Taxodione, a DNA-Binding Compound from *Taxodium distichum* L. (Rich.)

Ahmed M. Zaghloul, Ahmed A. Gohar*, Zein Al-Abdin M. Naiem, and Fatma M. Abdel Bar

Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt. E-mail: gohar1952@yahoo.com

* Author for correspondence and reprint requests

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8-β-Hydroxypimar-15-en-19-oic acid (**1**), taxodione (**2**), 6,7-dehydro-8-hydrotaxodone (**3**), quercetin-3-*O*-β-D-glucopyranoside (**4**), and shikimic acid (**5**) were isolated from the leaves of *Taxodium distichum* L. (Rich.) for the first time. Previously reported compounds [β-sitosterol (**6**), isorhamnetin (**7**), quercetin (**8**), isorhamnetin-3-*O*-α-arabinofuranoside (**9**), quercetin-3-*O*-α-arabinofuranoside (**10**)] have also been isolated. The activity of taxodione as an inhibitor for hepatic stellate cells was determined. The antitumour activity of **2**, **3**, and **5** using a DNA affinity probe was examined.

Key words: Taxodione, DNA-Binding Compounds, *Taxodium distichum*

Introduction

Taxodium distichum L. (Rich.) has been reported for its antitumour activity due to its seed content of the diterpenoid quinone methides taxodone and taxodione (Kupchan *et al*., 1968; Nakanishi *et al*., 1974). The diterpenes royleanone, taxoquinone, sugiol, Δ^5-dehydrosugiol, 11-hydroxymontbretol and ferruginol have also been isolated (Ahmed and El-Emary, 1999; Kupchan *et al*., 1969). The latter two compounds in addition to taxodione and taxodone were reported to have HIV-1 PR inhibition activity (Ahmed and El-Emary, 1999). The flavonoids apigenin, apigenin-7-glucoside, luteolin, luteolin-7-glucoside, kaempferol, kaempferol-3-arabinoside, quercetin, quercetin-3-arabinofuranoside, quercetin arabinopyranoside, quercetin-3-galactopyranoside, isorhamnetin, isorhamnetin-3-arabinofuranoside, isorhamnetin-3-arabinopyranoside, isorhamnetin-3-galactopyranoside, tricin-3′-methyl ether, tricin-3′-methyl-7-glucoside were reported in the leaves of *T. distichum* (Geiger and de Groot-Pfleiderer, 1979), as well as the biflavonoids amentoflavone, bilobetin, amentoflavone-7″,4‴-dimethyl ether, scaidopitysin, hinokiflavone, isocryptomerin, cryptomerin A and cryptomerin B (Geiger and de Groot-Pfleiderer, 1973). Other miscellaneous compounds including proanthocyanidins (Stafford and Lester, 1986) and sterols (Setsuko *et al*., 1984) were also reported.

Full investigation of the leaves of Egyptian *T. distichum* L. (Rich.) was not yet done. So, intensive phytochemical investigation of the aerial parts of the Egyptian plant as well as of the activity of taxodione as inhibitor for hepatic stellate cells together with the antitumour activity of taxodione, 6,7-dehydro-8-hydrotaxodone and shikimic acid using a DNA affinity probe are presented.

Results and Discussion

Isolation of compounds

Solvent partition of the methanol extract of *Taxodium distichum* leaves and chromatographic isolation of different fractions using suitable solvent systems and normal phase silica gel afforded compounds **1**–**10**. Compounds **1**–**5** (Fig. 1) were identified as 8-β-hydroxypimar-15-en-19-oic acid (**1**), taxodione (**2**), 6,7-dehydro-8-hydrotaxodone (**3**), quercetin-3-*O*-β-D-glucopyranoside (**4**), and shikimic acid (**5**) by comparing their IR, UV, MS, ^{1}H and ^{13}C NMR spectra with the available reported data (Silverstein *et al.*, 1991; Ramos *et al.*, 1984; Khabir *et al*., 1986; Simoes *et al.*, 1986; Rodriguez-Hahn *et al.*, 1989; Geiger *et al.*, 1995; Lambert *et al*., 1998; Ahmed and El-Emary, 1999; Gohar *et al*., 2000). These compounds were isolated from the leaves of *T. distichum* L. (Rich.) for the first time. The previously reported compounds β-sitosterol (**6**), isorhamnetin (**8**), quercetin (**7**), isorhamnetin-3-*O*-α-arabinofuranoside (**9**) and quercetin-3-*O*-α-arabinofuranoside (**10**) have been also isolated and identified by comparing their spectral

