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RESEARCH ARTICLE

Triterpenoids isolated from the rhizomes and roots of *Gentiana scabra* and their inhibition of indoleamine 2,3-dioxygenase

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Abstract *Gentiana scabra* Bunge (Gentianaceae) is an important traditional Chinese medicine commonly used as a stomachic or appetite stimulant. In this study, 21 triterpenoids (**1**–**21**) were isolated from a methanol extract of the rhizomes and roots of *G. scabra*. Their structures were elucidated by comparing spectroscopic data with reported values. Among the isolated triterpenoids, scabanol (**2**) was firstly isolated from natural sources. All isolated compounds were evaluated for their inhibitory activity against indoleamine 2,3-dioxygenase (IDO), which catalyzes the rate limiting reaction for the conversion of tryptophan to kynurenine. Compounds **10** and **11** showed significant inhibitory activities, with IC_{50} values of 12.5 and 9.5 μM, respectively. Compound **12** showed a moderate inhibitory effect, with an IC_{50} value of 18.7 μM. Compounds **2** and **13** showed weaker inhibitory effects, with IC_{50} values of 56.8 and 60.6 μM, respectively. Kynurenine is a potent immune modulator to suppress the functions of a variety of immune cells including T cells and natural killer cells. Given that, our results that a few selected triterpenoids inhibit IDO warrant further studies on their effects on the host immune system as natural immune stimulators.

Keywords *Gentiana scabra* · Gentianaceae · IDO inhibition · Triterpenoid

Introduction

The rhizomes and roots of *Gentiana scabra* Bunge (Gentianaceae) are traditional medicines used as a stomachic or appetite stimulant in China, Korea, and Japan (Ikeshiro and Tomita 1983). Previous phytochemical investigations of *G. scabra* resulted in the isolation of secoiridoids, triterpenoids, flavonoids, xanthones and alkaloids (Ikeshiro and Tomita 1983; Ikeshiro et al. 1990; Tan et al. 1996; Bergeron et al. 1997; Kakuda et al. 2001, 2002; Kim et al. 2009). Several secoiridoid glycosides exhibit smooth muscle relaxing, antibacterial, and free radical scavenging activities (Rojas et al. 2000; Kumarasamy and Nahar 2003). In particular, *G. scabra* has been shown to protect the liver, inhibit liver dysfunction, and promote gastric acid secretion, which makes it a popular component of Chinese herbal medicine and health products (Zhang et al. 2010). At this time, most studies have focused on secoiridoid, and the biological activities of triterpenoids from *G. scabra* are not well-charaterized. In the present study, we attempted to identify novel indoleamine 2,3-dioxygenase (IDO) inhibitors. A total of 21 triterpenoids (**1**–**21**) were isolated from a methanol extract of the rhizomes and roots of *G. scabra*.

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All isolated compounds were evaluated for their inhibitory activity against IDO.

IDO is an intracellular monomeric heme-containing protein that catalyzes the degradation of tryptophan, an essential amino acid, to *N*-formyl-kynurenine, which is further metabolized to kynurenine. The role of IDO in immunomodulation has been corroborated in studies with numerous animal models, including models of allograft tolerance, inflammation, and cancer (Mellor and Munn 2004). Recent studies have focused on the role of IDO in the induction of tumor immune tolerance (Liu et al. 2010). IDO mediated depletion of local tryptophan levels, and the production of kynurenine results in the suppression of T cell activation and induction of T-cell apoptosis (Curti et al. 2009). Thus, IDO is a promising molecular target of novel therapeutic agents for treating cancer and neurological disorders, as well as other diseases characterized by pathological tryptophan metabolism (Yue et al. 2009).

Materials and methods

General experimental procedures

The NMR spectra were recorded using a JEOL ECA 600 spectrometer (^{1}H, 600 MHz; ^{13}C, 150 MHz), electrospray ionization mass spectra (ESI-MS) using an Agilent 1200 LC-MSD Trap spectrometer. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were obtained using an Agilent 6530 Accurate-Mass Q-TOF LC/MS system. Column chromatography was performed using a silica gel (Kieselgel 60, 70–230, and 230–400 mesh, Merck, Darmstadt, Germany), YMC RP-18 resins, and thin layer chromatography (TLC) was performed using pre-coated silica-gel 60 F_{254} and RP-18 F_{254}S plates (both 0.25 mm, Merck, Darmstadt, Germany).

Plant material

Dried rhizomes and roots of *G. scabra* were purchased from herbal company, Naemome Dah, Ulsan, Korea in December 2013 and identified by one of the authors (Prof. Young Ho Kim). A voucher specimen (CNU 13109) was deposited at the Herbarium of College of Pharmacy, Chungnam National University.

