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Inhibition of DNA Topoisomerases I and II and Cytotoxicity of Compounds from

***Polygonum aviculare* L.**

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Abstract

Six flavonoid glycosides (**1–3**, **6–8**), one lignan (**4**), and one galloyl glycoside (**5**) were isolated based on the phytochemical study for the MeOH extract of *Polygonum aviculare* L. herb. The eight isolated compounds' structures were determined by comparing their physicochemical and spectral data with published ones. Compounds **5** and **6** were first isolated from this plant, and all the compounds were evaluated for DNA topoisomerase inhibitory activities and cytotoxicities. Among the purified compounds, **5** showed stronger inhibitory activity against topoisomerase I with an IC_{50} value of 12.0 μ M, while the value of positive control camptothecin (CPT) was 17.0 μ M. For compounds **1**, **3**, and **5–8**, the value ranged from 0.054 to 17.0 μ M, suggesting more potent inhibitory activity against topoisomerase II, and that of etoposide (VP-16) was 28.0 μ M. Particularly, compounds **6** and **8** (IC_{50} = 0.054 and 0.077 μ M, respectively) inhibited Topo II about 500 times stronger than VP-16, making them safer agents of DNA topoisomerase. Furthermore, all compounds demonstrated no cytotoxic activity against four cancer cell lines. This study is the first to report the inhibition of DNA topoisomerases of compounds from *P. aviculare*, suggesting that these compounds may be potential candidates for treating cancer.

Keywords *Polygonum aviculare* L. · Chemical constituents · Topoisomerase ·

Cytotoxicity

Introduction

Various treatments are available against life-threatening cancer, including surgery, chemotherapy or radiation, or combinations of these [1], among which chemotherapy is considered the most effective. As targets of chemotherapy drugs, DNA topoisomerases are enzymes that regulate DNA topology in the cell [2]. Topoisomerase I (Topo I) and topoisomerase II (Topo II) catalyze the passage of the DNA strand and double strands through a transient single-strand break without any high-energy cofactor and a transitory double-strand break with ATP, respectively [3]. CPT and VP-16 are well-known Topo I and II inhibitors, respectively [4], but their side effects have been reported, known as DNA damage in cancer cells and fast-growing normal cells [5], which highlights the necessity of finding a safer agent of DNA topoisomerase (without or with fewer side effects).

Studies have shown that many chemotherapeutic agents are derived from natural sources, especially classical medicinal plants. For thousands of years, natural products purified from natural sources have shown different therapeutic effects with few side effects based on pharmacological studies and clinical trials and can be crucial to searching topoisomerase inhibition [2, 6]. According to previous research, several

natural medicinal plants possess Topo I, II inhibitory activity and cytotoxicity, and some activity compounds have been screened out, such as *Illicium verum* Hook. Fil. [4], *Inula japonica* Thumb. [7], *Ulmus davidiana* var. *Japonica* (Rehd.) Nakai [2], *Polygonum multiflorum* Thumb. [8], *Tilia amurensis* Rupr. [9], and *Saururus chinensis* (Lour.) Baill. [10]. Furthermore, we found that the MeOH extract of *Polygonum aviculare* L. (Polygonaceae) showed Topo I and II inhibitory activities, which was never reported previously. *P. aviculare* is a perennial herbaceous plant widely distributed in countries like China, Korea, and Japan [11] and has been used for diuretic, antidotal, antiphlogistic, antipyretic, antiparasitic, and antidiarrheal effects in Chinese folk medicine to treat nephritis, urinary system infection, and gingivitis [12, 13]. Notably, in previous studies, several secondary metabolites of *P. aviculare* have been identified, such as flavonoids, phenols, and lignins [12, 14]. *P. aviculare* is characterized by a diversity of biological activities. Based on this, further research regarding the bioactivities of primary metabolites is essential. This study described the isolation and identification of eight compounds (**1–8**) from *P. aviculare* in detail. In addition, the solvent fractions and all purified compounds of *P. aviculare* were evaluated for their DNA Topo I and II inhibitory activities and anti-proliferation activities on four cancer cells.

