

Assessment of Enzyme Inhibitory and Antioxidant Activities of Lignans from *Taxus baccata* L.

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Phytochemical investigations of *Taxus baccata* L. by successive chromatographic methods resulted in the isolation of the lignans lariciresinol (**1**), taxiresinol (**2**), 3'-demethylsolariciresinol-9'-hydroxyisopropylether (**3**), isolariciresinol (**4**), and 3-demethylsolariciresinol (**5**) as well as taxoids. Compounds **1**–**5** were evaluated for their acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and lipoxygenase (LOX) inhibitory activities, which play a role in the pathogenesis of Alzheimer's disease (AD), by *in vitro* spectrophotometric methods, while they were also screened for their antioxidant capacity in 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferrous ion-chelating effect, and ferric-reducing antioxidant power (FRAP) at 125, 250, 500, and 1000 µg ml⁻¹. All compounds exhibited a moderate inhibition against both BChE and LOX, whereas they were inactive towards AChE. The compounds displayed a great scavenging activity against DPPH especially at 500 and 1000 µg ml⁻¹. Besides, they were found to exert noteworthy reducing antioxidant power on ferric ions. In particular, the FRAP of compounds **2** (3.552 ± 0.02), **4** (3.021 ± 0.71), and **5** (3.533 ± 0.01) were as high as that of the reference chlorogenic acid (3.618 ± 0.01) at 1000 µg ml⁻¹. None of the compounds exhibited chelating ability against ferrous ions.

Key words: *Taxus baccata*, Lignans, Biological Activity

Introduction

The genus *Taxus* L. (Taxaceae), known as “yew”, is widely distributed in the northern hemisphere, especially in Europe, North America, Eastern Asia and Asia Minor. There are eight *Taxus* species and two hybrids throughout the world, and *T. baccata* L. (European yew) is the single representative of the genus in Turkey (Davis and Cullen, 1965; Van Rozendall *et al.*, 1999). The discovery of paclitaxel (TaxolTM) from the bark of the Pacific yew (*Taxus brevifolia* Nutt.) and its introduction into cancer chemotherapy have attracted many scientists to investigate the chemical constituents of other *Taxus* species growing worldwide. Paclitaxel is the most important agent in human cancer chemotherapy and used for the treatment of several forms of breast, liver, lung, blood, and ovary cancers (Rowinsky, 1997). Up till now, isolation of a great number of taxoids as well as lignans, flavonoids, steroids, and sugar derivatives has been reported from different parts

of various *Taxus* species (Baloglu and Kingston, 1999; Parmar *et al.*, 1999).

Since all plant parts of yew, except arillus, contain toxic taxine alkaloids, the plant has been rarely documented as folk medicine. It was reported to be used as antimalarial and antirheumatic in historical documents (Bryan-Brown, 1932; Appendino, 1993). In Ayurvedic medicine, known indigenously as Talispatra, it is recommended to be used as emmenagogue, sedative, antispasmodic, and aphrodisiac (Bryan-Brown, 1932; Shanker *et al.*, 2002), as well as to treat asthma (Singh, 1995). Besides, a literature survey revealed that it was also listed in Avicenna's cardiac drugs, namely Zarnab (Tekol, 1989). In Turkish folk medicine, it is known to be used as sedative and stomachic (Baytop, 1999).

A great number of pharmacological effects have been ascribed to lignans such as antibacterial, antifungal, antiviral, antioxidant, anticancer, and anti-inflammatory (Rios *et al.*, 2002). Our phytochemical investigation on *T. baccata* growing in

Turkey by successive chromatographic methods resulted in the isolation of five lignans, namely lariciresinol (**1**), taxiresinol (**2**), 3'-demethylsolariciresinol-9'-hydroxyisopropylether (**3**), isolariciresinol (**4**), and 3-demethylsolariciresinol (**5**) (Erdemoglu *et al.*, 2003, 2004a). In our previous studies, *in vivo* anti-inflammatory and antinociceptive activities and *in vivo* antiulcerogenic potency of the lignans **1–5** isolated from *T. baccata* were investigated (Kupeli *et al.*, 2003; Gurbuz *et al.*, 2004). All compounds were shown to possess significant antinociceptive activity against *p*-benzoquinone-induced abdominal stretching and significantly inhibited carrageenan-induced hind paw edema in mice (Kupeli *et al.*, 2003). Besides, these compounds were shown to possess significant anti-ulcerogenic activity and among them, taxiresinol was found to be the most prominent (Gurbuz *et al.*, 2004). In our other previous study on bioactivity of lignans from *T. baccata*, three lignan derivatives, **2**, **3** and **5**, were evaluated for their cytotoxicity against the Oncology Cell Line Panel (breast, colon, ovary, prostate, lung and a normal adult bovine aortic endothelial cell line) as well as for their antimicrobial activity. None of the investigated compounds demonstrated much cytotoxic potency as compared to the reference drug etoposide. With the exception of compound **3**, the other two lignans **2** and **5** showed a remarkable antifungal activity, whereas none of the compounds displayed antibacterial activity (Erdemoglu *et al.*, 2004b).

