Bioorganic & Medicinal Chemistry Letters 19 (2009) 4684–4687

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Ulmosides A and B: Flavonoid 6-C-glycosides from *Ulmus wallichiana*, stimulating osteoblast differentiation assessed by alkaline phosphatase ☆

Preeti Rawat [a], Manmeet Kumar [a], Kunal Sharan [b], Naibedya Chattopadhyay [b], Rakesh Maurya [a,*]

[a] *Medicinal and Process Chemistry Division, Central Drug Research Institute, Lucknow 226 001, India*
[b] *Endocrinology Division, Central Drug Research Institute, Lucknow 226 001, India*

ARTICLE INFO

Article history:
Received 9 April 2009
Revised 2 June 2009
Accepted 20 June 2009
Available online 25 June 2009

Keywords:
Ulmus wallichiana
Flavonoid-6-C-glycosides
Osteogenic
Osteoblast
Differentiation

ABSTRACT

Chemical investigation of *Ulmus wallichiana* stem bark resulted in isolation and identification of three new compounds (2*S*,3*S*)-(+)-3′,4′,5,7-tetrahydroxydihydroflavonol-6-*C*-β-D-glucopyranoside (**1**), (2S,3S)-(+)-4′,5,7-trihydroxydihydroflavonol-6-*C*-β-D-glucopyranoside (**3**) and 3-*C*-β-D-glucopyranoside-2,4,6-trihydroxymethylbenzoate (**8**), together with five known flavonoid-6-C-glucosides (**2**, **4**–**7**). Their structures were elucidated using 1D and 2D NMR spectroscopic analysis. The absolute stereochemistry in compounds **1** and **3** were established with the help of CD data analysis and comparison with the literature data analysis. All the isolated compounds (**1**–**8**) were assessed for promoting the osteoblast differentiation using primary culture of rat osteoblast as an in vitro system. Compounds **1**–**3** and **5** significantly increased osteoblast differentiation as assessed by alkaline phosphatase activity.

Recently interests in assessing flavonoids as agents for prevention and treatment of osteoporosis have surged tremendously owing primarily to their presumed safety. Isoflavones, the classical phytoestrogens, have been tried in several clinical settings in menopausal women for potential beneficial effects on skeleton, but have met with limited success.[1,2] Other flavonoids, which are not isoflavones, too are targets of great interest for potential use in osteoporosis prevention and/or therapy; however, there are no reports of clinical trials with these molecules.

The major contentious issue regarding glycone and aglycone forms of the flavonoids *vis* a *vis* their in vivo functions. In addition, species difference in flavonoid metabolism makes it difficult for the observed efficacy in a given disease, such as osteoporosis in animal models to be translated to humans. O-glycosylation is a common metabolic fate for majority of flavonoids, an event that is also known to influence their stability. For example, rutin (quercetin-3-*O*-glucose rhamnose), distributed in many plants, dietary glycosides, are converted to aglycones, such as quercetin, in the large intestine in reactions catalyzed by the glycosidase of intestinal bacteria.[3] Rutin inhibits the ovariectomy-induced resorption of bone in rats[4] and quercetin has been reported to inhibit the osteoclastic resorption of bone in vitro.[5,6] So far, there is no report on C-glycosylated flavonoids for their potential bone forming effects. We hypothesized that C-glycosylated flavonoids will be better therapeutic candidates given their stability over aglycone or O-glycosylated flavonoids. *Ulmus wallichiana* plant known in traditional Indian medicinal practice to treat the bone fracture, we isolated flavonoid-C-glycosides and assessed their activity in stimulating osteoblast differentiation. Stimulation of osteoblast differentiation is a bone anabolic function that is desirable for osteoporosis therapy.[7] Our data show that four out of the seven 6-C-glycosylated flavonoids stimulate osteoblast differentiation. To the best of our knowledge, this is the first report identifying C-glycoside flavonoids as potential bone anabolic agents.

The *U. wallichiana* Planchon, belongs to the family Ulmaceae, distributed through Himalayas from Afghanistan to W. Nepal.[8] In India this plant is found in Kumaon and Garhwal Himalaya, locally called as Chamarmou, In and around Kumaon traditional healers use this plant for promoting fracture healing[9] but the effects on osteoporosis and total osteo-health and related disorders have not been scientifically explored. So far this plant has not been chemically and pharmacologically investigated. Thus, we have attempted to isolate compounds having stimulating activity on osteoblast differentiation from *U. wallichiana*.

