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Bioguided Fractionation of *Polygonum alpinum* and Isolation and Structure Elucidation of Active Compounds

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Abstract

Bioguided fractionation of the methanol extract of aerial parts from *Polygonum alpinum* L. (Polygonaceae) was investigated using the exudation phase of inflammation in aseptic arthritis model, which is produced by carrageenan and radical-scavenging properties. Flavonoid-containing fractions showed anti-inflammatory and radical-scavenging activities. Therefore, isolation and structure elucidation of flavonoids were carried out. The eight flavonol glycosides, which were quercetin 3-*O*-arabinofuranoside (=avicularin) **(1)**, quercetin 3-*O*-β-glucuronopyranoside **(2)**, quercetin 3-*O*-α-rhamnopyranosyl (1 → 6)-β-glucopyranoside **(3)**, quercetin 3-*O*-β-galacturonopyranoside **(4)**, quercetin 3-*O*-β-glucopyranoside **(5)**, kaempferol 3-*O*-β-galactopyranoside **(6)**, quercetin 3-*O*-β-galactopyranoside (=hyperoside) **(7)**, and myricetin 3-*O*-β-galactopyranoside **(8)**, were isolated from the methanol herb extract of *Polygonum alpinum*. The structures were established by spectroscopic methods.

Keywords: Anti-inflammatory activity, flavonol glycosides, Polygonaceae, *Polygonum alpinum*, radical scavenging effect.

Introduction

Since ancient times, plants have been used in traditional medicine. Even today, plant materials play an important role in primary health care as therapeutic agents in many developed countries. The genus *Polygonum* L. (Polygonaceae) comprises 33 species in Turkey and the East Aegean Islands (Davis, 1967). Some of them are used in traditional medicine to treat kidney stones and as antidiabetic, diuretic, and antidiarrheal agents (Baytop, 1984). A number of compounds have been isolated from *Polygonum* including sesquiterpene lactones (Isobe et al. 1980), chalcones (Maradufu & Oum, 1978; Rathore et al., 1987; Ahmed et al., 1988, 1990), anthraquinones (Kimura et al., 1983), naphthaquinones (Kimura et al., 1983), sesquiterpenoids (Fukuyama et al., 1985), lignans (Kim et al., 1994), coumarins (Petrescu et al., 1974), and stilbene glycosides (Layatilake et al., 1993). Some earlier work has been reported on the flavonoids of the same species (Isobe et al., 1987, 1979). Our recent investigation of the flavonoids of *Polygonum salicifolium* (Çalış et al., 1999) and *P. bistorta* subsp. *carneum* (Demirezer et al., 2000) revealed a rich mixture of flavonol glycosides. In this paper, we report the compounds isolated from *Polygonum alpinum* (which is widely distributed in Turkey) that are responsible for anti-inflammatory and radical scavenging effects.

Materials and Methods

General

NMR spectra were recorded on a Bruker AMX 300 NMR operating at 300 MHz for proton and 75.5 MHz for carbon by using TMS an internal standard. The solvent used was DMSO-d_6. TLC was carried out on precoated silica gel 60 F_{254} aluminum sheets (Merck). For column chromatography (CC), normal phase silica gel 60 (0.063–0.20 mm, Merck), reversed phase silica gel (LiChroprep RP-18, Merck), Sephadex LH-20 (Fluka), and Polyamid MN-Polyamid SC 6 Macherey-Nagel, Düren) were used.

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Compounds were detected by UV fluorescence and/or spraying with vanillin-H_2SO_4 reagent followed by heating at 100°C for 5–10 min and/or exposure to NH_3 vapor. For radical-scavenging TLC autographic assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH; Fluka) was used as autographic spray reagent.

Plant material

Polygonum alpinum was collected in July from Erzurum-Ispir (Turkey). A voucher specimen (HUEF 96044) has been maintained at the herbarium of the Hacettepe University, Turkey. Plant material was authenticated by K. Avci.

Extraction and fractionation

The dried powdered aerial parts of *P. alpinum* (160 g) were extracted twice with methanol (2×3.5 l) at 40°C. The MeOH extracts were combined and evaporated to dryness in *vacuo*. The crude extract was 22 g (PM). The methanol extract was chromatographed over polyamide, eluting with increasing concentrations of MeOH in H_2O (20%, 40%, 60%, 80%, and 100% MeOH; each mixture 200 ml; fraction volume 100 ml) to yield 21 fractions, which were combined into six main fractions (PM1-6).

Animals

For anti-inflammatory activity, 72 adult male Wistar albino rats, weighing between 185 and 200 g, from the experimental animal laboratory of Atatürk University were used. Animals were nourished under standard conditions.