Alzheimer's disease (AD) is a progressive, neurodegenerative disorder resulting in impaired memory and cognitive function. Since the populations of many developed countries are aging, AD represents an increasingly important social, health, and economic problem worldwide. According to the cholinergic hypothesis, memory impairments in patients suffering from AD is a result from a deficit of cholinergic function in brain (Lahiri *et al.*, 2002). Acetylcholinesterase (AChE) is the most important enzyme regulating the level of acetylcholine (ACh), whereas butyrylcholinesterase (BChE) also plays a role in the pathology of AD. It has been accepted that AChE and BChE inhibition is the most used treatment for the symptoms of AD and related dementias (Greig *et al.*, 2002; Scarpini *et al.*, 2003). In recent literatures, new therapies targeted at the underlying pathology of AD give the promise of disease-modifying approaches. The use of anti-inflamma-

tory agents has also been suggested to delay the progression of AD (Scarpini *et al.*, 2003; Reale *et al.*, 2004).

Lipoxygenases (LOXs) constitute a family of non-haem iron-containing dioxygenases that are widely distributed in animals and plants. In mammalian cells, these are key enzymes in the biosynthesis of a variety of bioregulatory compounds such as hydroxyecosatetraenoic acids (HETEs), leukotrienes, lipoxins and hepoxylines (Lands, 1985). It has been reported that these LOX products play a role in a number of disorders such as bronchial asthma, inflammation, and tumour angiogenesis. Thus, LOXs are potential targets for the rational drug design and discovery of mechanism-based inhibitors for the treatment of bronchial asthma, inflammation, cancer and autoimmune diseases (Steinhilber, 1999; Nie and Honn, 2002).

In continuation of our studies on screening of Turkish medicinal plants and their secondary metabolites for enzyme inhibitory activity to be used as potential leading compounds in the treatment of AD since the year 2000, this time, we have screened the lignans obtained from *T. baccata*. These lignans (Fig. 1) were evaluated for their AChE, BChE, and LOX inhibitory activities by *in vitro* spectrophotometric methods using an ELISA microplate reader. In addition, the compounds were screened for their antioxidant capacity in 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferrous ion-chelating effect, and ferric-reducing antioxidant power (FRAP) at 125, 250, 500, and 1000 $\mu\text{g ml}^{-1}$.

Material and Methods

General

Column chromatography (CC) was performed using Silica gel (Kieselgel 60, 0.063–0.200 mm, Art. 7734, Merck), and Kieselgel 60 F₂₅₄ (0.5 mm thickness, Art. 5554, Merck) was used for preparative thin layer chromatography (TLC). Analytical TLC was performed on precoated plates (Kieselgel 60 F₂₅₄, Art. 5554, Merck), visualized under UV light at 254 nm, and then sprayed with anisaldehyde reagent [76% methanol (Merck), 19% *ortho*-phosphoric acid (Riedel-De Haën, Buchs, Switzerland), 5% *p*-anisaldehyde (Merck)] and heated.

Plant material

Taxus baccata L. (Taxaceae) was collected from the vicinity of Çamlıhemşin-Rize (Turkey) at an altitude of 1400 m, in June 2000. A voucher specimen (GUE 1560) was deposited in the Herbarium of Faculty of Pharmacy, Gazi University, Ankara, Turkey.

Extraction and isolation

The heartwood of the plant was dried in the shade and powdered to a fine grade. The material (3078 g) was extracted with 95% ethanol (EtOH) at room temperature. The EtOH extract was evaporated under reduced pressure to give a reddish residue. The residue (308.91 g) was dissolved in distilled H₂O and extracted with chloroform (CHCl₃). The CHCl₃ phase was concentrated under reduced pressure to give a concentrate (63.54 g). A portion (49 g) of the CHCl₃ extract was subjected to silica gel column chromatography (CC). The column was eluted with solvents of increasing polarity using hexane, acetone, CHCl₃ and methanol (CH₃OH), which led to the collection of seven main fractions (I–VII). Each fraction was further purified by CC, followed by preparative thin layer chromatography (PTLC) or recrystallization.