Extraction and isolation

Dried rhizomes and roots (2.5 kg) of *G. scabra*were extracted with MeOH (10 L × 3) under reflux. The MeOH extract (670.0 g) was suspended in water and partitioned with $CHCl_3$ and *n*-BuOH. The $CHCl_3$ fraction (90.0 g) was subjected to silica gel (8 × 30 cm) column chromatography with *n*-hexane–EtOAc (20:1, 5:1), $CHCl_3$-acetone (5:1), and $CHCl_3$–MeOH–H_2O (6:1:0, 3:1:0.1) to give 5 fractions (Fr. 1A–1E). The fraction 1B (17.8 g) was subjected to silica gel (8 × 30 cm)column chromatography with *n*-hexane–EtOAc (50:1, 25:1, 15:1, 10:1, 5:1; 4.0 L for each step) elution solvent to give 15 sub-fractions (Fr. 1B-1–1B-15).The fraction 1B-7 was separated using an YMC (1 × 80 cm) column chromatography with a MeOH-acetone-H_2O (3:3:1–7:7:1, 2.5 L) elution solvent to give compounds **6** (5.0 mg),**7** (26.8 mg), **8** (9.0 mg), and **20** (12.6 mg). The fraction 1B-9 was separated using an YMC (1 × 80 cm) column chromatography with a MeOH-acetone-H_2O (6:6:1–10:10:1, 1.0 L) elution solvent to give compounds **1** (8.0 mg) and **5** (1.6 mg).The fraction 1B-10 was separated using an YMC (1 × 80 cm) column chromatography with a MeOH-acetone-H_2O (7:7:1, 650 mL) elution solvent to give compound **3** (7.0 mg).The fraction 1B-12 was separated using an YMC (1.5 × 80 cm) column chromatography with a MeOH-acetone-H_2O (3:3:1–10:10:1, 3.5 L) elution solvent to give compounds **2** (3.0 mg), **9** (75.2 mg), **10** (37.0 mg), **14** (6.2 mg), and **21** (3.7 mg). The fraction 1B-13 was separated using an YMC (1 × 80 cm) column chromatography with a MeOH-acetone-H_2O (5:5:1–8:8:1, 1.5 L) elution solvent to give compounds **11** (62.7 mg), **16** (2.1 mg), and **19** (5.5 mg).The fraction 1D (28.6 g) was subjected to silica gel (4 × 30 cm) column chromatography with $CHCl_3$–MeOH (20:1, 10:1, 7:1, 5:1, 3:1; 3.0 L for each step) elution solvent to give 8 sub-fractions (Fr. 1D-1–1D-8).The fraction 1D-1 was separated using an YMC (1.5 × 80 cm) column chromatography with a MeOH-acetone-H_2O (1:1:1–4:4:1, 2.5 L) elution solvent to give compounds **4** (17.0 mg), **12** (45.9 mg), **13** (42.3 mg), **15** (4.7 mg), **17** (5.2 mg), and **18** (12.6 mg).

*Scabranol (**2**)*

White powder; $C_{30}H_{50}O_3$; [α]: +12.78 (*c* 0.1, MeOH); ^{1}H-NMR (pyridine-d_5, 600 MHz) and ^{13}C-NMR data (pyridine-d_5, 150 MHz), see Table 1; HR-ESI-MS: *m/z* 481.3661 $[M+Na]^+$ (calcd for $C_{30}H_{50}O_3Na$, 481.3652).

IDO assay and determination of inhibition pattern of IDO inhibitors

IDO assays were performed mainly as described previously by Nakano et al. (2012). Briefly, a compound serially diluted in DMSO was mixed with 1 μg of purified human IDO in an IDO assay buffer (50 mM potassium phosphate buffer, pH 6.5). Then L-(+)-ascorbic acid, methylene blue, and catalase and L-tryptophan were added in order to the enzyme-compound mixture to final concentrations of 50 mM, 20 mM, 10 mM, 100 μg/mL, and 200 μM, respectively. The enzyme reaction mixture was incubated at 37 °C for 1 h. After

