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Isolation, structure elucidation and bioactivity of schischkiniin, a unique indole alkaloid from the seeds of *Centaurea schischkinii*

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Abstract—Reversed-phase HPLC analysis of the methanol extract of the seeds of *Centaurea schischkinii* afforded a novel indole alkaloid, named schischkiniin (**1**), together with four lignans, arctiin (**2**), matairesinoside (**3**), matairesinol (**4**), and arctigenin (**5**), and three flavonoids, astragalin (**6**), afzelin (**7**) and apigenin (**8**). While the structure of schiskiniin (**1**) was established unequivocally by UV, HRFABMS and a series of 1D and 2D NMR analyses, all known compounds were readily identified by comparison of their spectroscopic data with literature data. The free radical scavenging properties of these compounds were assessed using the DPPH assay, and their general toxicity and cytotoxicity were evaluated, respectively, by brine shrimp lethality and MTT cytotoxicity assays with CaCo-2 colon cancer cell lines. Arctigenin (**5**) exhibited promising in vitro anticancer activity ($IC_{50}=7$ μM) while with schischkiniin (**1**) the activity was of moderate level ($IC_{50}=76$ μM).

1. Introduction

Centaurea schischkinii Tzvelev (family: Asteraceae *alt*. Compositae), an erect perennial, is an endemic species distributed in the East Anatolian regions of Turkey.[1] To our knowledge, no report on the isolation of any plant secondary metabolites from *C. schischkinii* or any pharmacological properties of this plant is available to date. However, many species of the genus *Centaurea* have long been used in traditional medicine to cure various ailments, for example, diabetes, diarrhoea, rheumatism, malaria, hypertension etc., and a variety of secondary metabolites have been reported from different species of this genus.[2] As part of our on-going phytochemical investigation on the species of the genus *Centaurea*,[3–16] we now report on the isolation, structure elucidation and bioactivity of a series of compounds, including a novel indole alkaloid, named schischkiniin (**1**), four lignans, arctiin (**2**), matairesinoside (**3**), matairesinol (**4**), and arctigenin (**5**), and three flavonoids, astragalin (**6**), afzelin (**7**) and apigenin (**8**) from the seeds of *C. schichskinii*.

2. Results and discussion

Reversed-phase preparative HPLC analysis of the methanol extract of the seeds of *C. schischkinii* led to the isolation of a novel indole alkaloid, named schischkiniin (**1**), four lignans, arctiin (**2**), matairesinoside (**3**), matairesinol (**4**), and arctigenin (**5**), and three flavonoids, astragalin (**6**), afzelin (**7**), and apigenin (**8**). The spectroscopic data of the known lignans (**2–5**) and flavonoids (**6–8**) were in good agreement with literature data.[17–23] The structure of schischkiniin (**1**) was established unequivocally by UV, HRFABMS and a series of 1D and 2D NMR analyses.

Compound **1** was obtained as a gum and the molecular formula was determined as $C_{26}H_{24}N_6O_2$ from its HRMS spectrum where the $[M+Na]^+$ ion was observed at *m/z* 475.1857 ($C_{26}H_{24}N_6O_2Na$ requires *m/z* 475.1858). The compound showed positive colour reaction with Dragendorff's reagent. The UV absorption maxima at 220 and

Keywords: *Centaurea schischkinii*; Asteraceae; Afzelin; Apigenin; Arctigenin; Arctiin; Astragalin; Matairesinol; Matairesinoside; Schischkiniin; DPPH assay; Cytotoxiciy; MTT assay; Colon cancer.
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Table 1. ^{1}H (coupling constant J in Hz in parentheses) and ^{13}C NMR data, and ^{1}H–^{13}C long-range (^{2}J and ^{3}J) correlation observed in HMBC spectra of **1**

Position	δ_H in ppm	δ_C in ppm	HMBC correlation	
	1	**1**	^{2}J	^{3}J
2	7.15s	123.9	C-3	C-3a, C-7a, C-8
3	—	108.4	—	—
3a	—	127.3	—	—
4	7.65d (8.2)	118.1	C-3a	C-3, C-6, C-7a
5	7.01dd (8.2, 8.2)	118.9	—	C-3a, C-7
6	7.08dd (8.2, 8.2)	121.5	C-7	C-4, C-7a
7	7.32d (8.2)	111.2	—	C-3a, C-5
7a	—	137.2	—	—
8	3.47dd (4.4, 15.2), 3.10dd (9.2, 15.2)	27.3	C-3, C-9	C-2, C-3a, C-10
9	3.82[a]	55.5	C-8	C-12, C-3
10	—	174.0	—	—
12	3.86[a]	55.4	C-13, C-12′	C-10, C-13′
13	3.84[a]	55.5	C-12, C-13′	C-9, C-12′

[a] Overlapped peaks, assigned with the help of ^{1}H–^{13}C HSQC correlation.

