X-ray analysis in the case of compound (**2**). Vasodilator activity of compound (**2**) was demonstrated in isolated rat aorta contracted with high KCl or with noradrenaline.

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1. Introduction

Flavonoids constitute one of the largest group of polyphenols which are widely distributed throughout the plant kingdom (Bohm, 1998). To date, more than 4000 flavonoids have been described (Bravo, 1998). Flavonoids exhibit several biological activities and have free radical scavenging abilities and contribute to antioxidant capacity in fruits and vegetables (Morel et al., 1993; Cao et al., 1997). The antioxidant activity of some subclasses of the flavonoids, such as the flavonols, has been

reported to be greater than that of either vitamin C or E (Rice-Evans et al., 1995; Bors et al., 1990). Antihypertensive activity and direct vasodilator effect of flavonoids have been reported (Duarte et al., 1993; 2001; Ibarra et al., 2003; Perez-Vizcaino et al., 2002). Recent work demonstrates the importance of the chemical nature of these compounds to their bioactivity and bioavailability (Plumb et al., 1997). These findings suggest that the degree of hydroxylation is important in determining the antioxidant activity.

In previous phytochemical studies of *Calycotome villosa* (Poiret) Link subsp. *intermedia* (C.Presl) Quezel and Santa (Leguminosae), we have reported the isolation and characterization of flavone glucosides from the leaves and flowers (El Antri et al., 2004a) and alkaloids from the seeds (El Antri et al., 2004b,c). Further chemical investigation of the seeds of this species revealed the presence of a complex mixture of flavonoid compounds. In the present paper we describe the isolation and structural determination of two methoxyflavonols: 3,5,7,4′-tetrahydroxy-3′-methoxyflavone (**1**) and 3,5,7,4′-tetrahydroxy-8-methoxyflavone (**2**) and the vasodilator activity of flavonoid (**2**).

2. Experimental

2.1. Instrumentation

Melting points were measured in open capillary tubes in a Büchi 530 apparatus and are uncorrected. UV–visible spectra were obtained on a Varian Cary 3E spectrophotometer, and IR spectra were recorded on a Pye Unicam Perkin–Elmer spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 on a Bruker (Wiessembourg, France) AM 300 spectrometer (300 and 75 MHz, for ^{1}H and ^{13}C NMR respectively) and chemical shifts are given as δ value with TMS as an internal standard. EI-MS data were obtained on a Quattro II tandem quadripole mass spectrometer (Micromass, Manchester, UK) fitted with an electrospray ionisation. Silica gel GF_{254} was used for TLC. Spots were detected on TLC under UV light. Column chromatography (CC) was carried out on silica gel 60 (70–230 mesh).

2.2. Plant material

Seeds of *C. villosa* subsp. *intermedia* were collected from the aerial part of the plant in June 2002 and again in June 2003 from Zrireg valley, plateau of Tazzeka, area of Taza, Morocco. The plant was identified by Dr. Rachid El alami and Dr. Abdeslam Ennabili (Université Sidi Mohamed Ben Abdellah, Fès). A voucher specimen (no. EN008) has been deposited at the herbarium of the ''Institut National des Plantes Médicinales et Aromatiques'', Université Sidi Mohamed Ben Abdellah, Fès, Morocco.

2.3. Extraction and isolation

The powdered seeds of *C. villosa* subsp. *intermedia* (200 g) were extracted with MeOH in a soxhlet apparatus. After solvent evaporation under reduced pressure, the MeOH extract (3.50 g) was partioned between a sequence of solvents (Yang et al., 1989). The residue from the EtOAc (1.00 g) was subjected to silica gel CC using CH_2Cl_2 as solvent, followed by the gradual introduction of MeOH to give a flavonoids-rich fraction (0.450 g). Repeated chromatography of this fraction on silica gel column yielded pure compounds (**1**) (0.034 g, eluted with CH_2Cl_2–MeOH 99:1) and (**2**) (0.015 g, eluted with CH_2Cl_2–MeOH 95:5). Recrystallization of compound (**2**) from methanol yielded crystals suitable for X-ray crystal analysis.