Anti-inflammatory studies

The effect of the methanol extract (PM) and its six fractions (PM-1, PM-2, PM-3, PM-4, PM-5, PM-6) from the aerial parts of *Polygonum alpinum* was investigated on the exudation phase of inflammation in aseptic arthritis model that is produced by carrageenan. The ratio of anti-inflammatory activity of PM and PM-1, PM-2, PM-3, PM-4, PM-5, PM-6 was calculated by the following equation:

$$\text{Anti-inflammatory activity } (\%) = (1 - D/C) \times 100$$

where D represents the percentages of difference of the paw volume after PM and PM-1, PM-2, PM-3, PM-4, PM-5, PM-6 were administered to rats, and C represents the percentage difference of paw volume in the control group.

Carrageenan-induced edema in rats

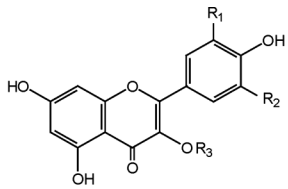
In this test, edema was induced in rats by injecting 0.2 ml of carrageenan (1% w/v) solution in distilled water into the subplantar region of the right hind paw. The volume of the paw was measured immediately after injection, six times with periods of 1 h, and once in 24 h, until the inflammation disappeared (Winter et al., 1962).

Statistical methods

Values reported are mean $\pm$ SEM. Student's *t*-test and probability level of $p < 0.05$ were chosen as the criterion of statistical significance.

Isolation of active compounds

The fractions eluted with 40% and 50% MeOH (fraction PM-3 and PM-4 were combined 1.736 g) were chromatographed over CC using polyamide as stationary phase eluting with 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 100% MeOH. Fractions 38–40 gave compound **1** (45 mg). Fractions 1–3 (195 mg) eluted with MeOH were repeatedly chromatographed over Sephadex LH-20 open CC to yield compound **2** (53 mg). Fractions 4–15 (150 mg) were chromatographed over CC using normal phase silica gel as stationary phase eluting with EtOAc/MeOH/H_2O mixture (100/16.5/13.5). Fractions 11–13 gave compound **3** (13 mg). Fractions 16–37 (782 mg) were chromatographed over polyamide eluting with 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 100% MeOH. The combined fractions 7–28 were further fractionated by MPLC (column dimensions 18.5×352 mm, LiChroprep RP-18) eluting with increasing amounts of MeOH in H_2O (20–100% MeOH) to give 47 fractions (15 ml/fraction). Fractions 21–27 gave compound **4** (30.5 mg), fraction 37 gave compound **5** (28 mg), and fractions 45–46 gave compound **6** (26.6 mg). Fractions 32–36 were purified by MPLC using MeOH in H_2O (20–100% MeOH), and compound **7** and compound **8** were obtained (Fig. 1).



Compound	R1	R2	R3
1	OH	H	Arabinose
2	OH	H	Glucuronic acid
3	OH	H	Rhamnosyl-1″→6′ Glucose
4	OH	H	Galacturonic acid
5	OH	H	Glucose
6	H	H	Galactose
7	OH	H	Galactose
8	OH	OH	Galactose
9	OH	H	Mannuronic acid

Figure 1. Structures of flavonoids in *Polygonum alpinum*.

Quercetin 3-*O*-*arabinofuranoside* (**1**): ^{1}H NMR (300 MHz) and ^{13}C NMR (75.5 MHz) data superimposable with those reported in the literature (Markham, 1982, 1993; Agrawal, 1989).

Quercetin 3-*O*-β-*glucuronopyranoside* (**2**): ^{1}H NMR (300 MHz) and ^{13}C NMR (75.5 MHz) data superimposable with those reported in the literature (Markham, 1982, 1993; Agrawal, 1989).

Quercetin 3-*O*-α-*ramnopyranosyl* (1 → 6)-β-*glucopyranoside* (**3**): ^{1}H NMR (300 MHz) and ^{13}C NMR (75.5 MHz) data superimposable with those reported in the literature (Markham, 1982, 1993; Agrawal, 1989).

Quercetin 3-*O*-β-*galacturonopyranoside* (**4**): ^{1}H NMR (300 MHz) and ^{13}C NMR (75.5 MHz) data superimposable with those reported in the literature (Markham, 1982, 1993).

Quercetin 3-*O*-β-*glucopyranoside* (**5**): ^{1}H NMR (300 MHz) and ^{13}C NMR (75.5 MHz) data superimposable with those reported in the literature (Markham, 1982, 1993; Agrawal, 1989).