Results and discussion

This study investigated the chemical constituents of 70% MeOH extract of the whole plants of *P. aviculare* yielded six flavonoid glycosides: avicularin (**1**) [15], juglanin (**2**) [16, 17], myricitrin (**3**) [18], agetiin (**6**) [19, 20], miquelianin (**7**) [21], and astragalin (**8**) [22]; Aviculin (**4**), one lignan [23] and 6-di-*O*-galloyl- β -D-glucopyranose (**5**), one galloyl glycoside [24]. Their chemical structures are shown in Fig. 1, showing that flavonoids may be the main component of this plant. Compounds **5** and **6** were isolated from this plant for the first time, enriching the study of this plant's chemical constituents.

The conversions of supercoiled pBR322 plasmid DNA to the relaxed DNA were investigated using calf thymus Topo I and II in the presence of solvent fractions and **1–8**. Table 1 shows that all solvent fractions had potent inhibitory activity against Topo II but only weak inhibitory activity against Topo I. Among the purified compounds, **4**, **5**, **6**, and **8** showed strong inhibitory activity against Topo I with IC₅₀ values of 63.0, 12.0, 46.0, and 69.0 μ M, respectively, and **5** showed more potent inhibitory activity than the positive control CPT (IC₅₀: 17.0 μ M, Fig. 2, Table 2). All compounds (**1–8**) showed inhibitory activity against Topo II with IC₅₀ values of 0.58, 35.0, 12.0, 35.0, 17.0, 0.054, 0.55, and 0.077 μ M, respectively. Except for **2** and **4**, other compounds exhibited great

inhibitory activity against Topo II, while the IC₅₀ of the positive control VP-16 was 28.0 μM (Figs. 3 and 4, Table 2). In particular, compounds **6** and **8** inhibited Topo II about 500 times stronger than the positive control, VP-16. Notably, compounds **4**, **5**, **6**, and **8** had strong inhibitory activity against both DNA Topo I and II, compound **5** (1, 6-di-*O*-galloyl-β-D-glucopyranose), a galloyl glycoside, had more potent inhibitory activity compared with the positive controls (CPT and VP-16), making it a safer agent of DNA topoisomerase. This study first researched and reported the Topo I and II inhibitory activities of compounds **1–8**, except that the Topo II inhibitory activity of **1**, **3**, and **8** had been reported previously [25-27].

Secondary metabolites are critical in controlling anticancer response pathways, such as structurally diverse flavonoids with significant bioactivity. Abundant in both traditional medicinal herbs and marine life, the secondary metabolites possess various biological and chemical properties like antioxidants [28], antimicrobial, anti-inflammatory [29], antimutagenic [30]. In particular, studies have been conducted to elucidate the role of flavonoids as topoisomerase inhibitors, contributing to studying highly selective chemotherapeutic agents.

Topoisomerase research on flavonoids has been widely concerned in recent years, and some scholars suggested that flavonoids inhibit both Topo I and II [31]. For

instance, quercetin and kaempferol were reported as traditional Topo II poisons, and the C4'-OH was believed to be essential for the compound to function as a traditional poison. The addition of -OH groups at C3' and C5' could increase the redox activity of the B ring, enabling the compound to function as a redox-dependent poison [32]. In this study, compounds (**1**, **3**, **6-7**) reported moderate inhibitory activity against Topo II, and the experimental results were consistent with the previous literature. Compound **8** showed strong inhibitory activity toward Topo II without -OH group at C3' and C5', which could result from the purity of the isolated compounds and/or the nature of topoisomerase and different concentrations test methods.

Tests against four cancer cell lines were conducted (A549, SK-OV-3, HepG2, and HT-29) to investigate whether the solvent fractions and all compounds from *P. aviculare* mediated the DNA topoisomerase inhibitory activity to induce cell death, whose results showed that *n*-hexane extract and H₂O extract exhibited cytotoxicity, while none of the compounds showed cytotoxicity against the selected cell lines (Tables 1 and 2).