2.3.1. 3,5,7,4′-tetrahydroxy-3′-methoxyflavone (*1*)

Yellowish amorphous powder (0.034 g); mp 301–303 °C; UV–visible λ_{max} nm: MeOH 255, 268 sh, 370; NaOMe 276, 320, 425; $AlCl_3$ 267, 360, 435; $AlCl_3$ + HCl 265 sh, 358, 423; NaOAc 260 sh, 275, 318 sh; NaOAc + H_3BO_3 260, 271 sh, 328 sh, 377; IR ν_{max} (KBr, cm^{-1}): 3280–3300, 2930, 1653, 1557, 1501, 1453, 1263, 1172, 1015; ^{1}H NMR (300 MHz, δH, DMSO-d_6): 11.59 (s, HO-C5), 7.67 (1H, d, J = 8.4, H-C6′), 7.74 (1H, s, H-C2′), 6.93 (1H, d, J = 8.4, H-C5′), 6.46 (1H, d, J = 1.8, H-C8), 6.18 (1H, d, J = 1.8, H-C6), 3.82 (3H, s, OCH_3) ppm; ^{13}C NMR (75 MHz, δC, DMSO-d_6): 175.8 (C-4), 164.0 (C-7), 160.6 (C-5), 156.1 (C-9), 148.7 (C-2), 147.3 (C-3′), 146.5 (C-4′), 135.8 (C-3), 122.0 (C-6′), 121.7 (C-1′), 115.5 (C-5′), 111.6 (C-2′), 102.9 (C-10), 98.2 (C-6), 93.6 (C-8), 55.7 (OCH_3) ppm; HRMS-FAB (m/z 317.0579; calc.: 317.0583); EI-MS m/z: 316 $[M]^+$.

2.3.2. 3,5,7,4′-tetrahydroxy-8-methoxyflavone (*2*)

Yellowish amorphous powder (0.015 g); mp 262–264 °C; UV–visible λ_{max} nm: MeOH 272, 326 and 376; NaOMe 284, 335, 429; $AlCl_3$ 261 sh, 272, 309 sh, 359, 436; $AlCl_3$ + HCl 261 sh, 271, 309 sh, 359, 435; NaOAc + H_3BO_3 273, 307 sh, 326, 377; IR ν_{max} (KBr, cm^{-1}): 3305, 2933, 1652, 1558, 1506, 1457, 1263, 1176, 1013; ^{1}H NMR (300 MHz, δH, DMSO-d_6): 12.19 (s, HO-C5), 8.08 (2H, d, J = 9.0, H-C2′, H-C6′), 6.98 (2H, d, J = 9.0, H-C3′, H-C5′), 6.30 (1H, s, H-C6), 3.85 (3H, s, OCH_3) ppm; ^{13}C NMR (75 MHz, δC, DMSO-d_6): 177.0 (C-4), 160.2 (C-7), 160.1 (C-5), 157.4 (C4′), 156.4 (C-2), 156.3 (C-9), 136.6 (C-8), 130.3 (C-3), 128.3 (C-2′, C-6′), 122.7 (C-1′), 116.4 (C-3′, C-5′), 103.9 (C-10), 99.3 (C-6), 61.8 (OCH_3) ppm; EI-MS m/z: 316 $[M]^+$.

2.4. X-ray diffraction analysis

The crystal structure of the title compound has been determined on single crystal. Data were collected on an Enraf-Nonius κ-CCD diffractometer equipped with an Oxford cryosystems low temperature device using a monochromator $Mo_{K\alpha}$ radiation (λ = 0.71073 Å). Shelx97 was used for the structure determination (Sheldrick et al., 1997), and full-matrix least squares refinements were based on F^2.

All the non-hydrogen atoms were anisotropically refined. H atoms were located in an electron difference map, but H atoms attached to C atoms were thereafter as riding atoms, with C–H distances of 0.98 (CH_3), 0.82 (OH) and 0.93 (C–H aromatic). The O–H bond length for water was restrained to 1.02(2) . No decay of intensity was observed. Pertinent crystallographic data and experimental conditions are summarized in Table 1.