280 nm indicated that this compound might be an indole alkaloid.[2] In the ^{1}H NMR spectrum of **1** (Table 1), a singlet at δ 7.15 and the signals at δ 7.65, 7.08, 7.32 and 7.01, were typical of a 3-substituted indole skeleton, and could be assigned to H-2, H-4, H-5, H-6 and H-7.[2] In the ^{13}C NMR spectrum (Table 1) all signals required for a 3-substituted indole skeleton were also present. In addition to the signals attributable to the 3-substituted indole skeleton, the ^{1}H and ^{13}C NMR spectra also showed signals for a methylene (δ_H 3.47 and 3.10, δ_C 27.3), three methines (δ_H 3.80–3.86, δ_C 55.5, 55.4 and 55.5) and an amide carbonyl (δ_C 174.0). All these signals formed the part structure **1a**. Taking the molecular formula and molecular mass into account, it was clear that the ^{1}H and ^{13}C NMR signals actually displayed signals for just one of the two identical parts of the molecule. Therefore, the molecule must be composed of two of these part structures **1a**. When combining two **1a** structures, only structure **1** could satisfy the molecular formula and molecular mass of this molecule. Further evidence to support the structure of **1** was obtained from a series of ^{1}H–^{13}C long-range couplings observed in its HMBC spectrum (Table 1). In the HMBC spectrum, H-12 showed ^{2}J correlation to C-13 and C-12′, and ^{3}J to C-10 and C-13′. Similarly, H-13 displayed ^{2}J correlation to C-12 and C-13′, and ^{3}J to C-9 and C-12′. Owing to overlapped ^{1}H NMR signals for H-9, H-12 and H-13, ^{1}H–^{1}H NOESY was not successful to establish the relative stereochemistry at C-9 (and C-9′), C-12 (and C-12′) and C-13 (and C-13′). Although it is difficult to completely determine the relative stereochemistry of this unique molecule, the use of biogenetic speculation in tandem with molecular mechanics may give an insight into the stereochemistry of schischkiniin (**1**). Simple diketopiperazines such as the Trp-Gly diketopiperazine (**9**) are common natural products (Fig. 1). On this occasion we are assuming that the naturally occurring L-Trp has been incorporated into **9**. The next steps would involve the reduction and dehydration of the Gly residue, resulting in the formation of **10**, followed or preceded by the dimerisation at the Trp indole *N* to give the dimer **11**. This dimer would then undergo a photochemically allowed 2+2 cycloaddition to give 4 possible products. Schischkiniin (**1**) is a symmetrical structure as is evident from the degeneracy of the resonances in the NMR spectra. For this reason two possible asymmetric structures can be ruled out leaving only possibilities **1b** and **1c**. Molecular mechanics calculations[24,25] to determine the global energy minima of these possible structures suggest that **1c** has the lowest total energy function 986.93 kcal/mol (Fig. 2). Although it is possible that a natural product is enzymetically biosynthesised in a high-energy conformation, in this case a photochemically driven cycloaddition reaction will result in the lowest energy product. We therefore, speculate that the relative stereochemistry of schischkiniin is as shown in **1c**. If we assume that the origin of the Trp residue is L-Trp then we may also predict the absolute stereochemistry as shown. Thus, this novel indole alkaloid was identified as schischkiniin (**1**), which is a tryptophan-derived alkaloid. In addition to the indole skeleton, compound **1** also possesses a distinct macrocyclic polyamine ($n=14$) structure.

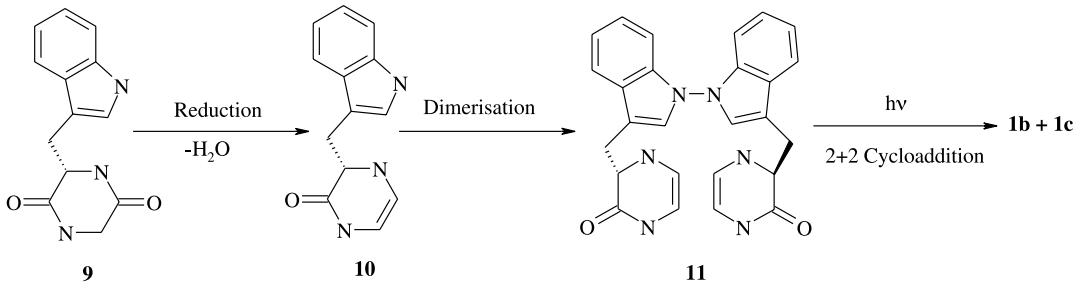
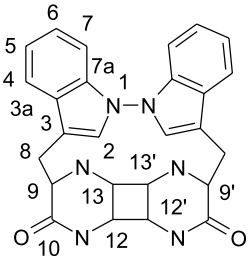
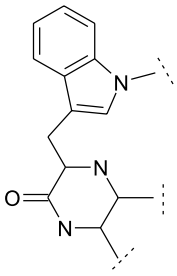


