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## Constituents of *Plantago major* subsp. *intermedia* with antioxidant and anticholinesterase capacities

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The methanol extract of *Plantago major* subsp. *intermedia* (Gilib.) Lange afforded 4 known compounds, namely isomartynoside (**1**), 10-hydroxymajoroside (**2**),  $\beta$ -sitosterol (**3**), and ursolic acid (**4**). Their structures were established by spectroscopic methods. After determination of the total phenolic and flavonoid contents of the methanol extract, the antioxidant potentials of the crude extract and isolated compounds **1-4** were determined by  $\beta$ -carotene bleaching, DPPH free radical and ABTS cation radical scavenging, and cupric reducing antioxidant capacity methods. The methanol extract, rich in phenolic contents, indicated the same DPPH free radical scavenging activity (72% inhibition) as a standard compound, butylated hydroxytoluene, at 100  $\mu\text{g/mL}$ . Isomartynoside (**1**), a phenylpropanoid glycoside, showed the best inhibition of lipid peroxidation, ABTS cation radical scavenging activity, and cupric reducing antioxidant capacity among the tested samples. The anticholinesterase effects of the methanol extract and isolated compounds **1-4** were established using the Ellman method. A triterpenic acid, ursolic acid (**4**), exhibited moderate acetyl- (54.01  $\pm$  0.82%) and butyryl-cholinesterase (68.74  $\pm$  0.36%) inhibitory activity at 200  $\mu\text{g/mL}$ . Isomartynoside (**1**) was isolated here for the first time from a *Plantago* species; the antioxidant and anticholinesterase activities of *P. major* subsp. *intermedia* and compounds **1-2** were not previously determined.

**Key Words:** *Plantago major* subsp. *intermedia*, Plantaginaceae, isomartynoside, phenylpropanoid glycoside, antioxidant, anticholinesterase

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## Introduction

In Turkey, the family Plantaginaceae is represented only by 1 genus, *Plantago* L., which has 22 species (24 taxa), 2 of which are endemic.<sup>1,2</sup> The leaves and seeds of *Plantago* species have been commonly used in traditional medicine since ancient times all around the world. Phytochemical studies have shown that the main constituents of *P. major* L. leaves (*P. major* subsp. *major* L.) are iridoids and caffeic acid derivatives, which have been suggested to be taxonomic markers in family Plantaginaceae.<sup>3</sup> *P. major* leaves have been used for the treatment of many diseases, such as digestive, respiratory, skin, and infectious diseases, in Turkey and throughout the world.<sup>4,5</sup> The aerial parts and seeds of *P. asiatica* L. have been used as antiinflammatory, antihypertensive, diuretic, antitussive, expectorant, and antiphlogistic treatments in Chinese and Japanese traditional medicines.<sup>6,7</sup>

In Turkey, there are 2 subspecies of *Plantago major*: *P. major* subsp. *intermedia* and *P. major* subsp. *major*.<sup>1</sup> A literature survey revealed that many phytochemical and biological studies have been carried out on *P. major* subsp. *major*.<sup>5</sup> The aim of this first study of *P. major* subsp. *intermedia* (Gilib.) Lange was to evaluate its phytochemical properties and in vitro antioxidant and anticholinesterase activities. Total phenolic and flavonoid contents of the methanol extract prepared from the aerial parts of *P. major* subsp. *intermedia* were determined as pyrocatechol and quercetin equivalents, respectively. Four known compounds, isomartynoside (**1**), 10-hydroxymajoroside (**2**),  $\beta$ -sitosterol (**3**), and ursolic acid (**4**), were obtained from the plant.<sup>8-11</sup> The antioxidant capacity of the methanol extract and isolated compounds **1-4** was established by  $\beta$ -carotene bleaching, DPPH free radical and ABTS cation radical scavenging, and cupric reducing antioxidant capacity methods. The anticholinesterase activity of the crude extract and isolated compounds **1-4** was determined using the Ellman method.

## Experimental

### General procedures

The UV spectra ( $\lambda_{max}$ ) were recorded on a Shimadzu UV-1601 in MeOH, IR spectra ( $\nu_{max}$ ) on a PerkinElmer One B in  $\text{CHCl}_3$ , NMR spectra on a Varian UNITY INOVA spectrometer operating at 500 MHz for  $^1\text{H-NMR}$  and 125 MHz for  $^{13}\text{C-NMR}$  (TMS as an internal standard) including APT, and HRESI-MS spectra on a Bruker Microsoft Q spectrometer. A Thermo pH meter, an Elma S15 ultrasonic bath, a vortex (LMS Co., Ltd.), and a BioTek Power Wave XS were used for the activity assays.