Supplementary tables of crystal structure and refinements, as the anisotropic thermal parameters have been deposited [CCDC 255530, these data can be obtained free of charge using the link: www.ccdc.cam.ac.uk or from the CCDC, 12 Union Road Cambridge CB2 1EZ, UK; fax: +44 1223 336033; E-mail: deposit@ccdc.cam.ac.uk].

Table 1 Selected geometrical (Å, °) features of (**2**).

Empirical formula	$C_{16}H_{12}O_7{\cdot}2H_2O$
Systematic name	3,5,7,4′-Tetrahydroxy-8-methoxyflavone
Formula weight (g/mol)	352.29
Temperature (K)	293(2)
Wavelength [λK_α (Å)]	0.71073
Crystal system	Monoclinic
Space group	$P2_1/c$
Unit cell dimensions (Å, °)	a = 3.8620(10) α = 90.00 b = 15.7570(5) β = 93.771(9) c = 25.2940(10) γ = 90.00
Volume (Å^3)	1535.90(9)
Z	4
d_{cal} (g/cm^3)	1.524
Absorption coefficient (mm^{-1})	0.127
F(000)	736
Crystal size (mm)	$0.2 \times 0.1 \times 0.05$
Reciprocal space	$-5 \leqslant h \leqslant 5$, $-18 \leqslant k \leqslant 20$, $-27 \leqslant l \leqslant 32$
Reflections collected	3437
Independent reflections	1899
Refined parameters	239
Final R indices [$I > 2\sigma(I)$]	R_1 = 0.0663, ωR_2 = 0.1801

2.5. Measurement of the contractile response of rat aorta

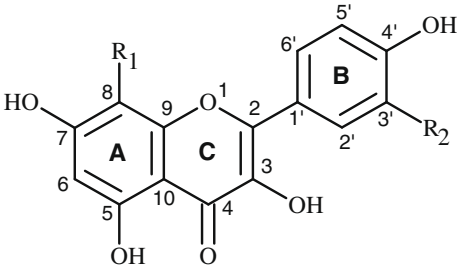
Male Wistar rats (250–300 g) were used. Contractions of isolated aorta were measured as previously described (El Bardai et al., 2003). Aortic rings (2 mm wide) were suspended under a resting tension of 20 mN, in 12.5 ml organ baths filled with a physiological solution (composition (mM): NaCl, 122; KCl, 5.9; $NaHCO_3$, 15; $MgCl_2$, 1.25; $CaCl_2$, 1.25; glucose, 11) bubbled with a gas mixture of 95% O_2, 5% CO_2 and maintained at 37 °C.

After an equilibration period, each preparation was contracted either by adding noradrenaline (1 μM) into the bath solution or by changing the physiological solution in the bath to a KCl-rich depolarizing solution, containing (in mM): KCl, 100; NaCl, 27; $NaHCO_3$, 15; $MgCl_2$, 1.25; $CaCl_2$, 1.25; glucose, 11. The fractions were added in the solution when the contraction had reached a plateau. In some experiments endothelium was removed by gentle rubbing.

The absence of acetylcholine (1 μM) induced relaxation in arteries contracted with noradrenaline (1 μM) was taken as an indicator that vessels were denuded successfully. Inhibition of the contractions was calculated as a percentage of the contractile force measured before the addition of the fraction and was corrected for time-matched controls. pD_2 values (−log IC_{50}, concentration producing 50% inhibition of the contraction) were calculated by non-linear regression of averaged data (GraphPad, Prism). Compound (**2**) was dissolved in DMSO immediately before use.