In the present study, we focus on a fractionation of ethanolic extract of stem bark of *U. wallichiana*. This led to the isolation[10,11] of two new flavonoid C-glucosides, five known flavonoid C-glucosides and one new phenolic-C-glucoside. The known compounds were characterized as naringenin-6-C-β-D-glucopyranoside[12] (**2**), eriodictyol-6-*C*-β-D-glucopyranoside[13] (**4**), kaempferol-6-*C*-β-D-glucopyranoside[14] (**6**), quercetin-6-*C*-β-D-glucopyranoside[15] (**5**) and

☆ CDRI Communication No. 7792.

* Corresponding author. Tel.: +91 522 2612411 18x4235; fax: +91 522 2623405/2623938/2629504.
E-mail address: mauryarakesh@rediffmail.com (R. Maurya).

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doi:10.1016/j.bmcl.2009.06.074

iso-orientin[16] (**7**) by direct comparison of NMR data with those reported in literature. All these compounds were isolated for the first time from this plant. Remarkably, *U. wallichiana* is clearly an abundant source of flavonoid 6-C-glucosides. In the present communication, we describe the isolation and characterization of new compounds (**1**, **3**, **8**) and determination of the absolute configuration at C-2 and C-3 positions of compounds **1** and **3**. Compounds (**1**–**8**) were evaluated for their osteogenic activity using neonatal (1–3 day old) rat calvaria derived primary osteoblast cultures. Compounds (**1**–**3**, **5**) showed a significant stimulative effect on differentiation of cultured osteoblast cells (Fig. 1).

Compound **1** was obtained as white amorphous powder, $[\alpha]_D^{25}$ +1.33 (*c* 0.098; MeOH). The positive FABMS showed molecular ion peak at *m/z* 467 $[M+H]^+$, analyzed for $C_{21}H_{22}O_{12}$. The UV absorption at 290, 327 nm was typical of a dihydroflavonol chromophore.[17] The inspection of **1**[18] by ^{1}H NMR, ^{13}C NMR and 2D NMR (^{1}H–^{1}H COSY, HSQC, HMBC) indicated it to be taxifolin-6-*C*-β-D-glucopyranoside and reported absolute stereochemistry at C-2 and C-3 is (2*R*,3*R*).[19] The absolute configuration of **1** at C-2 and C-3, the CD spectrum showed positive Cotton effect at 304 nm and negative Cotton effect at 332 nm, which is consistent with 2*S*,3*S* configuration.[20] Further, comparison with (2*R*,3*R*)-(+)-taxifolin[21] (negative Cotton effect at 297 nm and positive Cotton effect at 332 nm) and (2*S*,3*S*)-(+)-taxifolin[22,23] (positive Cotton effect at 297 nm and negative Cotton effect at 332 nm) and from the spectroscopic evidence, the structure of **1** was established to be (2*S*,3*S*)-(+)-3′,4′,5,7-tetrahydroxydihydroflavonol-6-*C*-β-D-glucopyranoside, has not been reported previously, named ulmoside A.

Compound **3** was obtained as yellow amorphous powder, $[\alpha]_D^{25}$ +48.69 (*c* 0.115, MeOH). The positive FABMS showed molecular peak at *m/z* 451 $[M+H]^+$, analyzed for $C_{21}H_{22}O_{11}$. The UV absorption at 296, 334 nm was typical of a dihydroflavonol chromophore.[17] The close structural relationship between compounds **1** and **3** was evident from similar spectral features. The most significant difference between the ^{1}H NMR spectra of compound **1** and **3**[24] was the replacement of the ABX spin system by an A_2B_2-type pattern in that of **3**. Further inspection of **3** by ^{1}H NMR, ^{13}C NMR and 2D NMR (^{1}H-^{1}H COSY, HSQC, HMBC) indicated it to be aromadendrin-6-*C*-β-D-glucopyranoside and the reported absolute stereochemistry at C-2 and C-3 is (2*R*,3*R*).[25,26] The compound **3** showed a CD curve with positive Cotton effect at 299 nm and negative Cotton effect at 328 nm, which is consistent with a 2*S*,3*S* configuration, comparison with established absolute configuration of the aromadendrin (2*R*,3*R*)[27] and aromadendrin (2*S*,3*S*).[28] Thus structure of **3** was elucidated to be (2*S*,3*S*)-(+)-4′,5,7-trihydroxydihydroflavonol-6-*C*-β-D-glucopyranoside, has not been reported previously, named ulmoside B.