Kaempferol 3-*O*-β-*galactopyranoside* (**6**): ^{1}H NMR (300 MHz) and ^{13}C NMR (75.5 MHz) data superimposable with those reported in the literature (Markham, 1982, 1993; Agrawal, 1989).

Quercetin 3-*O*-β-*galactopyranoside* (**7**): ^{1}H NMR (300 MHz) and ^{13}C NMR (75.5 MHz) data superimposable with those reported in the literature (Markham, 1982, 1993; Agrawal, 1989).

Myricetin 3-*O*-β-*galactopyranoside* (**8**): ^{1}H NMR (300 MHz) and ^{13}C NMR (75.5 MHz) data superimposable with those reported in the literature (Markham, 1982, 1993; Agrawal, 1989).

Reaction of DPPH radical: TLC autographic assay

After developing and drying, TLC plates were sprayed with a 0.2% DPPH solution in MeOH. The plates were examined 30 min after spraying. Active compounds appeared as yellow spots against a purple background (Takao et al., 1994; Cuendet et al., 1997). Quercetin was used as a reference compound.

Results and Discussion

The methanol extract of the herbs of *Polygonum alpinum* was fractionated over a polyamide column with increasing polarity of methanol in water. The anti-inflammatory activity and radical-scavenging effect were identified on the collected fractions. The carrageenan-induced edema test in rats was evaluated. Bioguided fractionation of *Polygonum alpinum* led to the isolation of six fractions (PM-1–PM-6). Their anti-inflammatory activity is shown in Table 1. It is evident from these data that the strongest anti-inflammatory activity was found in PM-3 and PM-6. Anti-inflammatory potential sequence of fractions was PM-6 > PM-3 > PM-4 > PM-2 > PM-1 > PM-5. From these fractions, eight flavonoids were isolated by repeated column chromatography (silica gel, RP-18, Sephadex LH-20). The chemistry of *Polygonum alpinum* seems to be typical of the genus. All of the structures were established by NMR and 2D-NMR techniques.

In the ^{1}H NMR spectrum, a 3,4′-dihydroxylation for ring B for compounds **2**, **5**, and **7** (for 2′-H a doublet at 6.80–6.90 ppm, $J = 8.33$–8.49 Hz interval, for 5′-H a doublet at 7.31–7.95 ppm, $J = 2.10$–2.19 Hz interval, for 6′-H a doublet at 7.19–7.59 ppm, $J = 2.12$–2.21 Hz range) was proved. A doublet at 6.18–6.21 ppm, $J = 1.62$–2.03 Hz interval for 6-H and at 6.35–6.40 ppm, $J = 1.55/2.04$ Hz interval for 8-H were detected. Anomeric protons for glucose, galactose, glucuronic acid, and galacturonic acid resonated similarly at 5.1–5.49 ppm, $J = 7.1$–7.66 Hz interval. A singlet at 5.49 ppm was seen for arabinose. As a second sugar, rhamnose bound on glucose resonated at 4.51 ppm. The ^{13}C NMR data clearly showed quercetin for six substances as genin and a C-3 glycosylation. The subspectrum of the sugars with high digital resolution, the results of HMQC, HMBC, and ^{1}H, ^{1}H-COSY experiments, and the absolute values of the

Table 1. The effect of methanol extract of *Polygonum alpinum*, its fractions, and indomethacin on inflammation produced by carrageenan.

Drugs	Dose (mg/kg)	Paw volume before infl.	Paw volume after 4 h infl.	Difference volume of paw (%)	Anti-inflammation (%)	p
MeOH	25	0.81	1.22	0.41 ± 0.050	29	<0.04
PM-1	25	0.96	1.38	0.42 ± 0.036	27	<0.03
PM-2	25	0.80	1.20	0.47 ± 0.039	31	<0.02
PM-3	25	0.87	1.21	0.34 ± 0.067	41	<0.03
PM-4	25	0.90	1.27	0.37 ± 0.045	36	<0.01
PM-5	25	0.87	1.39	0.52 ± 0.076	10.3	>0.5
PM-6	25	0.96	1.27	0.31 ± 0.039	45.7	<0.003
I	25	0.97	1.10	0.13 ± 0.053	77	<0.001
Control	–	0.83	1.41	0.58 ± 0.41	–	–

I, indomethacin.

Table 2. The effect of pure compounds on carrageenan-induced paw edema.