Table 1 The ^{1}H- and ^{13}C-NMR spectroscopic data of compound **2**

	$\delta_C^{a,b}$	$\delta_H^{a,c}$(*J* inHz)
1	39.6	1.68 m
		0.98 m
2	28.6	1.90 m
3	78.3	3.48 m
4	39.8	–
5	56.2	0.81 m
6	19.3	1.60 m
		1.37 m
7	34.2	1.39 m
		1.31 m
8	42.5	–
9	51.4	1.23 m
10	37.7	–
11	21.8	1.52 m
		1.27 m
12	24.4	1.53 m
		1.26 m
13	44.8	2.08 m
14	41.4	–
15	31.2	1.87 m
		1.39 m
16	36.0	2.49 m
17	216.6	–
18	50.5	–
19	34.0	2.11 m
		1.88 m
20	36.5	2.54 m
21	214.1	–
22	41.3	2.60 m
23	29.0	1.26 s
24	16.7	1.07 s
25	16.7	0.88 s
26	16.9	0.98 s
27	17.0	1.02 s
28	20.7	1.08 s
29	18.7	1.06d (7.5)
30	18.8	1.06d (7.5)

Assignments were done by HMQC and HMBC experiments; *J* values (Hz) are in parentheses

[a] Measured in pyridine-d_5

[b] 150 MHz

[c] 600 MHz

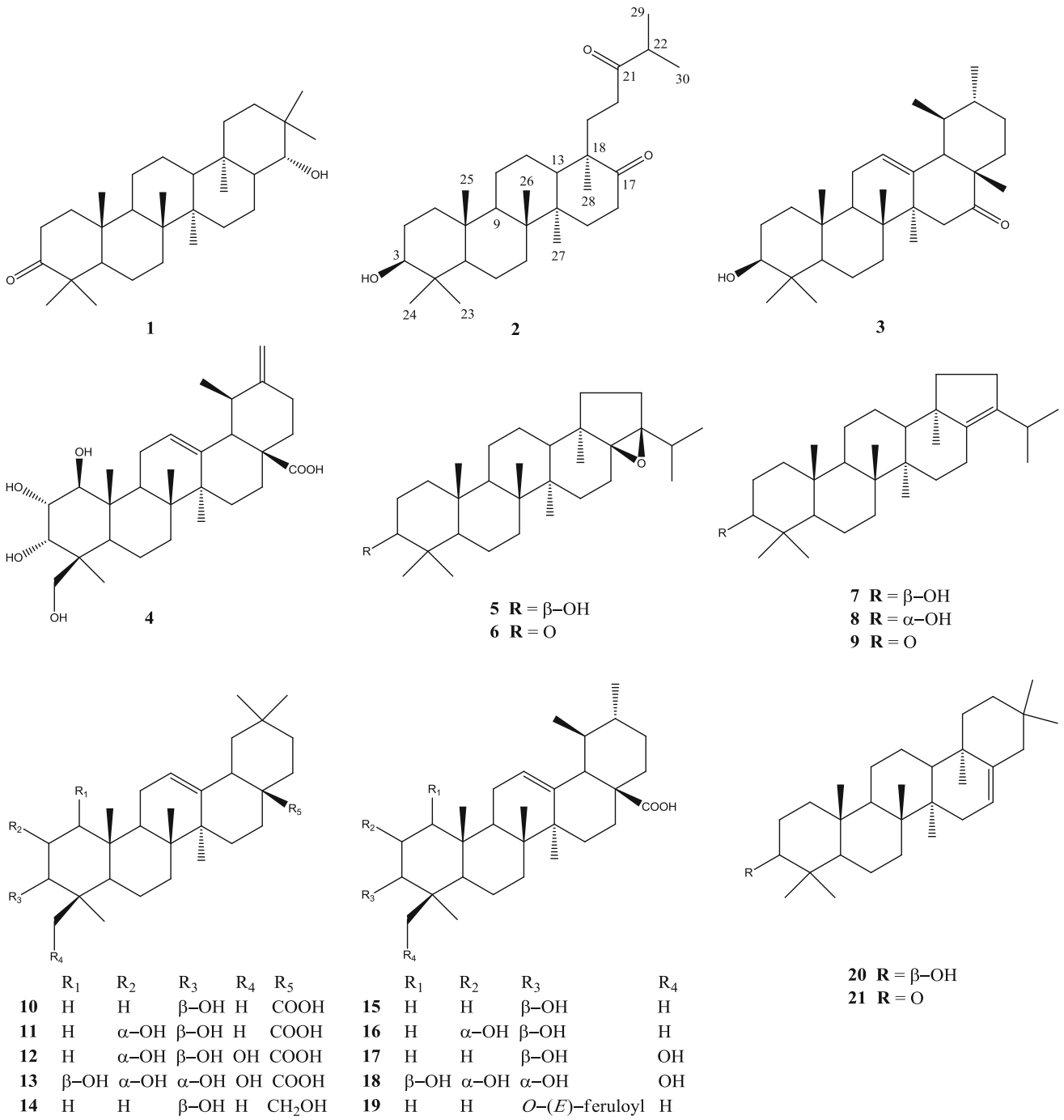
incubation, the reaction mixture was supplemented with 40 µL 30 % Trichloroacetic acid and heated for 15 min at 65 °C followed by centrifugation to remove the precipitate. The supernatant taken after centrifugation was mixed with an equal volume of Ehrlich's reagent (2 % *p*-dimethylaminobenzaldehyde in acetic acid) and incubated at RT. The intensity of the color developed, which represents the concentration of L-kynurenine produced during the enzyme reaction, was measured by reading the absorbance at 480 nm wavelength.

