ORIGINAL ARTICLE

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Rec. Nat. Prod. 4:2 (2010) 96-100

records of natural products

Free-radical Scavenging Flavonol 3-*O*-glycosides from the Leaves of *Ribes biebersteinii* Berl.

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(Revised February 23, 2010; Accepted Revised February 24, 2010)

Abstract: *Ribes biebersteinii* Berl. (Grossulariaceae), commonly known as 'reddish-black berry', is an Iranian medicinal plant found mainly in the region of the Arasbaran forests in Iran. Reversed-phase preparative HPLC analyses of the methanol extract of the leaves of this plant afforded four flavonol glycosides, e.g. quercetin 3-*O*-sophoroside (**1**), quercetin 3-*O*-sambubioside (**2**), kaempferol 3-*O*-sophoroside (**3**) and kaempferol 3,5-di-*O*-β-D-glucopyranoside (**4**). The free-radical-scavenging properties of the *n*-hexane, DCM and MeOH extracts, as well as the isolated compounds **1**-**4** were evaluated by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay.

Keywords: *Ribes biebersteinii* Berl.; Grossulariaceae; free-radical scavenging activity; flavonol glycosides; DPPH

1. Introduction

Ribes biebersteinii Berl. (Grossulariaceae), commonly known as 'reddish-black berry', is an Iranian medicinal plant found mainly in the region of the Arasbaran forests in Iran [1, 2]. In the East

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The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published 04/ 25/2010 EISSN:1307-6167

Azarbaiijan province of Iran, the fruits of *R. biebersteinii* (Persian name: 'Ghare-ghatt') are used as food and also in the traditional medicine as a hypotensive agent and as a retina-protectant [3]. No phytochemical studies on this species have ever been carried out previously. However, total fruit extract of this plant was reported to induce dose-dependant relaxation of rat aortic rings, pre-contracted with phenylephrine, by a mechanism independent of entothelium function or its production [3]. As part of our on-going studies on medicinal plants from the Iranian flora [4-15], we now report on the isolation and identification of four flavonol glycosides from the leaves *Ribes biebersteinii* for the first time, as well as their free radical scavenging properties.

2. Materials and Methods

2.1. Plant Material

The leaves of *Ribes biebersteinii* were collected from Arasbaran area of the East Azarbaijan province, Iran, during June-July 2007, and a voucher specimen (voucher no. TUM-ADE-674) representing this collection has been deposited in the herbarium of the School of Pharmacy, Tabriz University of Medical Sciences, Iran.

2.2 Extraction

The dried and ground leaves of *R. biebersteinii* (100 g) were Soxhlet-extracted, successively, with *n*-hexane, dichloromethane (DCM) and methanol (MeOH), 750 mL each. The extracts were dried using a rotary evaporator at a temperature not exceeding 50 ^{o}C.

2.3 Fractionation of the MeOH extract: solid-phase extraction

The MeOH extract (2 g) was subjected to Sep-Pack (C_{18}, 10 g cartridge) fractionation using a step gradient of MeOH-H_2O mixture to yield five fractions e.g. 20:80, 40:60, 60:40, 80:20 and 100:0. All fractions were dried using a rotary evaporator at a temperature not exceeding 50 ^{o}C.

2.4 Isolation of flavonol glycosides

The 40% MeOH-water Sep-Pak fraction was subjected to preparative reversed-phase HPLC analysis (Dr Maisch ODS preparative column 10 μm, 250 mm x 20 mm, solvent system: linear gradient 0-20 min, 12-20% acetonitrile (ACN) in water; isocratic 20% ACN in water during 20-24 min; linear gradient 24-29 min, 20-30% ACN in water; linear gradient 29-32 min, 30-100% ACN in water; isocratic 100% ACN during 32-42 min; flow rate 8 mL/min; detection at: 220 and 280 nm) to yield four flavonoid glycosides, e.g. quercetin 3-*O*-sophoroside (**1**, 18.0 mg, t_R = 17.4 min), quercetin 3-*O*-sambubioside (**2**, 15.2 mg, t_R = 19.4 min), kaempferol 3-*O*-sophoroside (**3**, 4.4 mg, t_R = 19.8 min) and kaempferol 3,5-di-*O*-β-D-glucopyranoside (**4**, 3.5 mg, t_R = 20.3 min) (Figure 1). The structures of these flavonoid glycosides were determined by UV (in MeOH and using various shift reagents [16], FABMS and NMR (^{1}H and ^{13}C) spectral analyses as well as by comparison with respective published data.

2.5 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) assay

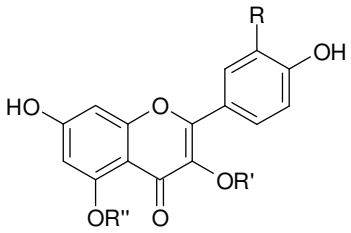
2,2-Diphenyl-1-picrylhydrazyl (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG, Bucks. Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Sigma-Aldrich, UK. The method used by Takao *et al.* [17] was adopted with appropriate modifications [18, 19].

Qualitative assay: Test compounds (**1-4**) were applied on a TLC plate and sprayed with DPPH solution (80 µg/mL) using an atomizer. It was allowed to develop for 30 min. The color change (purple on white) was noted.

Quantitative assay: Compounds **1**-**4** were dissolved in MeOH to obtain a concentration of 1 mg/mL. Dilutions were performed to obtain concentrations of $5x10^{-2}$, $5x10^{-3}$, $5x10^{-4}$, $5x10^{-5}$, $5x10^{-6}$ $5x10^{-7}$, $5x10^{-8}$, $5x10^{-9}$, $5x10^{-10}$ mg/mL. Diluted solutions (1 mL each) were mixed with DPPH (1 mL, 80 µg/mL) and allowed to stand for 30 min for any reaction to take place. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the positive control, a well known antioxidant Trolox®.