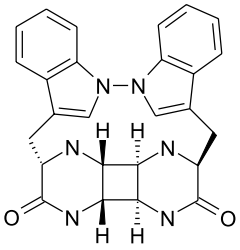
Figure 1. Proposed biogenetic pathway for the formation of schischkiniin (**1**) from a simple diketopiperazine **9**. The reduction/dehydration and dimerisation steps may be reversed.



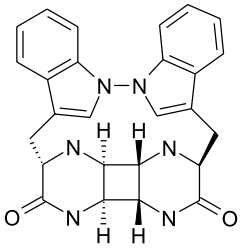
1



1a



1b



1c

The DPPH assay[26] is an easy and straightforward method for determining the free radical scavenging property of a compound. DPPH is a molecule containing a stable free radical. In the presence of an antioxidant, which can donate an electron to DPPH, the purple colour, which is typical of the free DPPH radical, decays, and the change in absorbance at 517 nm is monitored spectrophotometrically. All compounds (**1**–**8**) showed low to moderate levels of free radical scavenging activity ($IC_{50}=16.0\times10^{-2}$–$2.02\times10^{-3}$ mg/mL) (Table 2). Among four structurally related lignans **2**–**5**, matairesinol (**4**) showed the most prominent antioxidant property, which could be attributed to the presence of the highest number of phenolic hydroxyl groups (four –OH) in the molecule. Despite not having any phenolic hydroxyl group in the molecule, compound **1** still showed significant antioxidant activity ($IC_{50}=3.8\times10^{-3}$ mg/mL) (Table 1).

The brine shrimp lethality assay, which has been proven to be an effective and rapid assay method to screen compounds for potential general toxicity and cytotoxic activity[27] was used to determine the general toxicity of compounds **1**–**8**. The LD_{50} of these compounds were in between 1.4×10^{-1} and 2.0×10^{-3} mg/mL (Table 2). Arctigenin (**5**) was found to be the most toxic of all test compounds towards brine shrimp ($LD_{50}=2.0\times10^{-3}$ mg/mL) and displayed toxicity comparable to that of the positive control podophyllotoxin ($LD_{50}=2.79\times10^{-3}$ mg/mL), a well known cytotoxic lignan. The novel alkaloid (**1**) also showed significant general toxicity in this assay ($LD_{50}=7.2\times10^{-3}$ mg/mL).

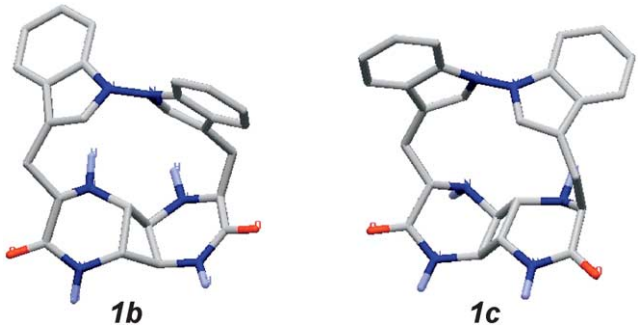


Figure 2. Global energy minima for structures **1b** and **1c** (heavy atoms and polar hydrogens only shown). The global minimum for **1b** was found 15 times ($E_{tot}=1022.68$ kcal/mol) and the global minimum for **1c** was found 37 times ($E_{tot}=986.93$ kcal/mol).