### Plant material

The aerial parts of *P. major* subsp. *intermedia* were collected from northeastern Turkey (Patnos-Ağrı) in June 2008 and identified by Assoc. Prof. Dr. Emine Akalın Uruşak. A voucher specimen was deposited in the herbarium of İstanbul University (ISTE 85778).

## Extraction, isolation, and identification

The dried and powdered aerial parts of the plant (1.2 kg) were macerated with 6 L of methanol at room temperature (25 °C) 3 times (24 h × 3). After filtration, the solvent was evaporated to dryness in a vacuum. The crude extract (35 g) was fractionated on a silica gel column (3.5 × 150 cm). The column was eluted with petroleum ether (40-60°) followed by a gradient of chloroform, and then methanol up to 100%. The similar fractions were combined with TLC control and then further subjected to preparative thin layer chromatography to yield compounds **1-4** using the following solvent systems: isomartynoside (**1**, 28 mg) and 10-hydroxymajoroside (**2**, 22 mg) (dichloromethane-methanol, 4:1),  $\beta$ -sitosterol (**3**, 15 mg) (toluene-diethyl ether, 7:1), and ursolic acid (**4**, 12 mg) (toluene-diethyl ether, 2:1). TLC plates were visualized by spraying with cerium(IV) sulfate dissolved in 10% sulfuric acid followed by illumination under UV light.

## Determination of total phenolic content

The concentrations of the phenolic content in the methanol extract were expressed as micrograms of pyrocatechol equivalents (PEs). The phenolic content was determined as described in the literature.<sup>12</sup> The concentration of phenolic compounds was calculated according to the following equation, obtained from the standard pyrocatechol graphic:

$$\text{Absorbance} = 0.0160 \text{ pyrocatechol } (\mu\text{g}) + 0.0246 \text{ (R}^2 = 0.9975\text{)}.$$

## Determination of total flavonoid content

The measurement of the flavonoid concentration was based on the method described by Moreno et al. with a slight modification and results were expressed as quercetin equivalents.<sup>13</sup> The concentration of flavonoid compounds was calculated according to the following equation:

$$\text{Absorbance} = 0.1341 \text{ quercetin } (\mu\text{g}) - 0.0956 \text{ (R}^2 = 0.9992\text{)}.$$

## Antioxidant activity

### Determination of antioxidant activity with the $\beta$ -carotene bleaching method

The antioxidant activity was established by using the  $\beta$ -carotene-linoleic acid test system.<sup>14</sup>

### DPPH free radical scavenging activity

The free radical scavenging activity of the samples was determined by the DPPH assay as described by Blois.<sup>15</sup>

### ABTS cation radical decolorization assay

The ABTS<sup>•+</sup> scavenging activity was determined according to the method of Re et al. with slight modifications.<sup>16</sup>

### Cupric reducing antioxidant capacity (CUPRAC)

Cupric reducing antioxidant capacity of the samples was determined according to the method described by Apak et al.<sup>17</sup>

### Anticholinesterase activity

Acetyl- (AChE) and butyryl-cholinesterase (BChE) inhibitory activities were established by slightly modifying the spectrophotometric method developed by Ellman et al.<sup>18</sup>

### Statistical analysis

The results were expressed as the mean  $\pm$  standard deviation (SD) of 3 parallel measurements. The statistical significance was estimated using Student's t-test;  $P < 0.05$  was regarded as significant.

## Results and discussion

Since *Plantago* species are well-known medicinal plants in every part of the world, and since some of them (*P. coronopus*, *P. lanceolata*, *P. serraria*) have been used for food and animal feeding, they have been investigated chemically and biologically by many research groups.<sup>19</sup> The results of these studies revealed that the extracts prepared from *Plantago* species possess significant antioxidant activity.<sup>19,20</sup> We report here for the first time the antioxidant and anticholinesterase potential of *P. major* subsp. *intermedia* and isolated compounds **1-2**.

A phenylpropanoid glycoside (isomartynoside, **1**), an iridoid glycoside (10-hydroxymajoroside, **2**), a steroid ( $\beta$ -sitosterol, **3**), and a triterpenic acid (ursolic acid, **4**) were isolated from the methanol extract of *P. major* subsp. *intermedia* (Figure 1). The structure determination of known compounds **1-4** was established by spectral methods (UV, IR, NMR, mass spectrometry) and their spectral data were compared to those of standard samples.