No noticeable correlation could be noticed between the cytotoxicity and DNA relaxation and decatenation inhibitory activity by DNA Topo I and II. It might be hypothesized that these compounds may inhibit Topo I and II reactions in enzyme assays, but transportation of these compounds via cell and nucleus membranes to the

target Topo I and II may be possible barriers in the MTT assay. Based on this newly reported biological activity, the studied plant could be a source of new anticancer agents, and further phytochemical inquiry should clarify the mechanism of action for the sake of drug discovery.

Conclusion

This is the first study to report the compounds **5** (1, 6-di-*O*-galloyl- β -D-glucopyranose) and **6** (agetiin) isolated from *P. aviculare*, and it also informs the inhibition of DNA Topo I and II of the plant. The inhibitory activities of all purified compounds against Topo I and II were reported for the first time, except that **1**, **3**, and **8** of Topo II were reported previously. Interestingly, compound **5** showed more potent inhibitory activity against both DNA Topo I and II than the positive control (CPT and VP-16). In addition, compounds **6** and **8** (astragalin) ($IC_{50} = 0.054$ and $0.077 \mu M$, respectively) inhibited Topo II about 500 times stronger than VP-16, which enabled them to be safer agents of DNA topoisomerase and serve as potential candidates for treating cancer illness. This experiment enriched and clarified the chemical compositions and bioactivities of *P. aviculare*, providing a scientific theoretical basis for further in-depth research and rational development and utilization of this plant in the future.

Materials and methods

General experimental procedures

The melting point was determined using the Fisher-Johns melting point apparatus and is unadjusted. The JASCO DIP-1000 (Tokyo, Japan) automatic digital polarimeter was used to detect optical rotations. The NMR spectra was acquired on a Bruker 250 MHz (DMX 250, Germany) spectrometer using Bruker's standard pulse program. After samples were dissolved in CDCl_3 , pyridine- d_5 , or CD_3OD , chemical shifts were determined in ppm downfield from TMS. A JEOL JMS700 spectrometer was used to get FABMS (JEOL, Japan). Merck KGaA provided the TLC plates (Silica gel 60 F254 and RP-18 F254, 0.25 mm) (Darmstadt, Germany) and stationary phases for column chromatography (Silica gel 200–300 mesh, Lichroprep RP-18 gel, 40–63 μm , SephadexTM LH-20). UV radiation and a spray of 10% H_2SO_4 followed by heating were used to identify spots. SIGMA Chemical Co. (St. Louis, MO, USA) provided CPT (purity 95%) and VP-16 (purity 98%). Supercoiled pBR322 plasmid DNA and calf thymus Topo I were given by MBI Fermentas, Inc. (Hanover, MD, USA), and one unit of the enzyme relaxes 1 μg of pBR322 DNA in 30 minutes at 37 °C. TopoGEN, Inc. (Columbus, OH, USA) supplied human Topo II and one unit of the enzyme relaxes 1 μg of pBR322 DNA in 30 minutes at 37 °C. The rest of the chemicals and solvents were analytical grade and were utilized without additional purification.

Biological material

P. Aviculare was collected in June 2019 from the Changbai Mountain area of Jilin Province, China, and was identified by Prof. Hui-zi Lv (School of Pharmaceutical Sciences, Yanbian University, China). A voucher specimen (20190607-BX) was placed at the Department of Pharmacognosy, School of Pharmaceutical Sciences, Yanbian University.