Compound **8** was obtained as dark brown amorphous powder. The EIMS displayed the molecular ion peak at *m/z* 367 $[M+Na]^+$ corresponding to the molecular formula $C_{14}H_{18}O_{10}$ supported by its NMR spectra.[29] The IR spectrum exhibited bands at 3404, 1720 and 1603 cm^{-1} indicated the presence of hydroxyl group, ester moiety, and aromatic ring. It shows UV absorption at λ_{max} 260 nm. The ^{1}H and ^{13}C NMR spectra (Table 1) showed the presence of one proton singlet at δ 5.94 (δ_C 97.0) and five aromatic quaternary carbons at δ_C 94.5, 105.1, 163.2 (2C), 165.3 indicating penta-substituted aromatic ring. The ^{1}H NMR spectrum attributable to one C-glucose moiety with the anomeric protons at δ 4.83 (d, *J* = 9.9 Hz) with corresponding carbon signals at δ 75.9, in the characteristic region of C-substituted glucoside. The assignments of carbon signals have been made by analysis of ^{1}H and ^{13}C NMR spectral data and comparison with the reported data[30] for aromatic C-glucosides. The coupling constant of the signal resulting from the anomeric proton of the glucopyranoside indicated the glucosidic linkage to have β-configuration. Further the position of glucosyl moiety in the compounds at C-3 was confirmed by HMBC correlation of anomeric proton to the C-2 and C-4. Using anomeric proton as starting point in ^{1}H–^{1}H COSY spectrum other sugar protons were assigned (Table 1). Further NMR spectra showed methyl proton singlet at δ 3.99 (δ_C 53.0) and one carbonyl at δ_C 172.0, revealed the presence of an ester group, supported by HMBC correlation between –OCH_3/CO. Thus, consideration of spectral data enabled us to construct compound **8** as 3-*C*-β-D-glucopyranoside-2,4,6-trihydroxymethylbenzoate, a new phenolic-C-glucoside named ulmoside C. This is not an artifact as it was detected by HPLC in the ethanol extract prepared by cold percolation of the plant.

Alkaline phosphatase (ALP) activity[31–34] is a phenotypic marker for the mature osteoblast. Osteoblast differentiation for compounds **1**–**8** was evaluated by measuring ALP activities, using *p*-nitrophenylphosphate (PNPP) as a substrate. Compounds **1**–**3** and **5** significantly stimulated osteoblast ALP activity compared with control (vehicle) (Fig. 2). Bone morphogenetic protein-2 (BMP-2) is a known stimulator of osteoblast differentiation.[35] We used BMP-2 as positive control to compare the differentiation promoting effects of compounds **1–3** and **5**. Our data in Figure 2 show that these four compounds have comparable level of stimulation on osteoblast differentiation to that of BMP-2. The compounds **4**, **6**–**8** exhibited neither stimulatory nor inhibitory effect on osteoblast ALP activity (data not shown). It is concluded that compounds **1**–**3** and **5** have bone anabolic function in vitro.

It is interesting to observe that flavonoid-6-C-glucosides of the type **1** and **3** possessing the (2*S*,3*S*) stereochemistry and **2** possessing the (2*S*) stereochemistry are important unit for eliciting better osteogenic activity. The same activity pattern was also observed in the compound **5** even in the absence of stereochemistry at position 2 and 3. Further, comparing the flavonoid-6-C-glucosides whether both 3 and 3′ hydroxyl groups are present or both are absent then

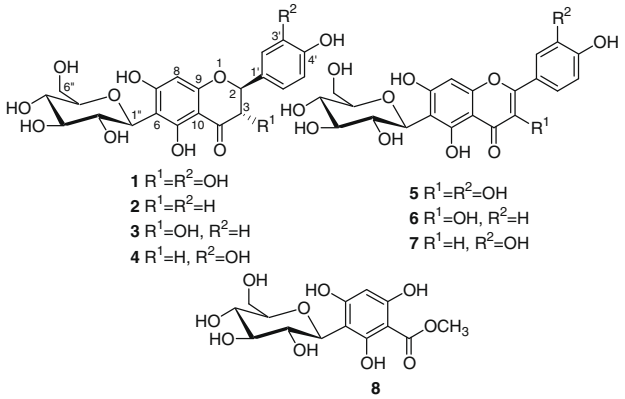

Figure 1. Isolated compounds.