Total phenolic and flavonoid contents of the methanol extract prepared from the aerial parts were determined as pyrocatechol and quercetin equivalents, respectively (Table 1). The methanol extract, which was rich in phenolic contents, showed the best DPPH free radical scavenging activity among the tested samples and exhibited the same effect with butylated hydroxytoluene (BHT), a standard compound, at 100  $\mu\text{g/mL}$  (Figure 2). The methanol extract indicated no inhibition against acetyl- and butyryl-cholinesterase at 200  $\mu\text{g/mL}$  (Table 2).

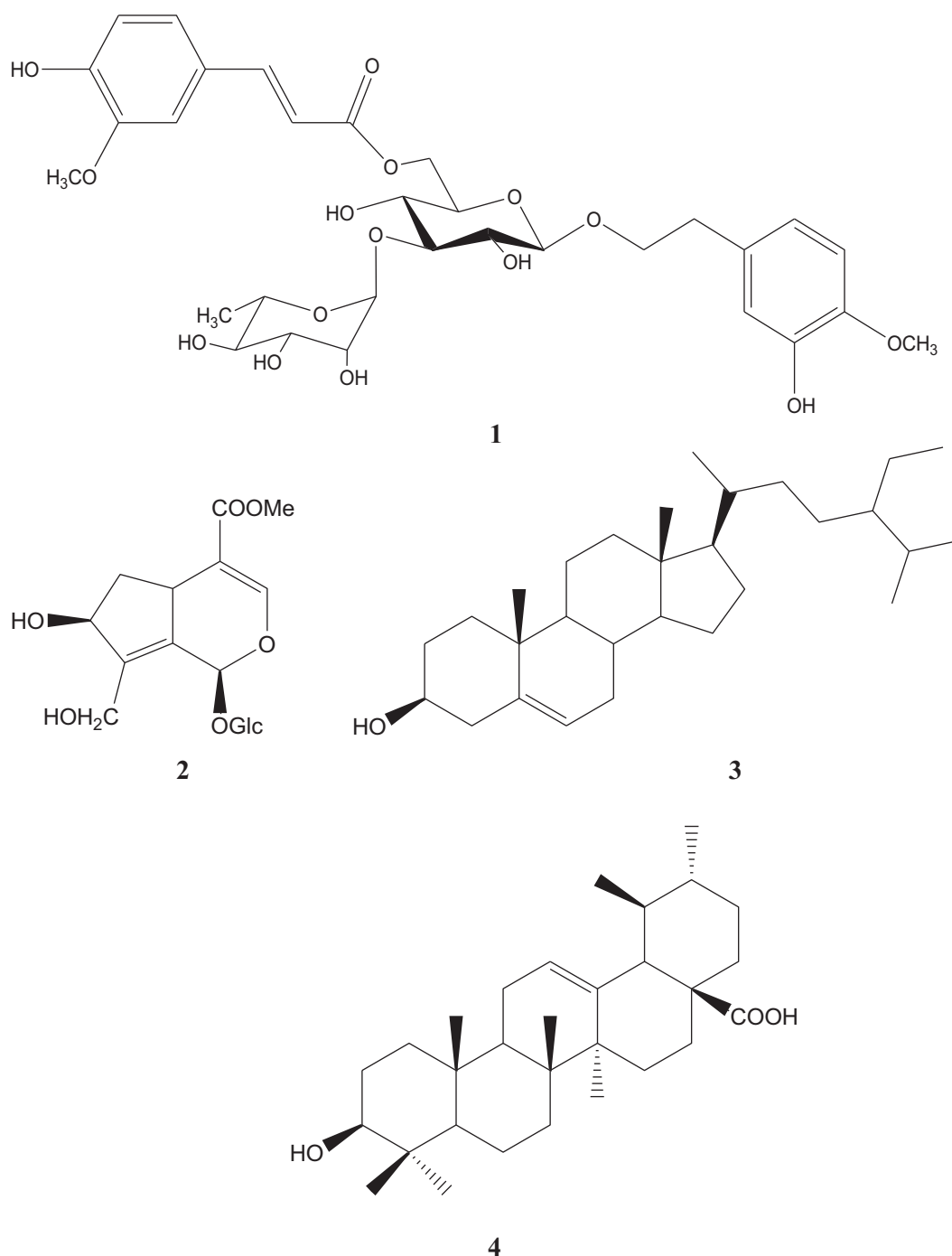
**Table 1.** Total phenolic and flavonoid contents of the methanol extract of *Plantago major* subsp. *intermedia* (PM).<sup>a</sup>

Extract	Phenolic content ( $\mu\text{g}$ PEs/mg extract) <sup>b</sup>	Flavonoid content ( $\mu\text{g}$ QEs/mg extract) <sup>c</sup>
PM	131.41 $\pm$ 0.22	15.76 $\pm$ 0.12

<sup>a</sup> Values are means  $\pm$  SD of 3 parallel measurements ( $P < 0.05$ ).