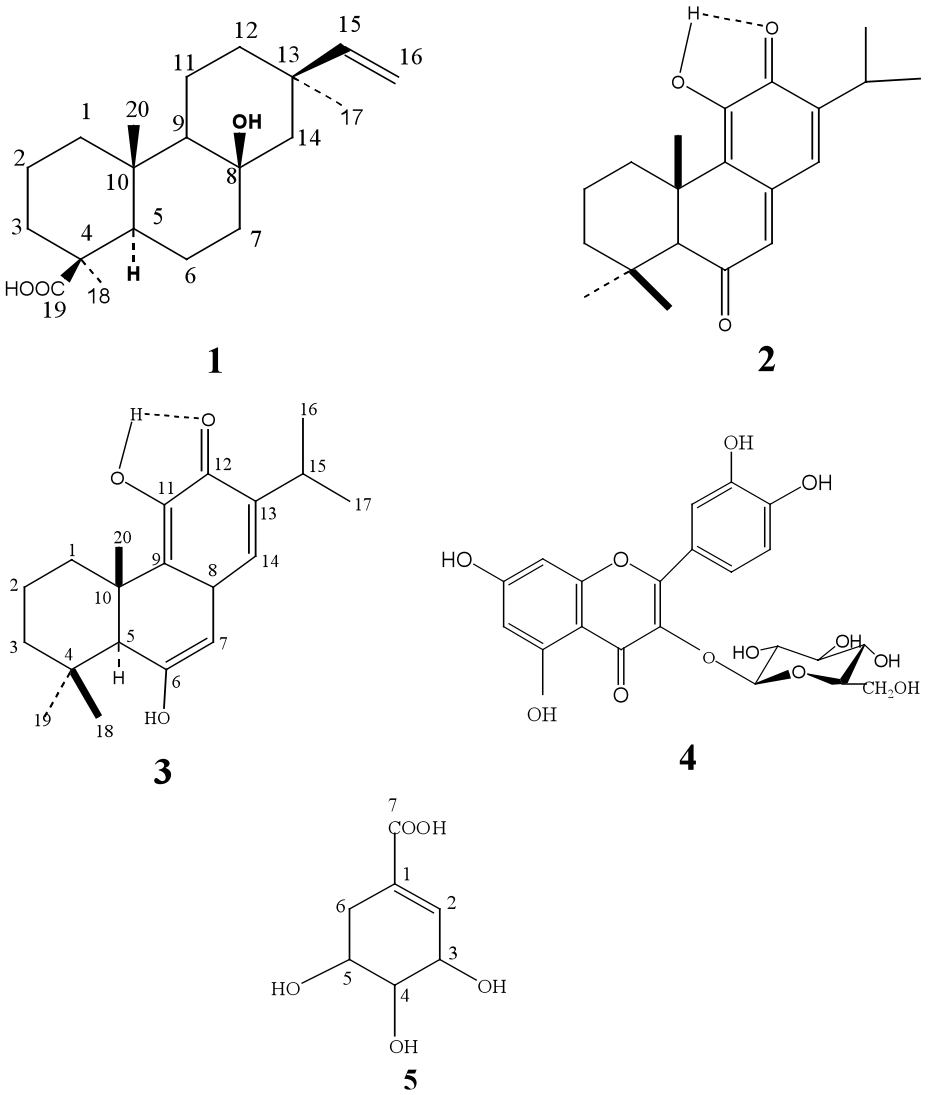

Fig. 1. Chemical structures of compounds **1**–**5**.

data with the reported ones (Geiger and de Groot-Pfleiderer, 1979) (data not shown).