Extraction and isolation

Air-dried *P. aviculare* (9.0 kg) was extracted by reflux with 70%, 90%, and 100% MeOH for 12 h, respectively. The crude extract (1.4 kg) was suspended in distilled water and further partitioned with *n*-hexane, EtOAc and *n*-BuOH (each 1.5 L $\times$ 3), successively, to obtain *n*-hexane, EtOAc, *n*-BuOH, and aqueous fractions, respectively. The EtOAc fraction (50.0 g) was eluted with *n*-hexane–EtOAc–MeOH (50:1:0–0:1:1, v/v/v) to afford 29 fractions (Fr. E1–Fr. E29) using silica gel column chromatography (CC). Fr. E20 was subjected to reverse-phase (RP) CC via gradient elution with MeOH–H₂O (3:7–1:0, v/v) to obtain compounds **1** (165.2 mg) and **2** (29.0 mg), respectively. Fr. E22 was eluted with MeOH–H₂O (2:8–1:0, v/v) for RP CC, yielding compounds **3** (78.0 mg). Fr. E24 was subjected to RP CC by gradient elution with MeOH–H₂O (2:8–1:0, v/v) to **4** (28.0 mg). The *n*-BuOH fraction (20.0 g) was separated into 17 fractions (Fr.

B1–B17) using silica gel CC with CH₂Cl₂–MeOH–H₂O (50:1:0–2:1:0.1, v/v/v). Fr. B12 was separated successively using a Sephadex LH-20 column by eluting with CH₂Cl₂–MeOH (3:7, v/v) and an RP column by eluting with MeOH–H₂O (1:9–1:0, v/v) to yield compounds **5** (38.6 mg) and **6** (4.2 mg). Fr. B14 was subjected to RP CC by gradient elution with MeOH–H₂O (1:9–1:0, v/v) to afford **7** (15 mg) and **8** (24.1 mg).

Physical and spectroscopic data of isolated compounds

Avicularin (1)

Yellow amorphous powder; mp 180–183 °C; $[\alpha]_D^{25}$ -150.3 (*c* 0.1, MeOH); Positive FABMS *m/z* 435.0 [M+H]⁺; ¹H NMR (Methanol-*d*₄, 250 MHz) δ 7.51 (1H, br s, H-6'), 7.47 (1H, d, *J* = 2.1 Hz, H-2'), 6.91 (1H, d, *J* = 8.1 Hz, H-5'), 6.38 (1H, br s, H-8), 6.19 (1H, br s, H-6), 5.46 (1H, br s, H-1''), 4.33 (1H, br s, H-2''), 3.48–3.92 (4H, m, H-3''–H-5''); ¹³C NMR (Methanol-*d*₄, 62.9 MHz) δ 180.1 (C-4), 166.2 (C-7), 163.2 (C-5), 159.5 (C-2), 158.7 (C-9), 150.0 (C-4'), 146.5 (C-3'), 135.0 (C-3), 123.2 (C-1'), 123.1 (C-6'), 116.9 (C-2'), 116.6 (C-5'), 109.6 (C-1''), 105.7 (C-10), 100.0 (C-6), 94.9 (C-8), 88.1 (C-4''), 83.5 (C-2''), 78.8 (C-3''), 62.6 (C-5'').

Juglanin (2)

Yellow amorphous powder; mp 181–183 °C; $[\alpha]_D^{25}$ -162.7 (*c* 0.1 MeOH); Positive FABMS *m/z* 419.0 [M+H]⁺; ¹H NMR (Methanol-*d*₄, 250 MHz) δ 7.97 (2H, d, *J* = 8.8 Hz, H-2',

H-6'), 6.93 (2H, d, $J = 8.8$ Hz, H-3', H-5'), 6.40 (1H, d, $J = 2.1$ Hz, H-8), 6.20 (1H, d, $J = 2.0$ Hz, H-6), 5.47 (1H, brs, H-1''); ^{13}C NMR (Methanol- d_4 , 62.9 MHz) δ 180.1 (C-4), 166.2 (C-7), 163.3 (C-5), 161.8 (C-4'), 159.6 (C-9), 158.7 (C-2), 135.1 (C-3), 132.2 (C-2', 6'), 122.9 (C-1'), 116.7 (C-3', 5'), 109.8 (C-1''), 105.8 (C-10), 100.0 (C-6), 94.9 (C-8), 88.1 (C-4''), 83.5 (C-2''), 78.8 (C-3''), 62.7 (C-5'').