<sup>b</sup> PEs: pyrocatechol equivalents.

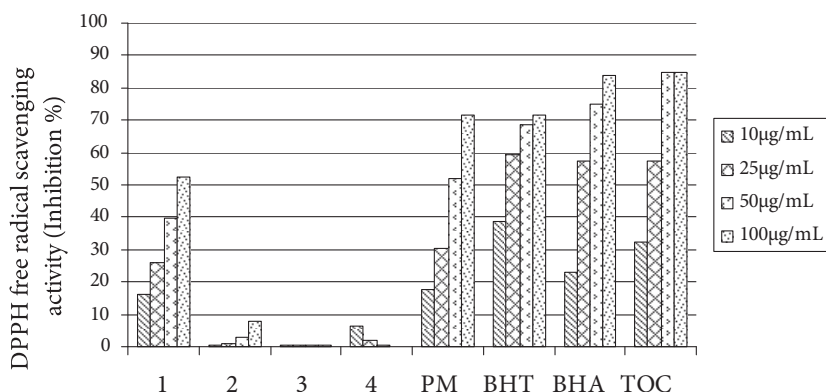
<sup>c</sup> QEs: quercetin equivalents.



**Figure 1.** Structures of compounds 1-4.

Isomartynoside (**1**) was previously obtained from *Galeopsis pubescens*, and then from *Prostanthera melissifolia*, *Clerodendron trichotomum*, and *Pedicularis rex*.<sup>8,21–23</sup> Isomartynoside (**1**) was isolated here for the first time from a *Plantago* species. Although isomartynoside (**1**) possessed a weak inhibition against acetyl- ( $21.84 \pm 3.84\%$ ) and butyryl-cholinesterase ( $25.66 \pm 2.90\%$ ), it showed the highest inhibition of lipid

peroxidation, DPPH free radical and ABTS cation radical scavenging activities, and cupric reducing antioxidant capacity among isolated compounds 1-4 at all concentrations (Table 2, Figures 2-5). Kim et al. investigated the HIV-1 integrase inhibitory activity of 7 phenylpropanoid glycosides and found that isomartynoside (1) exhibited no activity against HIV-1 integrase.<sup>22</sup> The inhibitory activities of the phenylpropanoid glycosides isolated from *Clerodendron trichotomum* against angiotensin-converting enzyme were determined by Kang et al.; isomartynoside (1) was found to be one of the active compounds.<sup>24</sup>



**Figure 2.** Free radical scavenging activity of compounds 1-4, PM, BHT, BHA, and  $\alpha$ -TOC. Values are means  $\pm$  SD, n = 3, P < 0.05, significantly different with Student's t-test.

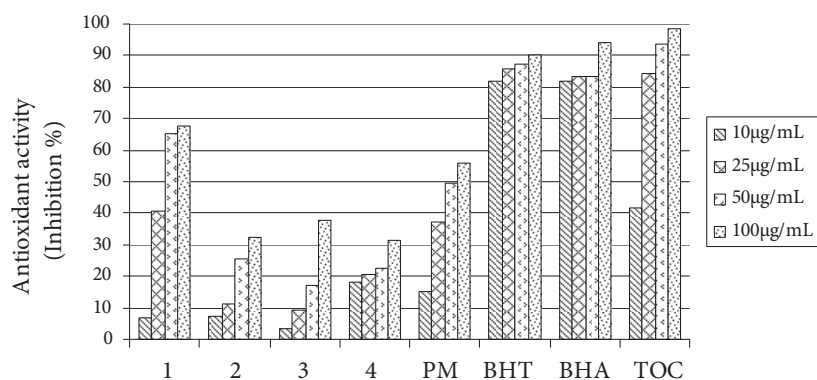
**Table 2.** Anticholinesterase activity of PM and compounds 1-4 at 200  $\mu$ g/mL.<sup>a</sup>