3. Results and Discussion

Among the three extracts (*n*-hexane, DCM and MeOH) of the dried and ground leaves of *R. biebersteinii,* the MeOH extract displayed significant level of free radical scavenging activity (RC_{50} = 9.1 x 10^{-3} mg/mL) in the DPPH assay (Table 1). Therefore, the MeOH extract was subjected to solid-phase extraction (Sep-Pak fractionation) to yield five fractions, out of which, the fraction eluted with 40% MeOH in water was the most active in the DPPH assay. The preparative reversed-phase HPLC analysis of the 40% Sep-Pak fraction led to the isolation of four flavonol glycosides, which were identified as quercetin 3-*O*-sophoroside (**1)** [16, 20-22], quercetin 3-*O*-sambubioside (**2)** [16, 20, 22], kaempferol 3-*O*-sophoroside (**3)** [16, 20, 21, 23] and kaempferol 3,5-di-*O*-β-D-glucopyranoside (**4)** [16, 24] on the basis of UV (in MeOH and using various shift reagents [16], FABMS and NMR (^{1}H and ^{13}C) spectral analyses as well as by comparison with respective published data.



Compounds	R	R’	R’’
1	OH	Sophorosyl (2-O-β-D-Glucopyranosyl-α-D-glucosyl)	H
2	OH	Sambubiosyl (β-D-Xylosyl-(1→2)-β-D-glucosyl)	H
3	H	Sophorosyl (2-O-β-D-Glucopyranosyl-α-D-glucosyl)	H
4	H	Glucosyl	Glucosyl

Figure 1. Flavonol glycosides (**1**-**4**) from *R. biebersteinii*

This is the first report on the phytochemical studies on *R. biebersteinii* revealing the presence of quercetin and kaempferol 3-*O*-glycosides (**1**-**4**). In addition to *O*-glycosylation at C-3 of flavonol skeleton, compound **4** also has *O*-glucosylation at C-5. While kaempferol and quercetin 3-*O*-glycosides were found in other species of the *Ribes*, kaempferol or quercetin sophoroside or sambubioside (**1**-**3**), have not been reported from any other species of this genus before. Also, kaempferol 3,5-di-*O*-β-D-glucopyranoside (**4**) has never been reported from this genus. In fact, this is

the first report on any 5-*O*-glycoside found in the *Ribes*. However, **4** was first isolated from *Dryopteris dickinsii* [24] and later from *Vicia calcarata* [25]. The incidences of 3-*O*-glycosylation on kaempferol or quercetin aglycones within the *Ribes* species might be chemotaxonomically significant.

All compounds (**1**-**4**) exhibited considerable levels of free radical scavenging activity in the DPPH assay [17-19]. Among the flavonol glycosides (**1**-**4**), quercetin 3-*O*-sophoroside (**1**) and quercetin 3-*O*-sambubioside (**2**) displayed similar free radical scavenging activity [concentration at which a test sample reduces 50% of the DPPH (80 μg/mL) absorbance at 517 nm, RC_{50} values, 5.1 x 10^{-3} and 6.2 x 10^{-3} mg/mL, respectively] which were comparable to that of the positive control Trolox (RC_{50} = 2.6 x 10^{-3} mg/mL). Kaempferol 3,5-di-*O*-β-D-glucopyranoside (**4**) was the least active free radical scavenger among the test compounds (RC_{50} = 7.9 x 10^{-2} mg/mL). Generally, the free radical scavenging activity of **1-4**, like other natural phenolic compounds, was the consequence of the phenolic moieties present in the structures. The free radical scavenging property of phenolic natural products is mainly owing to their ability to act as reducing agents, hydrogen donors and singlet oxygen quenchers, and to some extent, could also be due to their metal chelation potential [17-19]. The presence of these free radical scavengers in *R. biebersteinii* might be relevant in relation to this plant's various biological properties and medicinal uses. Also, the considerable levels of free radical scavenging property of **1**-**4** might provide plant with some protection against oxidative damages or stress.

Table 1. Free radical scavenging properties of the extracts, fractions and isolated compounds **1**-**4** from the leaves of *R. biebersteinii* in the DPPH assay

Extract/Fractions/Compound	**RC_{50} value (mg/mL)**
n-Hexane extract	1.4 x 10^{-1}
Dichloromethane extract	11.2 x 10^{-1}
Methanol extract	9.1 x 10^{-3}
Sep-Pak fraction 1 (20% MeOH in water)	9.7 x 10^{-2}
Sep-Pak fraction 2 (40% MeOH in water)	8.1 x 10^{-3}
Sep-Pak fraction 3 (60% MeOH in water)	15.3 x 10^{-2}
Sep-Pak fraction 4 (80% MeOH in water)	16.2 x 10^{-2}
Sep-Pak fraction 5 (100% MeOH)	1.0 x 10^{-1}
Quercetin 3-*O*-sophoroside (**1**)	5.1 x 10^{-3}
Quercetin 3-*O*-sambubioside (**2**)	6.2 x 10^{-3}
Kaempferol 3-*O*-sophoroside (**3**)	1.5 x 10^{-2}
Kaempferol 3,5-di-*O*-β-D-glucopyranoside (**4**)	7.9 x 10^{-2}
Trolox® (positive control)	2.6 x 10^{-3}

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