Myricitrin (3)

Yellow amorphous powder; mp 199-200°C; $[\alpha]_D^{25} -120$ (c 0.06 MeOH); Positive FABMS m/z 465.1 $[\text{M}+\text{H}]^+$; ^1H NMR (Methanol- d_4 , 250 MHz) δ 6.95 (2H, s, H-2', H-6'), 6.36 (1H, d, $J = 1.9$ Hz, H-8), 6.20 (1H, d, $J = 1.9$ Hz, H-6), 5.32 (1H, d, $J = 1.9$ Hz, H-1''), 3.35–3.93 (4H, m, H-2''–H-5''), 0.98 (3H, d, $J = 6.1$ Hz, H-6''); ^{13}C NMR (Methanol- d_4 , 62.9 MHz) δ 179.7 (C-4), 166.0 (C-7), 163.3 (C-5), 159.6 (C-2), 158.6 (C-9), 147.0 (C-3', C-5'), 138.0 (C-4), 136.4 (C-3), 122.0 (C-1'), 109.7 (C-2', C-6'), 106.0 (C-10), 103.7 (C-1''), 99.9 (C-6), 94.8 (C-8), 73.5 (C-4''), 72.2 (C-2'', C-3''), 72.0 (C-5''), 17.8 (C-6'').

Aviculin (4)

Brown amorphous powder; mp 155-157°C; $[\alpha]_D^{25} +20.3$ (c 0.074 MeOH); Positive FABMS m/z 506.1 $[\text{M}]^+$; ^1H NMR (Methanol- d_4 , 250 MHz) δ 6.75 (1H, d, $J = 7.5$ Hz, H-5'), 6.67 (1H, s, H-5), 6.64 (1H, d, $J = 1.5$ Hz, H-2'), 6.58 (1H, dd, $J = 1.8, 8.0$ Hz, H-6'), 6.17 (1H, s, H-8), 4.51 (1H, d, $J = 1.0$ Hz, H-1''), 3.81 (3H, 6-OCH₃), 3.78 (3H, H-

3'-OCH₃), 1.20 (1H, d, $J = 5.0$ Hz, H-6''); ¹³C NMR (Methanol-*d*₄, 62.9 MHz) δ 149.3 (C-6), 147.4 (C-3'), 146.3 (C-7), 145.4 (C-4'), 138.2 (C-9), 134.2 (C-1'), 129.1 (C-10), 123.3 (C-6''), 117.3 (C-8), 116.2 (C-5'), 113.7 (C-2'), 112.6 (C-5), 102.4 (C-1''), 74.0 (C-4''), 72.7 (C-3''), 72.5 (C-2''), 70.3 (C-5''), 68.1 (C-2 α), 65.5 (C-3 α), 56.5 (3'-OCH₃, 6-OCH₃), 50.0 (C-1), 45.6 (C-2), 40.2 (C-3), 33.8 (C-4), 18.1 (C-6'').

1, 6-Di-*O*-galloyl- β -D-glucopyranose (5)

Brown amorphous powder; mp 200-203 °C; $[\alpha]_D^{25} +28.4$ (*c* 0.1 MeOH); Positive FABMS m/z 467.0 [M-OH]⁺; ¹H NMR (Methanol-*d*₄, 250 MHz) δ 7.11 (2H, s, H-2', H-2''), 7.06 (2H, s, H-6', H-6''), 5.67 (1H, d, $J = 7.25$ Hz, H-1), 4.56 (1H, dd, $J = 1.4, 10.4$ Hz, H-6 α), 4.37 (1H, dd, $J = 4.8, 12.2$ Hz, H-6 β), 3.50–3.80 (4H, m, H-2–5); ¹³C NMR (Methanol-*d*₄, 62.9 MHz) δ 168.3 (C-7''), 167.0 (C-7'), 146.5 (C-3', C-3'', C-5', C-5''), 140.4 (C-4''), 140.1 (C-4'), 121.3 (C-1''), 120.6 (C-1'), 110.6 (C-6', C-6''), 110.2 (C-2', C-2''), 95.9 (C-1), 78.0 (C-3), 76.5 (C-5), 74.1 (C-2), 71.2 (C-4), 64.4 (C-6).