Elution with CHCl₃/CH₃OH (80:20) afforded fraction Fr. VII (5.65 g), which was again subjected to silica gel CC employing CHCl₃/CH₃OH (100:0 → 92:8, v/v) as the eluting solvent mixture to afford sixty-four subfractions. Subfractions 11–13 were subjected to PTLC with CHCl₃/CH₃OH (90:10, v/v) to yield **1** (45.7 mg, 0.0028%) and **3** (20.2 mg). Compound **3** (42.6 mg) was also obtained from subfractions 14–22 through recrystallization using CHCl₃, and the total yield of **3** was 62.8 mg (0.0039%). Subfractions 47–52 were further separated by PTLC using CHCl₃/CH₃OH (80:20, v/v) to afford **2** (84.6 mg, 0.0052%). Subfractions 28–33 were crystallized from CHCl₃ to give **4** (41.3 mg, 0.0026%). Subfractions 58–64 were subjected to CC on silica gel and eluted with CHCl₃/CH₃OH (100:0 → 80:20, v/v) to give fractions 4–14, which were purified by PTLC developed with CHCl₃/CH₃OH (80:20, v/v) to afford **5** (71.4 mg, 0.0044%).

LOX inhibition

LOX-inhibiting activity was measured by modifying the spectrophotometric method developed by Tappel (1962). LOX (EC 1.13.11.12, type I-B), baicalein, and linoleic acid were purchased from Sigma (St. Louis, MO, USA). Baicalein was used as the reference drug. All other chemicals were of analytical grade. The reaction mixture contained 165 μ l (100 mM) sodium phosphate buffer (pH 8.0), 5.0 μ l of test compound solution and 20 μ l of enzyme solution, and was incubated for 10 min at 25 °C. The reaction was then initiated by the addition of 10 μ l of linoleic acid (substrate). Due to the formation of (9Z,11E)-(13S)-13-hydroperoxy-octadeca-9,11-dienoate, the change of absorbance at 234 nm was followed for 6 min. Test compounds and the control were dissolved in CH₃OH. All reactions were performed in 96-well microplates using an ELISA microplate reader (SpectraMax Plus³⁸⁴, Molecular Devices, Sunnyvale, CA, USA). The percentage (%) inhibition was calculated as $(E - S)/E \cdot 100$, where *E* is the activity of the enzyme without test compound, and *S* is the activity of enzyme with test compound. The IC₅₀ values were then calculated using the EZ-Fit enzyme kinetics software program (Perrella Scientific Inc., Amshert, USA).

AChE and BChE inhibition

Electric eel AChE (EC 3.1.1.7, type VI-S), horse-serum BChE (EC 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and eserine (the reference) were purchased from Sigma. Buffers and other chemicals were of extrapure analytical grade. Galanthamine (Reminyl[®], Johnson & Johnson, Langhorne, PA, USA) was used as another reference drug. AChE- and BChE-inhibiting activities were measured by slightly modifying the spectrophotometric method developed by Ellman *et al.* (1961). The reaction was carried out in 100 mM sodium phosphate buffer, pH 8.0, at 25 °C. In a typical assay, 140 μ l buffer, 20 μ l enzyme preparation, and 20 μ l test compound solution were mixed and incubated for 30 min. 10 μ l of DTNB were then added, and the reaction was started by adding 10 μ l of acetylthiocholine as substrate for AChE. Butyrylthiocholine chloride was used as substrate for BChE, while all other reagents and conditions were the same as previously described (Orhan *et al.*, 2008). The hydrolysis of

acetylthiocholine or butyrylthiocholine was monitored at 412 nm through the formation of the yellow 5-thio-2-nitrobenzoate anion because of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine or butyrylthiocholine. Test compounds and the control were dissolved in CH₃OH, which was used as negative control. The concentrations of the test compounds that inhibited the hydrolysis of the substrates (acetylthiocholine and butyrylcholine) by 50% (IC₅₀ values) were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The IC₅₀ values were then calculated using the EZ-Fit enzyme kinetics software program (Perella Scientific Inc.).