Samples	Inhibition % against AChE	Inhibition % against BChE
1	21.84 $\pm$ 3.84	25.66 $\pm$ 2.90
2	16.68 $\pm$ 4.23	9.83 $\pm$ 1.87
3	NA	12.15 $\pm$ 0.73
4	54.01 $\pm$ 0.82	68.74 $\pm$ 0.36
PM	NA	27.83 $\pm$ 3.86
Gаланthamine <sup>b</sup>	89.98 $\pm$ 0.61	92.47 $\pm$ 0.63

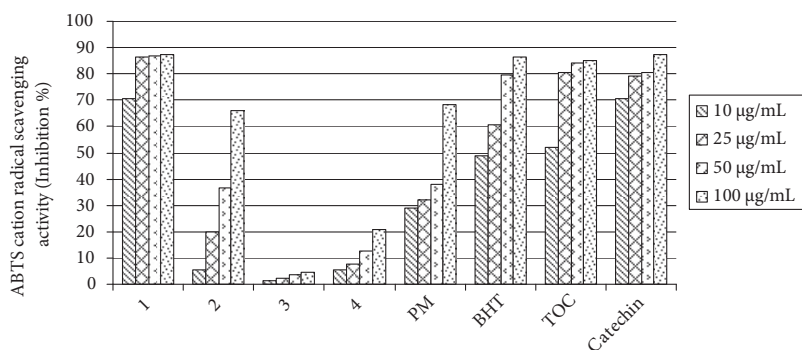
<sup>a</sup> Values expressed are means  $\pm$  SD of 3 parallel measurements (P < 0.05).

<sup>b</sup> Standard drug.

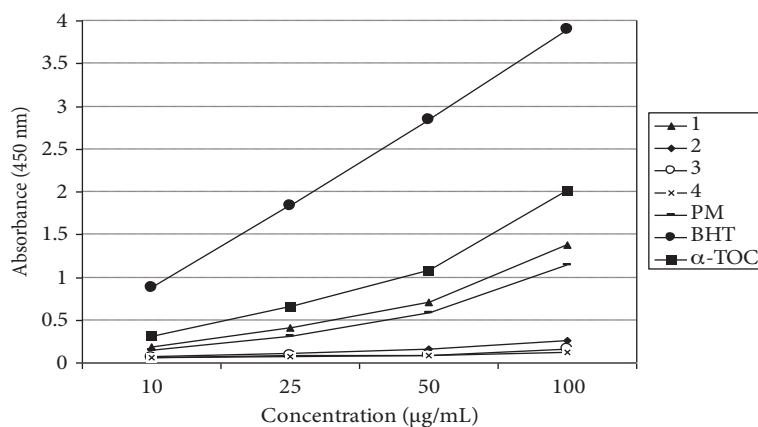
10-Hydroxymajoroside (2), which was obtained previously from *Plantago cornuti* Gouan L., showed 66% inhibition at 100  $\mu$ g/mL in the ABTS cation radical scavenging activity method; it was inactive in other activity tests (Figure 4).<sup>9</sup>  $\beta$ -Sitosterol (3) and ursolic acid (4) were found to be inactive at all concentrations in the activity assays, although ursolic acid (4) indicated moderate anticholinesterase activity (Table 2). Ursolic acid is a triterpenic acid with various activities, including antitumorigenic and trypanocidal activities.<sup>25,26</sup> Ringbom et al. showed that ursolic acid (4) isolated from a hexane extract of *P. major* possessed a significant cyclooxygenase-2 (COX-2) inhibitory effect.<sup>27</sup>



**Figure 3.** Inhibition (%) of lipid peroxidation of compounds 1-4, PM, BHT, BHA, and  $\alpha$ -TOC by  $\beta$ -carotene bleaching method. Values are means  $\pm$  SD, n = 3, P < 0.05, significantly different with Student's t-test.



**Figure 4.** ABTS cation radical scavenging activity of compounds 1-4, PM, BHT,  $\alpha$ -TOC, and (+)-catechin. Values are means  $\pm$  SD, n = 3, P < 0.05, significantly different with Student's t-test.



**Figure 5.** Cupric reducing antioxidant capacity of compounds 1-4, PM, BHT, and  $\alpha$ -TOC. Values are means  $\pm$  SD, n = 3, P < 0.05, significantly different with Student's t-test.



## Conclusion

This study showed that the antioxidant activity of *P. major* subsp. *intermedia* can be related to the presence of the phenylpropanoid glycoside, isomartynoside (**1**). Therefore, other *Plantago* species could be investigated phytochemically and biologically to find their active compounds.

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