Tagetiin (6)

Yellow amorphous powder; mp 196-198 °C; $[\alpha]_D^{25} -108.2$ (*c* 0.1 MeOH); Positive FABMS m/z 481.0 [M+H]⁺; ¹H NMR (Methanol-*d*₄, 250 MHz) δ 7.85 (1H, d, $J = 2.0$ Hz, H-2'), 7.78 (1H, dd, $J = 2.1, 8.5$ Hz, H-6'), 6.91 (1H, d, $J = 8.5$ Hz, H-5'), 6.66 (1H, s, H-8), 4.98 (1H, d, $J = 7.2$ Hz, H-1''), 3.96–3.47 (6H, m, H-2''–H-6''); ¹³C NMR (Methanol-*d*₄,

62.9 MHz) δ 180.1 (C-4), 153.5 (C-7), 151.7 (C-2), 149.1 (C-5), 148.8 (C-9), 146.2 (C-3'), 145.6 (C-4'), 137.5 (C-3), 132.5 (C-6), 125.3 (C-1'), 122.2 (C-6'), 116.5 (C-5'), 116.2 (C-2'), 106.5 (C-10), 103.3 (C-1''), 99.6 (C-8), 78.2 (C-3''), 77.3 (C-5''), 74.5 (C-2''), 70.9 (C-4''), 62.0 (C-6'').

Miquelianin (7)

Brown amorphous powder; mp 190-192°C; $[\alpha]_D^{25} -24.5^\circ$ (*c* 0.66 MeOH); Positive FABMS *m/z* 460.1 [M-OH+H]⁺; ¹H NMR (Methanol-*d*₄, 250 MHz) δ 7.80 (2H, m, H-2', H-6'), 6.84 (1H, d, *J* = 9.0 Hz, H-5'), 6.38 (1H, d, *J* = 2.0 Hz, H-8), 6.19 (1H, d, *J* = 2.0 Hz, H-6), 5.33 (1H, d, *J* = 7.4 Hz, H-1''), 3.77 (1H, d, *J* = 9.5 Hz, H-5''), 3.42–3.62 (3H, m, H-2''–H-4''); ¹³C NMR (Methanol-*d*₄, 62.9 MHz) δ 179.2 (C=O), 172.3 (C-6''), 166.0 (C-7), 163.0 (C-5), 159.0 (C-2), 158.4 (C-9), 150.0 (C-4'), 146.0 (C-3'), 135.4 (C-3), 123.5 (C-6'), 122.8 (C-1'), 117.2 (C-5'), 116.0 (C-2'), 105.6 (C-10), 104.3 (C-1''), 99.9 (C-6), 94.8 (C-8), 77.6 (C-3''), 77.1 (C-5''), 75.4 (C-2''), 72.9 (C-4'').

Astragalin (8)

Yellow amorphous powder; mp 218-220°C; $[\alpha]_D^{25} +15.5^\circ$ (*c* 0.2 MeOH); Positive FABMS *m/z* 430.2 [M-H₂O]⁺; ¹H NMR (Methanol-*d*₄, 250 MHz) δ 8.00 (2H, d, *J* = 7.0 Hz, H-2', H-6'), 6.86 (2H, d, *J* = 7.1 Hz, H-3', H-5'), 6.38 (1H, br s, H-8), 6.19 (1H, br s, H-6), 5.21 (1H, d, *J* = 7.4 Hz, H-1''), 3.42–3.88 (6H, m, H-2''–H-6''); ¹³C NMR