DPPH radical scavenging activity

The DPPH radical scavenging activity was determined by Blois's method (1958). The samples and references dissolved in ethanol (75%) were mixed with DPPH solution (1.5 · 10⁻⁴ M). Remaining DPPH was measured at 520 nm using a Unico 4802 UV-visible double beam spectrophotometer (Dayton, NJ, USA). Gallic acid was employed as the reference. Inhibition of DPPH in percent (*I*%) was calculated as follows: $I\% = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \cdot 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the compounds/reference.

Ferrous ion-chelating effect

The ferrous ion-chelating effect by the Fe²⁺-ferrozine test system was estimated by the method of Chua *et al.* (2008). Briefly, the samples dissolved in methanol were incubated with 2 mM FeCl₂ solution. The reaction was initiated by the addition of ferrozine solution, and the mixture was left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm. The ratio of inhibition (*I*%) of Fe²⁺-ferrozine complex formation was calculated as follows: $I\% = [(\text{absorbance of control} - \text{absorbance of test sample}) / \text{absorbance of control}] \cdot 100$. The control contained only FeCl₂ and ferrozine.

Ferric-reducing antioxidant power (FRAP) assay

The ferric-reducing power was tested using the assay of Oyaizu (1986). According to the method,

the compounds as well as chlorogenic acid as reference for comparative purposes were added to phosphate buffer (pH 6.6) and potassium ferricyanide. Later, the mixture was incubated at 50 °C for 20 min, and then trichloroacetic acid was added. After the mixture was shaken vigorously, this solution was mixed with distilled water and FeCl₃ (0.1%, w/v). After 30 min of incubation, absorbance was read at 700 nm using a Unico 4802 UV-visible double beam spectrophotometer. Analyses were achieved in duplicate. Increased absorbance of the reaction meant increased reducing power.

Statistical analysis

Results were expressed as means ± S.E.M. (standard error mean) of five replicates for enzyme inhibition tests and triplicates for antioxidant assays.

Results and Discussion

Five lignans, namely lariciresinol (**1**), taxiresinol (**2**), 3'-demethylisolariciresinol-9'-hydroxyisopropylether (**3**), isolariciresinol (**4**), and 3-demethylisolariciresinol (**5**) (Fig. 1), were isolated from the heartwood of *T. baccata*, and their structures were identified using extensive spectroscopic techniques (Erdemoglu *et al.*, 2003, 2004a). The isolated lignans were tested for AChE, BChE, and LOX inhibitory activities as well as their antioxidant potentials since AD is associated with oxidative stress (Table I).

As shown in Table I, compounds **1–3**, and **5** exerted inhibitory activity against BChE to some extents. Compound **4** was completely inactive in this assay. They showed moderate inhibitory activity against LOX, and their IC₅₀ values varied between 161.0 and 300.0 μM. However, since they displayed AChE inhibitory activity below 50%, their IC₅₀ values were not worth to calculate for this assay. Among the tested compounds, 3'-demethylisolariciresinol-9'-hydroxyisopropylether (**3**) possessed the highest inhibitory activity against both BChE and LOX with IC₅₀ values of (86.0 ± 0.2) and (161.0 ± 3.6) μM, respectively.

The antioxidant capacity of the compounds was tested in three assays, DPPH radical scavenging, ferrous ion-chelating effect, and ferric-reducing antioxidant power (FRAP) tests at 125, 250, 500, and 1000 μg ml⁻¹. As shown in Table II, the

compounds had a remarkable scavenging effect against DPPH radicals, compounds **5** and **2** were the best scavengers at 1000 $\mu\text{g ml}^{-1}$ as compared

to gallic acid, a natural antioxidant used as reference. However, none of the compounds had ability to chelate ferric ions.