Inhibition of hepatic stellate cells

Hepatic stellate cells (HSCs) play a central role in liver fibrogenesis. There are many factors, which may disturb the HSCs and convert them into myofibroblasts (Kawada, 2001). After liver injury, HSCs undergo an activation process, which represents a transition state into proliferation. Therefore, substances that inhibit the activation of the HSCs are of major importance in attenuating their fibrogenic response. The results of inhibition of HSCs proliferation of the tested samples are shown in Table I.

It was found that compound **2** (taxodione) has a good inhibitory effect on HSCs proliferation.

Antitumour activity assays using a DNA affinity probe

DNA qualitative binding assay

The tested compounds **2**, **3** and **5** showed binding affinity to DNA, as demonstrated by retaining

Table I. Effect of certain compounds isolated from *Taxodium distichum* on the proliferation of HSCs-T6.

Compound	OD_{595}	Inhibition (%)
1	0.89 ± 0.15	3.2
2	0.64 ± 0.02	83.8
Negative control	0.59 ± 0.02	
Positive control	0.90 ± 0.05	

the complex at the application site of the TLC plates.

Colourimetric assay for compounds that bind DNA

Methyl green reversibly binds to DNA, and the coloured complex is stable at neutral pH, whereas free methyl green fades at this pH value. DNA-binding active compounds displace DNA from its methyl green complex. The displacement was determined by a spectrophotometric assay as a decrease in the absorbance at 630 nm. The assay results are shown in Table II.

Table II. DNA/methyl green colourimetric assay of the DNA-binding compounds.

DNA-active compound	DNA/methyl green (IC_{50}, μg/ml)
2	40 ± 2
3	51 ± 4
5	38 ± 3

IC_{50} values represent the concentration (mean ± SD, $n = 3-5$ separate determinations) required for a 50% decrease in the initial absorbance of the DNA/methyl green solution.

Experimental

Materials

Taxodium distichum L. (Rich.) was collected in May 1998 from trees grown on the banks of the River Nile, Mansoura, Egypt. The identity of the plant was confirmed by Prof. Dr. N. Sharaf El-Din, Faculty of Agriculture, Department of Horticulture, Mansoura University, Egypt. The leaves of the plant were air-dried, powdered, sieved (sieve N°. 10) and kept in an airtight container.

Rat hepatic stellate cells (HSCs-T6) were provided as a gift by Prof. Scott Friedman (New York Hospital, New York, USA). Dulbeco medium eagle (DME), calf serum, trypsin, platelet-derived growth factor (PDGF), 10% formalin in saline, 0.05% crystal violet in 20% aqueous methanol, DMSO, RP-18 TLC plates, methanol, DNA, anisaldehyde reagent, ethidium bromide, DNA methyl green (Sigma, St. Louis, MO, USA), Tris-HCl buffer, $MgSO_4$, ethanol were used in the assays.

*Extraction and isolation of compounds **1–10** from the leaves*

3 kg of powdered leaves were extracted with MeOH (18 l). The concentrated methanol extract was mixed with 3 l distilled water and successively partitioned with petroleum ether, chloroform, ethyl acetate and *n*-butanol.

The petroleum ether extract (220 g) was further fractionated using a silica gel column and two solvent systems, petroleum ether/EtOAc and petroleum ether/$CHCl_3$, to afford three compounds. Fractions 24–30 were refractionated on silica gel using petroleum ether/$CHCl_3$ to afford compound **6** (40 mg; R_f 0.56, eluted with petroleum ether/$CHCl_3$ 20%). Fractions 31–69 eluted with petroleum ether/EtOAc 2% were refractionated on silica gel using petroleum ether/EtOAc to afford compound **2** (10 mg; R_f 0.55, eluted with petroleum ether/EtOAc 1%). Fractions 88–121 eluted with 5% EtOAc afforded compound **1** [100 mg; R_f 0.15, TLC, silica gel, petroleum ether/EtOAc (9:1 v/v)].