The in vitro cytotoxicities (IC_{50} μM) of all the compounds isolated and characterised in this work were determined by the MTT assay against colon cell lines, CaCo-2 (Table 1).[29] The novel indole alkaloid schischkiniin (**1**) exhibited moderate in vitro anticancer activity with an IC_{50} of 76 μM. The flavanoids astragalin (**6**), afzelin (**7**) and apigenin (**8**) showed low cytotoxicities with IC_{50} values 302, 316 and 133.1 μM, respectively. Similarly all isolated lignans demonstrated low levels of activity, for example, arctiin (**2**, $IC_{50}=220$ μM), matairesinoside (**3**, $IC_{50}=288$ μM), matairesinol (**4**, $IC_{50}=124$ μM), with the exception of arctigenin (**5**), which gave an IC_{50} value 7 μM, and therefore, can be considered to be an active compound against colon cancer cells in vitro. In the lignans and flavanoids described above, the presence of a sugar moiety in the molecule tends to reduce significantly the anticancer activity of these compounds. It can be assumed that the presence of a sugar group could prevent the effective transport of these compounds through the cell membrane, hence their reduced biological activities.

The degree of general toxicity displayed by the test compounds in the brine shrimp lethality assay corresponded well with the cytotoxic potentials of these compounds observed in the MTT assay using colon cancer cell line.

Table 2. Antioxidant (DPPH assay) and cytotoxic (MTT assay) activities, and brine shrimp toxicity (brine shrimp lethality assay) of compounds **1–8**

Compounds	Antioxidant activity IC_{50} (mg/mL)	Cytotoxicity IC_{50} (μM)	Brine shrimp toxicity LD_{50} (mg/mL)
1	3.8×10^{-3}	76	7.2×10^{-3}
2	16.0×10^{-2}	220	9.8×10^{-2}
3	2.19×10^{-3}	288	1.65×10^{-2}
4	2.02×10^{-3}	124	5.5×10^{-3}
5	1.88×10^{-2}	7	2.0×10^{-3}
6	8.0×10^{-2}	315	1.4×10^{-1}
7	11.6×10^{-2}	427	8×10^{-1}
8	1.44×10^{-2}	133	9.3×10^{-3}
Quercetin	2.88×10^{-5}	—	—
Podophyllotoxin	—	6×10^{-2}	2.79×10^{-3}

3. Experimental

3.1. General procedures

UV spectra were obtained in MeOH using a Hewlett-Packard 8453 UV–vis spectrometer. MS analyses were performed on a Quattro II triple quadrupole instrument. NMR spectra were recorded in CD_3OD on a Varian Unity INOVA 400 MHz NMR spectrometer 400 (400 MHz for ^1H and 100 MHz for ^{13}C) using the residual solvent peaks as internal standard. HPLC separation was performed using a Dionex prep-HPLC system coupled with Gynkotek GINA50 autosampler and Dionex UVD340S Photo-Diode-Array detector. A Luna C_{18} preparative (10 μM, 250 mm×21.2 mm) and/or a Luna C_{18} semi-preparative HPLC column (5 μM, 250 mm×10 mm) were used. Sep-Pak Vac 35 cc (10 g) C_{18} cartridge (Waters) was used for pre-HPLC fractions. HMBC spectra were optimised for a long range J_{H-C} of 9 Hz and the NOESY experiment was carried out with a mixing time of 0.8 s.

3.2. Plant material

The seeds of *C. schischkinii* were collected from East Anatolia, Turkey (B7 Erzincan, 30.5 km from Erzincan to Gumushane, 2024 m, 39°53′29″ N 39°21′6″ E), during September–October 2002. The voucher specimens, 10 VII.2002 and PH800001, have been maintained, respectively, in the herbarium of the Plant Ecology Laboratory, Science Faculty, Anadolu University, Turkey, and of the Plant and Soil Science Department, University of Aberdeen, Scotland (ABD).

3.3. Extraction and isolation of compounds

Ground seeds of *C. schischkinii* (80 g) were Soxhlet-extracted, successively, with *n*-hexane, dichloromethane and methanol (MeOH) (1 L each). The MeOH extract was fractionated by solid phase extraction method using a Sep-Pak C_{18} (10 g) cartridge eluting with a step gradient: 40, 60, 80 and 100% MeOH in water (200 mL each). Preparative-HPLC (eluted with a linear gradient-water: MeOH=65:25–30:70 over 50 min followed by 70% MeOH for 10 min, 15 mL/min, monitored by photo-diode-array detector) of the Sep-Pak fraction, which was eluted with 40% MeOH, afforded **1**, (7.5 mg; t_R=8.1 min), lignans **2** (59.2 mg; t_R=24.4 min), **3** (518.6 mg; t_R=28.5 min) and **4** (29.9 mg, t_R=33.4 min) and flavonoids **6** (11.7 mg, t_R=32.2 min) and **7** (11.5 mg, t_R=36.1 min). Compound **1** was further purified by semiprep HPLC (eluted with a linear gradient- water:acetronitrile (ACN)=10:90–60:40 over 50 min followed by 40% ACN for 10 min, 2 mL/min, monitored by photo-diode-array detector). Similar HPLC purification of the 60% Sep-Pak fraction resulted in the isolation of **6** (13.7 mg) and **8** (27.3 mg).