3. Results and discussion

The seeds of *C. villosa* subsp. *intermedia* were extracted with methanol. After the partition of the methanolic residue between water and a series of organic solvents, the ethyl acetate fraction was investigated and yielded two flavonols (**1**) and (**2**) in a pure forme (Scheme 1). Compounds (**1**) and (**2**) displayed



1 R_1= H R_2= OCH_3

2 R_1= OCH_3 R_2= H

Scheme 1

UV absorption and ^{1}H-, ^{13}C NMR data typical of flavonoids (Mabry et al., 1970). IR bands in the range of 1660–1000 cm^{-1} are typical of a flavone skeleton. The assignment of protons and carbons in (**1**) and (**2**) are based on those given in the literature (Markham et al., 1978). Both compounds showed a carbon signal at δ 175.8 (**1**) and 177.0 (**2**).

This indicated that there are flavones with a hydroxyl group at C-5. The UV spectral data of both flavones with diagnostic shifts reagents indicated flavonols with free 3, 5, 7 and 4′ hydroxyl groups (Mabry et al., 1970). Absence of a singlet relative to C-3 proton in the ^{1}H NMR spectra indicated that compounds (**1**) and (**2**) are flavonols. The spectral properties of flavonols (**1**) and (**2**) were verified by comparison of their spectral data with those previously described in the literature (Mabry et al., 1970; Markham et al., 1978; Harborne et al., 1975).

Flavonoid (**1**) was obtained as a yellow powder. Its molecular formula was determined to be $C_{16}H_{12}O_7$ on the basis of positive ion EI-MS (m/z 316 [M^+]) and HRMS-FAB (m/z 317.0579; calc.: 317.0583). The MS-fragmentation ion at m/z = 151 as well as the absence of m/z = 137 [if o-di-OH ring B] were in accordance with a B ring with one hydroxyl and one methoxyl group (Harborne et al., 1975).

The UV spectrum exhibited absorption maxima at 255 nm (band II) and 370 nm (band I) that are characteristic absorption bands of a flavone skeleton (Mabry et al., 1970). The UV spectral data showed the presence of a free 7-OH group. Its IR spectrum showed absorption bands due to hydroxyl (3280–3300 cm^{-1}), aromatic keto (1653 cm^{-1}) and phenyl and double bonds (2930, 1501, 1453 cm^{-1}) groups.

The ^{1}H NMR spectrum of (**1**) displayed signals for two meta-coupled protons at δ 6.18 (1H, d, J = 1.8 Hz, H-6) and δ 6.46 (1H, d, J = 1.8 Hz, H-8). The multiplicities and the weak coupling constants of H-6 and H-8 were in agreement with the existence of the hydroxyl group at C-7 (δ 164.0). The chemical shifts at δ 7.74, 7.67 and 6.93 were assigned to protons in the B ring and indicated that one methoxyl group was at 4′- or 3′-position.

The HMBC spectrum of compound (**1**) suggested that this methoxyl group was attached to the 3′-position. The UV shifts and ^{1}H, ^{13}C NMR spectra of flavonoid (**1**) were in agreement with a quercetin skeletal pattern (Mabry et al., 1970; Harborne et al., 1975). The only difference was that compound (**1**) showed the presence of a methoxyl group at δ 3.82 in the ^{1}H

NMR spectrum. Consequently, the structure of flavonoid (**1**) was established as known flavonol 3,5,7,4′-tetrahydroxy-3′-methoxyflavone (named isorhamnetin) (Simonsen et al., 2003; Nishida, 1994).

Flavonoid (**2**) was isolated as a yellow powder. Its molecular formula was established as $C_{16}H_{12}O_7$ by positive ion EI-MS (*m*/*z* 316 [M^+]). In the UV spectrum of (**2**), the maximum bands were observed at 272, 326 and 376 nm. No shift in band I of compound (**2**) with the addition of $AlCl_3$ and $AlCl_3$/HCl. These UV data indicated an absence of an ortho-dihydroxyl pattern in B ring (Markham, 1982).

The IR spectrum, which was very similar to that of flavonoid (**1**), showed absorption bands due to hydroxyl (3305 cm^{-1}), aromatic keto (1652 cm^{-1}) and aromatic (1457 and 1419 cm^{-1}) groups. The ^1H NMR spectrum of compound (**2**) displayed the characteristic signals of the kaempferol nucleus: one singlet aromatic proton at δ 6.30 (1H, s, H-6), ortho coupled A_2B_2-type aromatic protons at δ 6.98 and 8.08 (2H each, both d, J = 9.0 Hz, H-3′, H-5′ and H-2′, H-6′ respectively) and a chelate hydroxyl proton at δ 12.19 (1H, broad s, OH-5) Mabry et al., 1970; Harborne et al., 1975.