Inhibition patterns of IDO inhibitors were determined with Lineweaver–Burk plot, for which IDO assays were run at 5 different L-tryptophane concentrations. The assays were also run in the presence or absence of an IDO inhibitor of interest. Ki of IOD inhibitors were calculated from the y intercept of Lineweaver–Burk plot, which is denoted by (1+ [I]/Ki)/Vmax. (Kudo and Boyd 2000; Dolušic 2011a).

Results and discussion

During the screening of *G. scabra* for IDO inhibitory activity, we subjected the MeO Hextract to combined chromatographic separation and isolated 21 triterpenoids (**1**–**21**). Their structures were identified as duruvillonol (**1**; Huneck 1984), scabranol (**2**), 3β-hydroxy-urs-12-en-16-one (**3**; Laird et al. 1960), 1β,2α,3α,24-tetrahydroxyursa-12,20(30)-dien-28-oic acid (**4**; Fan et al. 2010), 17β,21β-epoxyhopan-3β-ol (**5**; Tanaka et al. 1990), 17β,21β-epoxyhopan-3-one (**6**; Tanaka et al. 1990), hop-17(21)-en-3β-ol (**7**; Kakuda et al. 2002), hop-17(21)-en-3α-ol (**8**; Ahmed and Bibi 1981), hopenone (**9**; Kakuda et al. 2002), oleanolic acid (**10**; Li 2013), masilinic acid (**11**; Sanchez-Quesada et al. 2013), urjinolic acid (**12**; Yang et al. 2014), 1β,2α,3α,24-tetrahydroxyolean-12-en-28-oic acid (**13**; Fan et al. 2010), 3β-erythrodiol (**14**; Szakiel et al. 2012), ursolic acid (**15**; Kim et al. 2014), corosolic acid (**16**; Kukina et al. 2014), 3β,24-dihydroxyurs-12-en-28-oic acid (**17**; Fan et al. 2010), pygenic acid C (**18**; Fan et al. 2010), 3-O-β-feruloylursolic acid (**19**; Tanachatchairatana et al. 2008), chiratenol (**20**; Kakuda et al. 2002), and chiratenone (**21**; Kakuda et al. 2002) (Fig. 1). Their structures were elucidated by comparing the spectroscopic data to reported values. Compounds **1**, **3**–**5**, **8**, **13**, **18**, and **19** were isolated from *G. scabra* for the first time. This is the first comprehensive report on triterpenoid components in *G. scabra* rhizomes and roots.

Compound **2** was obtained as a white powder. The molecular structure of **2** was inferred to be $C_{30}H_{50}O_3$ based on the pseudo molecular ion peak $[M+Na]^+$ at *m/z* 481.3661 in the HR-ESI-MS spectrum (calcd for $C_{30}H_{50}O_3Na$, 481.3652). The ^{1}H-NMR spectrum of **2** (Table 1) revealed 8 methyl groups [δ_H0.88 (s, H-25), 0.98 (s, H-26), 1.02 (s, H-27), 1.06 (d, $J = 7.5$, H-29, 30), 1.07 (s, H-24), 1.08 (s, H-28), 1.26 (s, H-23)], 5 methine groups [δ_H0.81 (m, H-5), 1.23 (m, H-9), 2.08 (m, H-13), 2.60 (m, H-22), 3.48 (m, H-3)],and 10 methylene groups (δ_H 0.98–2.54, H-1, 2, 6, 7, 11, 12, 15, 16, 19, 20). The^{13}C-NMR spectrum (Table 1), obtained using the DEPT spectrum, showed 30 signals



	R_1	R_2	R_3	R_4	R_5
10	H	H	β–OH	H	COOH
11	H	α–OH	β–OH	H	COOH
12	H	α–OH	β–OH	OH	COOH
13	β–OH	α–OH	α–OH	OH	COOH
14	H	H	β–OH	H	CH_2OH