Table 1
^{1}H and ^{13}C data of compound **8** in CD_3OD (300 MHz)

Position	δ_H (*J* in hertz)	δ_C	HMBC (H to C)
1	—	94.5	
2	—	165.3	
3	—	105.1	
4	—	163.2	
5	5.94 s	97.0	C-1, C-3
6	—	163.2	
1′	4.83 d (9.9)	75.9	C-2, C-4, C-3′, C-5′
2′	4.05 t (9.4)	73.0	C-3, C-4′
3′	3.44 m	80.2	C-1′, C-5′
4′	3.44 m	71.8	C-2′, C-6′
5′	3.39 m	82.6	C-1′, C-3′
6′a	3.85 dd (12.2, 1.8)	62.9	C-4′
6′b	3.71 dd (12.2, 4.9)		
CO	—	172.0	
OMe	3.39 s	53.0	CO

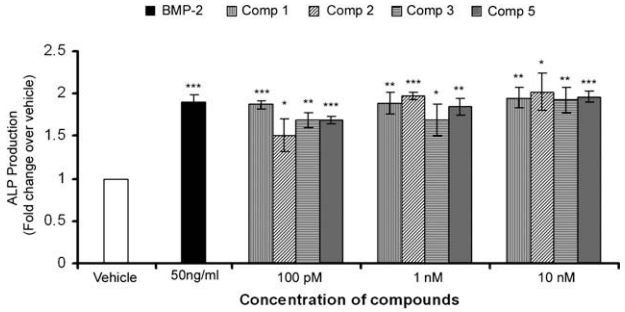

Figure 2. Effect of compounds **1**–**3** and **5** on osteoblast differentiation assessed by ALP production. 2000 cells/well were plated in 96-well plates. After 24 h, cells were treated with compounds **1**–**3** and **5** at varying concentrations for additional 48 h in complete growth media. BMP-2 (50 ng/ml) was used as positive control. All four compounds stimulated ALP production as measured by incubating with substrate solution (0.2 mM PNPP, 5 mM $MgCl_2$ in 1 M diethanolamine) and taking OD at 405 nm in an ELISA plate reader. Data expressed as mean ± SEM (***—$p \leqslant 0.001$, **—$p \leqslant 0.01$, *—$p \leqslant 0.05$) of fold change from three independent experiments.

compounds (**1**, **2**, **5**) are active. It seems that the presence of the hydroxy at C-3 or C-3′ is not important for the activity as compounds **4**, **6**, and **7** were found inactive. On the other hand, 4′-hydroxy group in ring-B and presence or absence of hydroxyl group at position 3 as in **3** possessing (2*S*,3*S*) stereochemistry and in **2** possessing (2*S*) stereochemistry also seems important for the osteogenic activity, the compound **6** do not bear the required stereochemistry, found inactive. In phenolic-C-glycosides **8**, no activity was observed, indicating that if flavonoid-C-glycosides is cleaved there is loss in activity, suggesting that flavonoid with 6-C-glycoside and the presence of hydroxyl groups at positions 5 and 7 in ring-A are important for the osteogenic activity.

In conclusion, the present study supports the use of *U. wallichiana* for treatment of fracture healing as claimed by traditional practitioners. The compounds **1**–**3** and **5** are the active principles of the plant. These compounds may serve as the starting point for design of novel semi-synthetic and synthetic compounds as osteogenic agents in the future.

Acknowledgments

Preeti Rawat and Manmeet Kumar are thankful to the CSIR, New Delhi for Senior Research fellowship and Kunal Sharan is thankful to Department of Biotechnology, Government of India for Junior Research Fellowship. Thanks to Dr. K. R. Arya, Botany Division, Central Drug Research Institute, Lucknow, for providing identified stem bark of *Ulmus wallichiana*.

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non-essential amino acid solution and sodium pyruvate. The medium was changed every alternate day. Test compounds were initially dissolved in DMSO and then diluted with serum-free medium, so that the final DMSO concentration was less than 0.1% in the conditioned medium.

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