The chloroform fraction (11 g) was fractionated on a silica gel column using $CHCl_3$ as eluent. The residue of the pure chloroform eluate was rechromatographed on a silica gel column using petroleum ether/$CHCl_3$ as solvent to afford compound **3** [10 mg, orange needles; R_f 0.55, TLC, petroleum ether/$CHCl_3$ (6:4 v/v)].

The *n*-butanol extract (15 g) was fractionated on a silica gel column using CH_2Cl_2/EtOAc/MeOH (40:40:20); 200 ml fractions were collected. Fraction 7 (2 g) was rechromatographed on a silica gel column and eluted with CH_2Cl_2/MeOH 20% to afford compound **5** [70 mg; R_f 0.40, TLC, silica gel, CH_2Cl_2/MeOH (7:3 v/v)].

The ethyl acetate fraction (56 g) was fractionated on a silica gel column; CH_2Cl_2/MeOH mixtures were used as solvent and 200 ml fractions collected. Fractions 18–20 eluted with CH_2Cl_2/MeOH 5% (300 mg residue) were rechromatographed on a silica gel column using CH_2Cl_2/MeOH 2% (10 ml fractions). Fractions 8–16 afforded compound **7** (20 mg; R_f 0.50, TLC, silica gel, CH_2Cl_2/MeOH 5%). Fractions 38–50, mother

column, eluted with CH_2Cl_2/MeOH 5% were rechromatographed on a silica gel column using the same solvent (100 ml fractions). Fractions 13–16 afforded compound **8** (150 mg; R_f 0.23, TLC, silica gel, CH_2Cl_2/MeOH 5%). Fractions 108–144, mother column, eluted with CH_2Cl_2/MeOH 12% were rechromatographed on a silica gel column using EtOAc/MeOH (200 ml fractions). Fractions 5–14 afforded compound **9** (37 mg; R_f 0.8) and compound **10** (36 mg; R_f 0.52) by repeated chromatography using a silica gel RP-18 column and elution with H_2O/MeOH (7:3 v/v). Fractions 15–18 afforded compound **4** (30 mg; R_f 0.32, TLC, silica gel, CH_2Cl_2/MeOH 15%).

8-β-Hydroxypimar-15-en-19-oic acid (**1**): Colourless needles (EtOAc). – M. p. 200 °C. – Positive FAB$^+$-MS: *m/z* = 321 [M+1], 303 [(M+1)–H_2O], 289 [(M+1)–H_2O–CH_2], 257 [M–COOH–H_2O]. – ^{1}H NMR: δ = 0.82 (m, H1α), 1.16 (m, H1β), 1.44 (m, H2α), 1.88 (m, H2β), 0.96 (ddd, *J* = 4.2, 13.2, 13.2 Hz, H3α), 2.14 (d, *J* = 13.2 Hz, H3β), 0.84 (m, H5, 1.74 m, H6α), 2.06 (m, H6β), 1.71 (m, H7α), 1.79 (m, H7β), 1.08 (dd, *J* = 2.4, 12.6 Hz, H9), 1.40 (m, H11α), 1.55 (m, H11β), 0.88 (m, H12α), 2.00 (m, H12β), 1.23 (m, H14α), 1.70 (m, H14β), 5.96 (dd, *J* = 10.8, 18 Hz, H15), 5.15 (dd, *J* = 0.6, 10.8 Hz, $H16_A$), 5.17 (d, *J* = 18 Hz, $H16_B$), 0.90 (s, H17), 1.21 (s, H19), 0.81 (s, 3H20). – ^{13}C NMR: see Table III.