3.3.1. Schischkiniin (1). Gum; 7.5 mg; UV (MeOH) λ_{max} 220, 280 nm; HRFABMS: $C_{26}H_{24}N_6O_2Na$ $[M+Na]^+$ requires *m/z* 475.1858 (found 475.1857); ^1H and ^{13}C NMR (Table 1).

3.4. Free radical scavenging activity: DPPH assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG, Bucks. Quercetin was obtained from Avocado Research Chemicals Ltd, Shore road, Heysham, Lancs. The method used by Takao et al.[26] was adopted with appropriate modifications.[10,11] DPPH (4 mg) was dissolved in MeOH (50 mL) to obtain a concentration of 80 μg/mL.

3.4.1. Qualitative assay. Test compounds (**1–8**) were applied on a TLC plate and sprayed with DPPH solution using an atomiser. It was allowed to develop for 30 min. The colour change (purple on white) was noted.

3.4.2. Quantitative assay. Test compounds (**1–8**) were dissolved in MeOH to obtain a concentration of 0.5 mg/mL each. Dilutions were made to obtain concentrations of 5×10^{-2}, 5×10^{-3}, 5×10^{-4}, 5×10^{-5}, 5×10^{-6}, 5×10^{-7}, 5×10^{-8}, 5×10^{-9}, 5×10^{-10} mg/mL. Diluted solutions (1 mL each) were mixed with DPPH (1 mL) and allowed to stand for 30 min for any reaction to occur. 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, quercetin, a well known natural antioxidant.

3.5. Brine shrimp lethality assay

Shrimp eggs were purchased from The Pet Shop, Kittybrewster Shopping Complex, Aberdeen, UK. The bioassay was conducted following the procedure described by Meyer et al.[27] The eggs were hatched in a conical flask containing 300 mL artificial seawater. The flasks were well

aerated with the aid of an air pump, and kept in a water bath at 29–30 °C. A bright light source was left on and the nauplii hatched within 48 h. The compounds **1**–**8** were dissolved in 20% aq DMSO to obtain a concentration of 1 mg/mL. These were serially diluted two-times, and seven different concentrations were obtained. A solution of each concentration (1 mL) was transferred into clean sterile universal vials with pipette, and aerated sea-water (9 mL) was added. About 10 nauplii were transferred into each vial with pipette. A check count was performed and the number alive after 24 h was noted. LD_{50}s were determined using the Probit analysis method.[28]

3.6. MTT cytotoxicity assay

CaCo-2 cells were maintained in Earle's minimum essential medium (Sigma), supplemented with 10% (v/v) foetal calf serum (Labtech Int.), 2 mM L-glutamine (Sigma), 1% (v/v) non-essential amino acids (Sigma), 100 IU/mL penicillin and 100 μg/mL streptomycine (Sigma). Exponentially growing cells were plated at 2×10^4 cells cm^{-2} into 96-well plates and incubated for 72 h before the addition of drugs. Stock solution of compounds were initially in DMSO or H_2O and further diluted with fresh complete medium.

The growth-inhibitory effects of the compounds (**1**–**8**) were measured using standard tetrazolium MTT assay.[29] After 72 h of incubation at 37 °C, the medium was removed, and 100 μL of MTT reagent (1 mg/mL) in serum free medium was added to each well. The plates were incubated at 37 °C for 4 h. At the end of the incubation period, the medium was removed and pure DMSO (200 μL) was added to each well. The metabolised MTT product dissolved in DMSO was quantified by reading the absorbance at 560 nm on a micro plate reader (Dynex Technologies, USA). The IC_{50} values were calculated from the equation of the logarithmic line determined by fitting the best line (Microsoft Excel) to the curve formed from the data. The IC_{50} value was obtained from the equation $y=50$ (50% value).

3.7. Modelling conditions

Minimisations (2000 steps) were carried out using MacroModel version 6.5[24] using the Merck Molecular Force Field. The generalised Born solvent accessible area continuum solvent model[25] was used to simulate H_2O solvent, due to the unavailability of parameters for MeOH. The minimisations were followed by 1000 steps of Monte Carlo conformational searching to give the global energy minima shown in the figures. The global energy minima were found 15 times for **1b** (1022.68 kcal/mol) and 37 times for **1c** (986.93 kcal/mol).

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