This functionality was confirmed by a batochromic shift in the UV–visible spectrum of (**2**) after addition of $AlCl_3$. A methoxyl was indicated with a signal at δ 3.85 which is typical for an 8-methoxyl group in many (but not all) flavonols (Star et al., 1975). This was confirmed by the ^{13}C NMR which showed absence of signals around δ 90–96 (C-8) replaced by signal at δ 136.6. The ^{13}C NMR spectrum corresponds well to the shifts of kaempferol (Harborne, 1988).

MS, IR and ^1H, ^{13}C NMR data suggested a flavonol substituted with four hydroxyl groups and one methoxyl group. Therefore, the structure of flavonol (**2**) was established to be 3,5,7,4′-tetrahydroxy-8-methoxyflavone previously identified in several samples of Spanish honey (Ferreres et al., 1991), and obtained by acid hydrolysis of *Sedum sexangulare*'s leaves (Combier et al., 1968) and of 8-methoxykaempferol-3-sophoroside isolated from almond pollen (*Prunus amygdalus*) Ferreres et al., 1989. However, as far as we know, this is the first reported occurrence of a 3,5,7,4′-tetrahydroxy-8-methoxyflavone aglycone in plant.

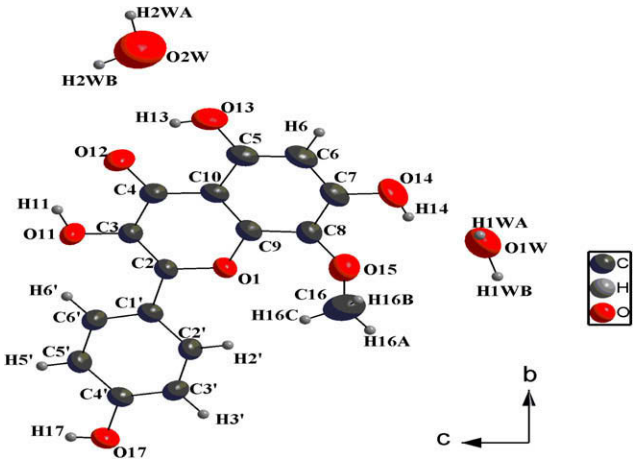


Figure 1 Perspective view, (Diamond Brandenburg, 1999), of the molecule of flavonoid (**2**). Thermal ellipsoids drawn at 50% probability levels.

3.1. X-ray structure determination

The structure of flavonoid (**2**) was unambiguously determined by single crystal X-ray diffraction analysis (Fig. 1). Suitable crystals for X-ray studies were obtained from methanol by slow evaporation. Selected geometrical parameters, bond lengths and angles are listed in Table 2 according to the numbering scheme displayed in Fig. 1.

Flavonoid (**2**) crystallizes in the monoclinic $P2_1/c$ space group with one organic molecule and two molecules of water comprising the asymetric unit (Fig. 1). The structure has revealed the basic flavone core similar to the other flavonoids reported in the litterature, i.e. a benzene ring (A) condensed with a six-membered ring (C), which in the 2-position caries a phenyl ring (B) as a substituent, see Scheme 1 (Mabry et al., 1970; Markham et al., 1978; Harborne et al., 1975).

The squeletal atoms of the two fused six-membered rings define good planes which are substantially coplanar with the associated phenyl ring. The corresponding dihedral angle being 169.6°. This configuration is in favour of a possible conjugation between the electronic systems of the two units, which

Table 2 Selected geometrical (Å, °) features of (**2**).