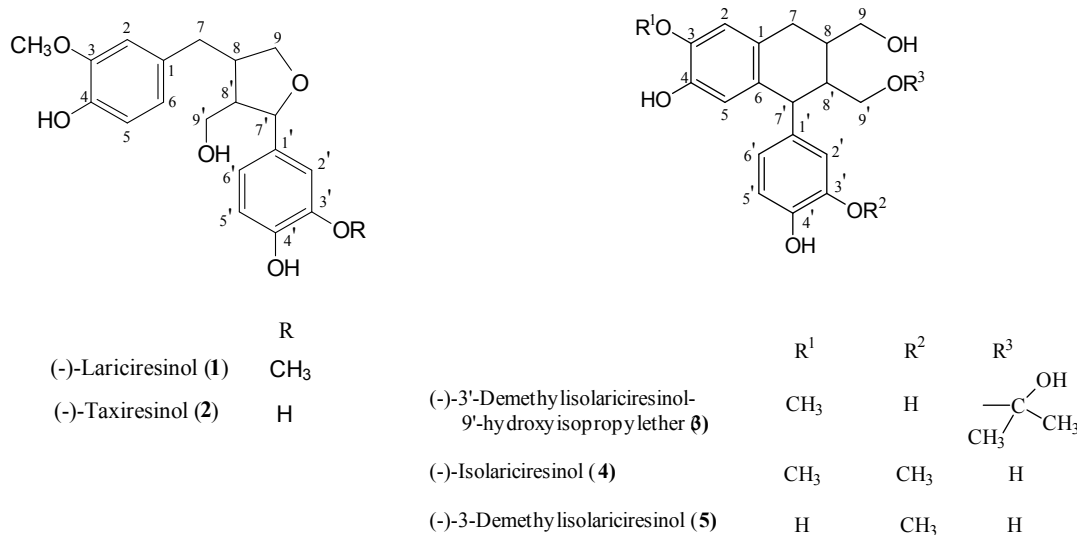


Fig. 1. Chemical structures of the investigated compounds **1–5**.

Table I. IC₅₀ values of compounds **1–5** against BChE and LOX.

Compound	IC ₅₀ ± S.E.M. ^a [μM]	
	BChE	LOX
1	117.5 ± 1.5	242.1 ± 1.5
2	100.0 ± 0.1	244.4 ± 0.5
3	86.0 ± 0.2	161.0 ± 3.6
4	– ^b	275.0 ± 0.5
5	193.7 ± 0.5	300.0 ± 2.0
Eserine ^c	0.9 ± 0.01	
Galanthamine ^d	8.5 ± 0.01	
Baicalin ^e		22.6 ± 0.5

^a Standard error mean. ^b No activity. ^{c,d} Reference drugs for AChE and BChE. ^e Reference drug for LOX.

Table II. Percentage of inhibition of compounds **1–5** against DPPH radicals.

Compound	Percentage of inhibition ± S.E.M. ^a			
	125 $\mu\text{g ml}^{-1}$	250 $\mu\text{g ml}^{-1}$	500 $\mu\text{g ml}^{-1}$	1000 $\mu\text{g ml}^{-1}$
1	38.73 ± 0.01	61.74 ± 0.09	75.79 ± 0.14	83.40 ± 0.01
2	24.56 ± 0.35	41.93 ± 0.55	71.71 ± 1.46	86.17 ± 2.57
3	17.52 ± 0.42	28.70 ± 0.49	43.06 ± 0.62	61.81 ± 0.28
4	25.54 ± 1.05	41.39 ± 0.76	60.73 ± 0.01	76.72 ± 0.07
5	25.69 ± 0.55	42.32 ± 1.45	63.29 ± 1.20	87.06 ± 0.21
Gallic acid	ND ^b	ND	91.61 ± 0.06	92.57 ± 0.10

^a Standard error mean. ^b Not determined.

In the FRAP test, the compounds exhibited quite noteworthy activity (Table III). Particularly, at a concentration of $1000 \mu\text{g ml}^{-1}$, compounds **2**, **4**, and **5** had an effect comparable to that of the reference, chlorogenic acid.

Lignans are one of the important classes of secondary metabolites. They have been so far reported with many desired biological activities (Rios *et al.*, 2002); however, in our literature survey, there was a limited number of reports on inhibitory activities of lignan derivatives against AChE, BChE, or LOX enzymes. For instance, fourteen lignans isolated from *Schizandra chinensis* were evaluated for their AChE inhibitory effects and, among them, lignans containing both aromatic methylenedioxy and hydroxy groups on their cyclooctadiene ring, such as gomisin C, gomisin G, gomisin D, schisandrol B, and gomisin A, were found to inhibit AChE totally in a dose-dependent manner, with IC_{50} values of (6.71 ± 0.53), (6.55 ± 0.31), (7.84 ± 0.62), (12.57 ± 1.07), and (13.28 ± 1.68) μM , respectively (Tran *et al.*, 2007). In a previous study, aryl-naphthalene-type lignans (diphyllin acetylapioside, especially) acting as LOX inhibitors were stated to be responsible for the topical anti-inflammatory activity of *Haplophyllum hispanicum* (Prieto *et al.*, 2002). On the other hand, two new lignans named negundins A and B along with (+)-diasyringaresinol and (+)-lyoniresinol isolated from *Vitex negundo* were tested for their LOX and BChE inhibitory effects, and negundin B was found to have potent inhibitory activity against LOX (Azhar-ul-Haq *et al.*, 2004). Several other lignan derivatives, namely (+)-3,4,3',4'-tetrahydroxy-9,7' α -epoxy-lignano-7a,9'-lactone, (+)-3,3'-bisdemethylpinogool, (-)-pinoresinol, and (-)-3,3'-bisdemethylpinoresinol, obtained from the fruits of noni (*Morinda citrifolia*) collected in Tahiti were