Taxodione (**2**): Orange plates (petroleum ether/$CHCl_3$). – M. p. 100–102 °C. – IR: ν_{max} = 3400 (broad O–H), 2917.9, 2852.7 (C–H), 1670.3, 1618.2 (2 C=O in two rings), 1550.1, 1460.3, 1428.1, 1419.8, 1354.8, 1182.1, 1148.8, 1076.5 (C–O), 973.8, 715, 637.3 cm^{-1}. – UV: λ_{max} = 333.4, 321.8, 205 nm. – Positive FAB-MS: *m/z* = 315 [M+1], 307, 289, 245, 176. – ^{1}H NMR: δ = 1.75 (m, H1α), 2.93 (m, H1β), 1.58 (m, H2α), 1.70 (m, H2β), 1.21 (dd, *J* = 4.2, 13.2 Hz, H3α), 1.40 (d, *J* = 13.2 Hz, H3β), 2.59 (s, H5), 6.21 (s, H7), 6.88 (s, H14), 3.07 (m, H15), 1.16 (d, *J* = 6.9 Hz, 3H16), 1.18 (d, *J* = 6.9 Hz, 3H17), 1.27 (s, 3H18, 3H19), 1.12 (s, 3H20). – ^{13}C NMR: see Table III.

6,7-Dehydro-8-hydrotaxodone (**3**): Orange needles (MeOH). – IR: ν_{max} = ~3500 (broad O–H), 3353 (O–H), 1736 (C=O), 1626.5 cm^{-1} (conjugated C=O, quinone). – UV: λ_{max} = 335.8, 238.0, 211 nm. – ^{1}H NMR: δ = 1.61 (m, H1α), 2.87 (m, H1β), 1.58 (m, H2α), 1.70 (m, H2β), 1.21 (dd, *J* = 4.2, 13.2 Hz, H3α), 1.40 (d, *J* = 13.2 Hz, H3β), 3.66 (s, H5), 6.46 (d, *J* = 8.7 Hz, H7), 2.29 (s, H8), 6.80 (d, *J* = 8.9 Hz, H14), 3.16 (m, H15), 1.21 (s, 3H16,

Table III. ^{13}C NMR data (150 MHz, $CDCl_3$) of compounds **1**–**5**.

C/H ≠	**1**	**2**	**3**	**5**	C/H ≠	**4**
1	40.0	37.1	35.6	128.4	2	158.4
2	19.6	18.6	19.1	138.9	3	135.8
3	38.1	42.7	39.7	65.5	4	179.6
4	43.7	32.9	33.0**	70.3	5	163.5
5	55.7	63.1	52.5	66.9	6	100.6
6	19.0	201.1	151.6	30	7	166.7
7	41.7	134.1	110	168	8	95.3
8	73.3	140.0	33.7		9	158.7
9	57.2	125.7	123.0		10	105.9
10	36.3	42.9	40.9		1′	123.1
11	17.6	145.4	140.9		2′	118.6
12	36.5	181.8	186.5		3′	147.5
13	37.8	145.1	138.9		4′	151.6
14	53.1	136.2	140.1		5′	117.0
15	147.3	27.2	24.5		6′	123.7
16	112.8	21.3	20.2		β-D-Glucose	
17	32.5	22.2	20.4		1″	106.3
18	28.9	21.9	23.1		2″	74.2
19	182.0	21.7	23.2		3″	76.3
20	13.6	33.3	32.3**		4″	70.6
					5″	78.5
					6″	62.7

** May be interchanged.

3H17), 1.25 (s, 3H18, 3H19), 0.97 (s, 3H20). – ^{13}C NMR: see Table III.

Quercetin-3-O-β-D-glucopyranoside (**4**): Yellow amorphous powder. – IR: ν_{max} = 3453.9, 2985.7, 2885.7, 1653.3 (C=O), 1603, 1557.2, 1500.2, 1469.9, 1254.6, 1297.5, 1203, 1157.1, 1126.5, 1003.6 (C–O) 941, 869.4, 829, 792.8, 647 cm^{-1}. – UV: λ_{max} (CH_3OH) = 258, 362; (NaOMe) 272, 329, 410; ($AlCl_3$) 271, 410; ($AlCl_3$/HCl) 268, 361, 398; (NaOAc) 222, 273, 322, 391; (NaOAc/H_3BO_3) 222, 262, 378 nm. – ^{1}H NMR: δ = 6.63 (d, J = 2 Hz, H6), 6.69 (d, J = 2 Hz, H8), 8.44 (d, J = 2.1 Hz, H2′), 7.25 (d, J = 8.4 Hz, H5′), 8.13 (dd, J = 8.4, 2.1 Hz, H6′), 6.07 (d, J = 7.7 Hz, H1″), 4.8 (m, H2″), 4.29 (m, H3″), 4.61 (d, J = 3.1 Hz, H4″), 4.16 (m, H5), 4.41 (m, H6α), 4.29 (m, H6β). – ^{13}C NMR: see Table III.