Drugs	Dose (mg/kg)	Paw volume before infl.	Paw volume after 4 h infl.	Anti-inflammation (%)	p
Comp. **1**	10	0.97	1.30 ± 1.47	26.7	>0.05
Comp. **2**	10	1.10	1.52 ± 2.30	0	–
Comp. **3**	10	1.12	1.57 ± 2.60	0	–
Comp. **4**	10	1.00	1.60 ± 1.90	0	–
Comp. **5**	10	0.97	1.60 ± 2.00	0	–
Comp. **6**	10	1.17	1.72 ± 1.80	0	–
Comp. **7**	10	1.10	1.67 ± 2.30	0	–
Comp. **8**	10	1.10	1.45 ± 1.90	22.0	–
Comp. **9**	10	1.17	1.60 ± 1.33	0	–
Control	10	1.17	1.75 ± 2.70	0	–

coupling constants indicated the presence of a glucopyranosyl, galactopyranosyl, glucuronopyranosyl, and galacturonopyranosyl moiety with β-configuration at the anomeric carbon. For uronic acids, the ^{13}C NMR shifts of position 6 are the 172–176 interval. The separations yielded quercetin 3-*O*-arabinofuranoside (= avicularin) (**1**), quercetin 3-*O*-β-glucuronopyranoside (**2**), quercetin 3-*O*-α-rhamnopyranosyl (1→6)-β-glucopyranoside (**3**), quercetin 3-*O*-β-galacturonopyranoside (**4**), quercetin 3-*O*-β-glucopyranoside (**5**), kaempferol 3-*O*-β-galactopyranoside(**6**), quercetin 3-*O*-β-galactopyranoside (= hyperohyperoside) (**7**), and myricetin 3-*O*-β-galactopyranoside (**8**).

Active fraction PM-3 contained quercetin 3-*O*-α-rhamnopyranosyl (1→6)-β-glucopyranoside **(3)** and quercetin 3-*O*-β-glucuronopyranoside **(2)**, and active fraction PM-6 contained quercetin 3-*O*-arabinofuranoside **(1)**. Furthermore, anti-inflammatory effect of these three compounds were tested. Quercetin 3-*O*-α-rhamnopyranosyl (1→6)-β-glucopyranoside **(3)** and quercetin 3-*O*-β-glucuronopyranoside **(2)** showed no activity, whereas quercetin 3-*O*-arabinofuranoside **(1)** showed good activity. Quercetin 3-*O*-galacturonopyranoside **(4)**, quercetin 3-*O*-glucopyranoside **(5)**, kaempferol 3-*O*-galactopyranoside **(6)**, and quercetin 3-*O*-galactopyranoside did not show any activity, while myricetin 3-*O*-galactopyranoside **(8)** showed moderate activity (Table 2).

Radical-scavenging properties of the fractions and compounds (**1–8**) were evaluated against the DPPH radical (Takao et al., 1994; Cuendet et al., 1997). By using DPPH as a TLC spray reagent, compounds **1–8** and fractions PM-3, PM-4, and PM-6 appeared as yellow spots against a purple background, whereas fractions PM-1, PM-2 and PM-5 did not react with the radical. Quercetin glycosides and myricetin glycoside were more active in all concentrations applied, whereas the kaempferol glycoside showed lower activity. These results indicate that *ortho*-hydroxyl groups are an essential feature for the antioxidant properties of the flavonoid type compounds.

Previously, the following flavonoids were reported from *Polygonum* species: quercetin 3-*O*-arabinofuranoside (Kim et al., 1994), quercetin 3-*O*-α-L-rhamnopyranosyl (1→6)-β-glucopyranoside, quercetin 3-*O*-glucopyranoside, quercetin 3-*O*-galactopyranoside (= hyperoside) (Midiwo et al., 1994; Demirezer et al., 2000; Smolarz, 2002), quercetin 3-*O*-β-glucuronopyranoside (Çalıs et al., 1999; Demirezer et al., 2000; Smolarz, 2002; Peng et al., 2003), quercetin 3-*O*-α-rhamnopyranoside (Çalıs et al., 1999; Demirezer et al., 2000; Smolarz, 2002; Peng et al., 2003), kaempferol 3-*O*-α-rhamnopyranoside (Demirezer et al., 2000), kaempferol 3-*O*-glucopyranoside (= astragalin) (Çalis et al., 1999; Smolarz, 2002, Peng et al., 2003), kaempferol 3-*O*-galactopyranoside, and quercetin 3-*O* (2″-*O*-galloyl) β-glucopyranoside (Çalis et al. 1999). This is the first report of the isolation of quercetin 3-*O*-galacturonopyranoside and myricetin 3-*O*-β-galactopyranoside from the *Polygonum* genus.

In conclusion, phytochemical and biological activity studies were performed on the herbs of *Polygonum alpinum*. Although we carried out an activity test with a small amount of substances, we observed anti-inflammatory activity. If we could increase the quantity of compounds, the response would probably be more intense.

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