Bond lengths (Å)			
C9 O1	1.367(3)	C2 C1′	1.467(3)
C2 O1	1.379(3)	C2 C3	1.357(4)
O12 C4	1.266(3)	C3 C4	1.436(4)
O13 C5	1.359(4)	C4 C10	1.426(4)
Bond angles (°)			
C9 O1 C2	121.1(2)	C10 C4 C3	117.3(2)
O1 C2 C3	119.8(2)	C9 C10 C4	119.2(2)
C2 C3 C4	121.3(2)	C9 C10 C5	118.1(3)
O12 C4 C10	122.9(2)	C4 C10 C5	122.7(3)

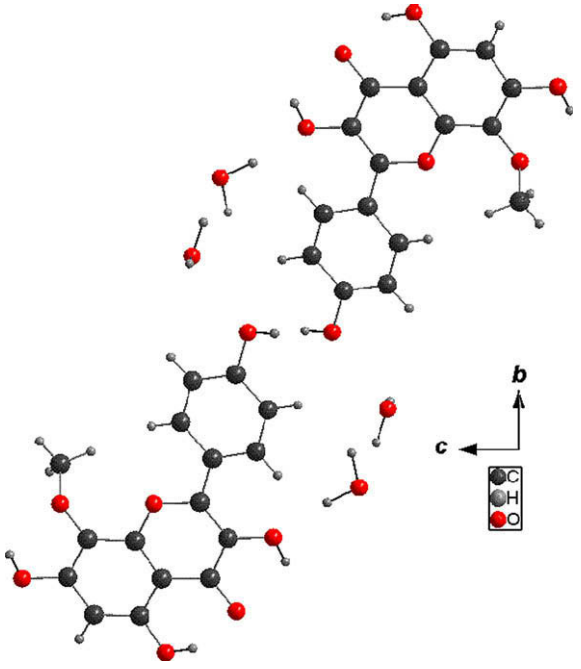


Figure 2 Dimer $[(C_{16}H_{12}O_7)\cdot 2H_2O]_2$ in the crystal of flavonoid (**2**).

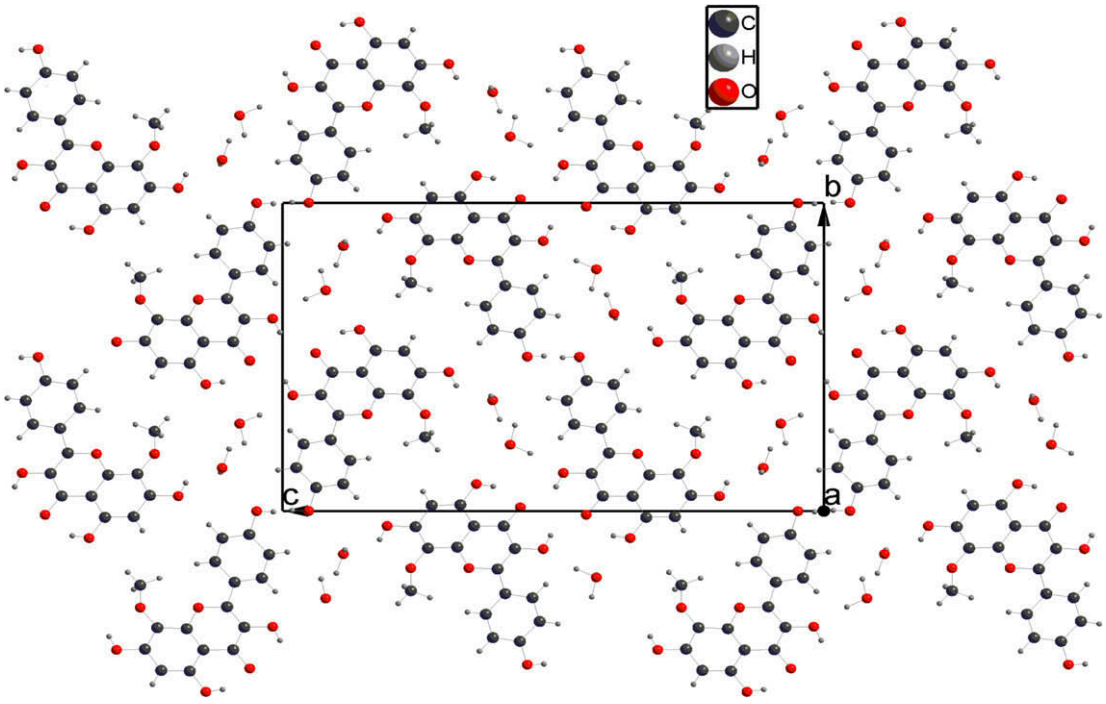


Figure 3 A projection, along [1 0 0], of the crystal structure of flavonoid (**2**).

could be checked by the relatively short value of the distance C1′-C2 [1.467(3) Å].