Shikimic acid (**5**): White amorphous powder. – IR: ν_{max} = 3474, 3375, 3221, 2870, 1680, 1642, 1448, 1120, 1065, 1024, 929, 863, 830, 746, 674 cm^{-1}. – UV: λ_{max} (CH_3OH) = 238, 224.6 nm. – ^{1}H NMR: δ = 6.57 (brs, H2), 4.2 (brs, H3), 3.53 (m, H4), 3.81 (m, H5), 1.99 (ddd, J = 1.7, 3.1, 18 Hz, H6β), 2.37 (ddd, J = 1.7, 3.1, 17.9 Hz, H6α). – ^{13}C NMR: see Table III.

Hepatic stellate cells inhibitory assay (Friedman, 1997)

A HSCs suspension ($4 \cdot 10^4$ cells/ml) in 10% calf serum in DME was prepared from confluent dishes (full growth culture) by brief trypsinization with 0.05% trypsin in DME supporting the detachment from the dishes. In 96-well culture trays, l00 μl of HSCs suspension was added to each well. After 24 h of incubation in 5% CO_2 in air at 37 °C, media in each well were replaced with 100 μl of 0.4% calf serum in DME. After 48 h of incubation, platelet-derived growth factor (PDGF) was added to each well to the final concentration 100 μg/ml. Tested samples were also added, each in 3 wells, to the final concentration 50 mmol/ml. Negative control wells (without PDGF) and positive control wells (without samples) were also prepared. The volume in each well was adjusted to 200 μl with DME. After 24 h of incubation, media were shaken off and cells fixed with 10% formalin/saline for 30 min and stained with crystal violet.

Unbound stain was rinsed away, while bound stain was dissolved by adding 200 μl DMSO to each well. Then the optical density (OD) was measured in a plate reader at 595 nm and the inhibition calculated according to

$$\% \text{ inhibition} = \frac{OD_{595}\,(\text{positive control}) - OD_{595}\,(\text{drug})}{OD_{595}\,(\text{positive control}) - OD_{595}\,(\text{negative control})} \times 100.$$

Antitumour activity assays using a DNA affinity probe

DNA qualitative binding assay (Pezzuto *et al.*, 1991)

RP-18 TLC plates (RP-18 F_{254}; 0.25 mm; Merck) were pre-developed with MeOH/H_2O (8:2). Test compounds were then applied (5 mg/ml in MeOH) at the base line, followed by the addition of an equal volume of DNA solution (1 mg/ml in H_2O/MeOH, 1:1 v/v) at the same application positions. The plates were developed with the same solvent and the position of the DNA was determined by spraying with anisaldehyde reagent (blue colour with DNA). Ethidium bromide was used as positive control (interacts with DNA).

Colourimetric assay for compounds that bind DNA (Burres *et al.*, 1992)

DNA methyl green (20 mg) was suspended in 100 ml of 0.05 M Tris-HCl buffer (pH 7.5) containing 7.5 mM $MgSO_4$; the mixture was stirred at 37 °C with a magnetic stirrer for 24 h. Test samples (10, 100, 1000 g) were dissolved in ethanol in Eppendorf tubes, solvent was removed under vacuum, and 200 μl of the DNA/methyl green solution were added to each tube. Samples were incubated in the dark at ambient temperature. After 24 h, the final absorbance of the samples was determined at 642.5–645 nm. Readings were corrected for initial absorbance and normalized as the percentage of the untreated standard.

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