(Methanol-*d*₄, 62.9 MHz) δ 179.3 (C-4), 166.0 (C-7), 163.0 (C-5), 161.6 (C-4'), 159.2 (C-9), 158.4 (C-2), 135.3 (C-3), 132.3 (C-2', 6'), 122.4 (C-1'), 116.1 (C-3', 5'), 105.6 (C-10), 104.5 (C-1''), 100.0 (C-6), 94.8 (C-8), 78.2 (C-5''), 77.5 (C-3''), 75.4 (C-2''), 72.9 (C-4''), 62.5 (C-6'').

Assay for DNA Topo I inhibition *in vitro*

The relaxation of supercoiled pBR322 plasmid DNA was measured using the isolated compounds in the DNA Topo I inhibition experiment, which was slightly modified from the formerly described procedure. The reaction mixture contains 72 mM KCl, 5 mM MgCl₂, 35 mM Tris-HCl (pH 8.0), 5 mM DTT, 2 mM spermidine, 0.01% bovine serum albumin (BSA), 250 ng pBR322 plasmid DNA, and 0.3 U calf thymus DNA Topo I, as well as the test compounds at the indicated concentrations (less than 0.25% DMSO), in a definite volume of 10 μ l. After 30 min of incubation at 37 °C, the reaction solutions were finished with a dye solution containing 2.5% SDS, 15% ficoll-400, 0.05% xylene cyanole, 0.05% bromophenol blue, and 25 mM EDTA (pH 8.0). Electrophoresis at 1.5 V/cm for 10 h on a 1% agarose gel in a TBE (Tris-borate-EDTA) running buffer was utilized to identify the reaction products. The gels were stained for 30 min with ethidium bromide (0.5 μ g/mL) before being destained in water for 30 min. The gels were scanned directly with an image analyzer for visualization and quantification of

DNA Topo I activity, with the area representing supercoiled DNA being computed [33].

Each sample was tested in triplicate.

Assay for DNA Topo II inhibition *in vitro*

The relaxation of supercoiled pBR322 plasmid DNA was used to assess DNA Topo II activity. The reaction mixtures contained 50 mM Tris-HCl (pH 8), 120 mM KCl, 10 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM ATP, 300 ng pBR322 plasmid DNA, and 0.3 U human DNA Topo II, as well as the test compounds at the indicated concentrations (less than 0.25% DMSO), in a final volume of 20 µL. After 30 min at 37°C, the reaction mixtures were aborted by adding 5 µL of a mixture including 0.77% SDS, 77 mM EDTA (pH 8.0), 30% sucrose, 0.5% bromophenol blue, and 0.5% xylene cyanole. Electrophoresis at 1.5 V/cm for 10 h on a 1% agarose gel in a TBE (Tris-borate-EDTA) running buffer was used to determine the reaction products. The gels were stained with 0.5 µg/mL ethidium bromide for 30 min before being destained in water for 30 min. The gels were scanned directly with an image analyzer to visualize and quantify DNA Topo II activity, and the area representing supercoiled DNA was then measured [33]. Each sample was tested three times.

Cytotoxicity in human tumor cell lines

A tetrazolium-based colorimetric assay (MTT assay) was used to determine the

cytotoxicity of human lung cancer (A549), ovarian cancer (SK-OV-3), human liver hepatoblastoma (HepG2), and colon adenocarcinoma (HT-29) cell lines [34]. All samples were tested in triplicate.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Fig. 1 Chemical structures of compounds 1–8 from *P. aviculare* herb

Fig. 2 DNA Topo I inhibitory activity of compounds **4**, **5**, **6**, and **8**

D: supercoiled DNA only; T: supercoiled DNA + Topo I (human); C1: supercoiled DNA + Topo I (human) + camptothecin (1 μ M) – positive control; C2: supercoiled DNA + Topo I (human) + camptothecin (100 μ M) – positive control; a: supercoiled DNA + Topo I (human) + compound (1 μ M); b: supercoiled DNA + Topo I (human) + compound (100 μ M)