screened against LOX, and all of them showed LOX inhibition to some extent (Deng *et al.*, 2007). Nevertheless, we have not encountered any report on inhibitory activities of the lignans tested herein, lariciresinol (**1**), taxiresinol (**2**), 3'-demethylsolariciresinol-9'-hydroxyisopropylether (**3**), isolariciresinol (**4**), and 3-demethylsolariciresinol (**5**), on AChE, BChE, or LOX enzymes.

Up to date, a number of studies have been reported on antioxidant activities of lignan derivatives. In a study by Baderschneider and Winterhalter (2001), the antioxidant activity of six novel lignans, *i.e.*, lariciresinol 4-*O*-glucoside, three isolariciresinol derivatives, and two secoisolariciresinols isolated from wine, were tested in the Trolox-equivalent antioxidant capacity (TEAC) test, and they were found to have a good activity (1.9 – 2.5 mmol TEAC mmol^{-1}). Five pure wood-derived lignans were assayed in the antioxidant potency and the superoxide and peroxy radical scavenging capacity tests, and they were found to possess a high activity in those tests (Willfoer *et al.*, 2003). Four lignans, including (+)-9-acetoxyisolariciresinol, lariciresinol, 9-acetoxyariciresinol, and isolariciresinol, isolated from *Ephedra viridis* exhibited moderate antioxidant activity without any cytotoxicity (Pullela *et al.*, 2005). Considering the structure-activity relationship, Yamauchi *et al.* (2007) stated that the benzylic structure without oxygen in the lignan structure, such as in (+)-secoisolariciresinol and 3,4-bis(4-hydroxy-3-methoxybenzyl)tetrahydrofuran, played a major role in the occurrence of the highest antioxidant activity. Therefore, high antioxidant activity of compounds **1** and **2** may be due to this reason. In another study, four lignans [(+)-taxiresinol, (+)-isotaxiresinol, (+)-isolariciresinol, and (-)-secoisolariciresinol] isolated from *Taxus cuspidata* were tested in DPPH radical scavenging and linethol oxidation

Table III. Ferric-reducing antioxidant power (FRAP) (absorbance at 700 nm) of compounds **1**–**5**.

Compound	FRAP \pm S.E.M. ^a			
	125 $\mu\text{g ml}^{-1}$	250 $\mu\text{g ml}^{-1}$	500 $\mu\text{g ml}^{-1}$	1000 $\mu\text{g ml}^{-1}$
1	1.055 \pm 0.29	1.174 \pm 0.12	1.202 \pm 0.29	1.908 \pm 0.13
2	0.634 \pm 0.03	0.758 \pm 0.04	0.773 \pm 0.05	3.552 \pm 0.02
3	0.552 \pm 0.27	0.695 \pm 0.10	0.714 \pm 0.08	0.779 \pm 0.02
4	0.980 \pm 0.06	0.995 \pm 0.05	1.005 \pm 0.19	3.021 \pm 0.71
5	0.702 \pm 0.04	0.745 \pm 0.01	2.310 \pm 0.02	3.533 \pm 0.01
Chlorogenic acid	ND ^b	ND	3.547 \pm 0.06	3.618 \pm 0.01

^a Standard error mean. ^b Not determined.

test systems (Veselova *et al.*, 2007). Among them, (+)-isotaxiresinol was the most active against DPPH radicals, and showed a comparable effect to that of α -tocopherol. (+)-Taxiresinol and (+)-isolariciresinol were found to have a moderate scavenging effect in this study as compared to the reference α -tocopherol. In our study, we found these two lignans to be highly active in both antioxidant tests, whose references were different from α -tocopherol. The authors stated that

high antioxidant activity of these lignans is associated with the molecular structure containing two *ortho* hydroxy groups in the aromatic nucleus and having additional aliphatic hydroxy groups.

To the best of our knowledge, we herein report the AChE, BChE, and LOX inhibitory activities as well as the antioxidant capacity by the above-mentioned methods of the lignans **1–5** for the first time.

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