The methoxy group at C8 being not planar with the associated ring because a coplanar disposition would interfere sterically with O14 and H2′. Torsion angle O15 C8 C7 C6 being of 176.3°. The smaller C5–O13–H13 angle of 109.5° indicates that intramolecular attractive forces are possible (O13–H13.....O12). The usual intramolecular O14–H14...O15 (methoxy), O11–H11.....O12 (ketone) and O13–H13.....O12 (ketone) interactions are respectively 2.314(6) Å, 2.249(6) Å and 1.874(5) Å.

Moreover, there is an important intermolecular interactions [2.003(8) Å], via the dipole O17–H17, contributing to the formation of pair of molecules $[(C_{16}H_{12}O_7)\cdot 2H_2O]_2$ in the crystal of the title compound (Fig. 2). The defined "dimers" interact via the remaining hydroxyl groups to propagate the crystal in the three dimensional as can be seen on Fig. 3.

The dimers form tunnels, parallel to [1 0 0], where locate water molecules. These laters reinforce the cohesion between the molecules by adding supplementary H bonds. As might be expected with such almost planar molecules, as it is the case here, the lattice array is dominated by a parallel disposition of molecular planes, with appreciable overlap characteristic of the presence of charge-transfer interactions and numerous non-hydrogen atom contacts <4 Å.

The vasodilator activity of flavonol (**2**) was tested in rat aorta. Flavonol (**2**) inhibited the contractions evoked by high KCl or by noradrenaline in a concentration-dependent manner as shown in Fig. 4. In endothelium-denuded aorta, pD_2 (-logIC50) values for the inhibition of the contraction were 4.37 ± 0.04 and 4.58 ± 0.03 for contractions evoked by 100 mM KCl and noradrenaline, respectively.

The inhibitory potency of flavonol (**2**) against KCl-evoked contraction was not significantly affected by endothelium removal (pD_2 4.52 ± 0.05). The present results indicate that flavonol (**2**) exhibits similar vasodilator potency as that of quercetin (Ibarra et al., 2003).

Contraction was evoked by 100 mM KCl solution, or by noradrenaline (Nad, 1 μM) in rings of endothelium-intact (E(+)), or endothelium-denuded (E(−)) aorta. Data are means from 4 to 6 determinations.

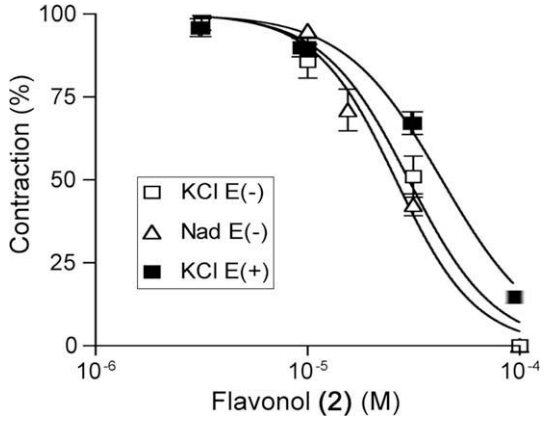


Figure 4 Effect of flavonol (**2**) on the contraction of isolated rat aorta.

4. Conclusion

This is the first report of the isolation of these flavonols from *C. villosa* subsp. *intermedia*. It is worthy to notice that it is also the first reported occurrence of a 3,5,7,4′-tetrahydroxy-8-methoxyflavone (**2**) aglycone in plant and its first X-ray crystal structure determination.

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