Fig. 3 DNA Topo II inhibitory activity of compounds **2**, **3**, **4**, and **5**

D: supercoiled DNA only; T: supercoiled DNA + Topo II (human); C1: supercoiled DNA + Topo II (human) + etoposide (1.0 μM) – positive control; C2: supercoiled DNA + Topo II (human) + etoposide (100.0 μM) – positive control; a: supercoiled DNA + Topo II (human) + compound (1.0 μM); b: supercoiled DNA + Topo II (human) + compound (100.0 μM)

Fig. 4 DNA Topo II inhibitory activity of compounds **1**, **6**, **7**, and **8**

D: supercoiled DNA only; T: supercoiled DNA + Topo II (human); C1: supercoiled DNA + Topo II (human) + VP-16 (10.0 μ M) – positive control; C2: supercoiled DNA + Topo II (human) + VP-16 (1000.0 μ M) – positive control; a: supercoiled DNA + Topo II (human) + compound (10.0 μ M); b: supercoiled DNA + Topo II (human) + compound (1000.0 μ M)

Table 1 Inhibitory and cytotoxic effects of the MeOH extract of *P. aviculare*, and its solvent fractions, for DNA Topo I and II (% Inhibition ratio of relaxation)

Extract / fractions	Inhibition ratio for	Inhibition ratio for	Cytotoxicity (% , 50 µg/mL)			
	Topo I	Topo II				
	(% , 50 µg/mL)	(% , 50 µg/mL)	A549 ^a	SK-OV-3 ^b	HepG2 ^c	HT-29 ^d
MeOH extract	2	91	18	0	0	6
<i>n</i> -Hexane extract	16	88	20	13	10	18
EtOAc extract	16	97	7	0	0	0
<i>n</i> -BuOH extract	1	89	1	0	0	0
H ₂ O extract	8	82	10	3	6	7

^aA549: human lung cancer; ^bSK-OV-3: Ovarian cancer; ^cHepG2: Human liver hepatoblastoma; ^dHT-29: Human colon adenocarcinoma

Table 2 Inhibitory effects of compounds **1–8** for DNA Topo I and II (% Inhibition ratio of relaxation), and their IC₅₀ values against A549, SK-OV-3, HepG2 and HT-29 cell lines

Comp.	Inhibition ratio for Topo I			Inhibition ratio for Topo II					Cytotoxicity IC ₅₀			
	(%)			(%)					(μM)			
	20	100	IC ₅₀	0.2	1.0	20	100	IC ₅₀	A549 ^a	SK-OV-3 ^b	HepG2 ^c	HT-29 ^d
	μM	μM	μM	μM	μM	μM	μM	μM				
1	0	0	0	13	100	0	0	0.58	>100	>100	>100	>100
2	0	0	0	0	0	48	100	35	>100	>100	>100	>100
3	0	0	0	0	0	80	88	12	>100	>100	>100	>100
4	31	71	63	0	0	45	62	35	>100	>100	>100	>100
5	84	100	12	0	0	81	100	17	>100	>100	>100	>100

6	15	100	46	88	98	-	-	0.054	>100	>100	>100	>100
7	0	0		10	96	-	-	0.55	>100	>100	>100	>100
8	2	76	69	76	94	-	-	0.077	>100	>100	>100	>100
CPT ^e	51	77	17	- ^g	-	-	-	-	10.0	1.0	0.7	1.7
VP-16 ^f	-	-	-	-	-	46	64	28	-	-	-	-

^aA549: human lung cancer; ^bSK-OV-3: Ovarian cancer; ^cHepG2: Human liver hepatoblastoma; ^dHT-29: Human colon adenocarcinoma;

^eCPT (camptothecin): positive control for Topo I; ^fVP-16 (etoposide): positive control for Topo II; ^g -: not applicable

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