	R_1	R_2	R_3	R_4
15	H	H	β–OH	H
16	H	α–OH	β–OH	H
17	H	H	β–OH	OH
18	β–OH	α–OH	α–OH	OH
19	H	H	*O*–(*E*)–feruloyl	H

Fig. 1 Structures of compounds **1**–**21** from the rhizomes and roots of *G. scabra*

including two carbonyl groups [δ_C214.1 (C-22), 216.6 (C-17)], 5 quaternary carbons [δ_C37.7 (C-10), 39.8 (C-4),41.4 (C-14),42.5 (C-8),50.5 (C-18)], 5 methine carbons [δ_C41.3 (C-22), 44.8 (C-13), 51.4 (C-9), 56.2 (C-5), 78.3 (C-3)], 10 methylene carbons [δ_C19.3 (C-6), 21.8 (C-11), 24.4 (C-12), 28.6 (C-2), 31.2 (C-15), 34.0 (C-19), 34.2 (C-7), 36.0 (C-16), 36.5 (C-20), 39.6 (C-1)], and 8 methyl carbons [δ_C16.7 (C-24, 25), 16.9 (C-26), 17.0 (C-27), 18.7 (C-29), 18.8 (C-30), 20.7 (C-28), 29.0 (C-23)]. Analyses of the ^{1}H- and ^{13}C-NMR spectral data, as well as DEPT spectrum, indicated that **2** was similar to 17β,21β-epoxyhopan-3β-ol (**5**), which is a hopane-type triterpenoid. The 17,21-epoxy group was not observed in **2**, but was split to form two carbonyl groups (C-17 and 21). Key HMBC correlations between H-28 (δ_H1.08)/C-19 (δ_C34.0) and C-17 (δ_C216.6); H-19 (δ_H1.88, 2.11)/C-21 (δ_C214.1) and C-28 (δ_C20.7); and H-20 (δ_H2.54)/C-18 (δ_C50.5) and C-22 (δ_C41.3)

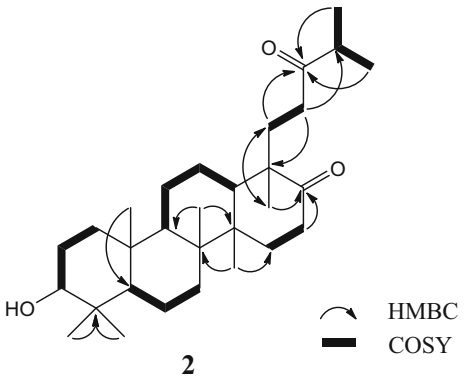


Fig. 2 ^{1}H–^{1}H COSY and HMBC correlations of compound **2**

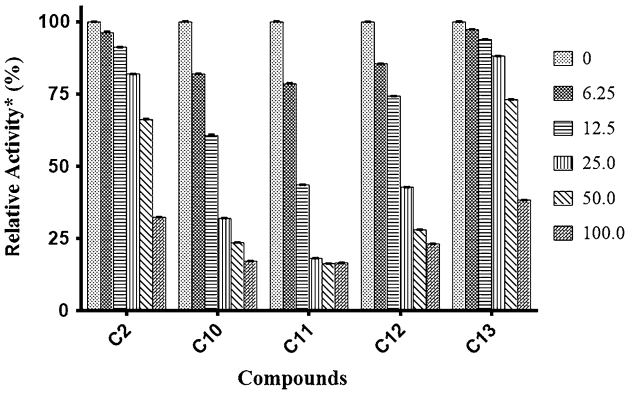


Fig. 3 IDO inhibitory effects of compounds **2** and **10**–**13**. *Asterisk* activities relative to that obtained in the absence of IDO inhibitor were plotted. Concentrations (μM) of inhibitors used in the experiment are as shown

Table 2 IC_{50} values of selected triterpene IDO inhibitors

Compound	IC_{50} (μM)[a]
2	56.8 ± 4.7
10	12.5 ± 3.4
11	9.5 ± 2.4
12	18.5 ± 1.1
13	60.6 ± 2.2
Menadione[b]	3.7 ± 0.5

[a] IC_{50} values are means ± SDs (n = 3). Other compounds are considered to be inactive (IC_{50} > 100 μM)

[b] Positive control

indicated that the E ring of aglycone was split to a chain (C-19–22, 29, and 30) (Fig. 2). Clear NOE correlations were observed between H-1α (δ_H1.68) and H-3α (δ_H3.48) and H-1β (δ_H0.98) and Me-25 (δ_H0.88), indicating that the hydroxyl group was in the 3β-orientation. Comparison of the chemical shifts of C-26 (δ_C16.9), C-27 (δ_C17.0), and C-28 (δ_C20.7) of **1**

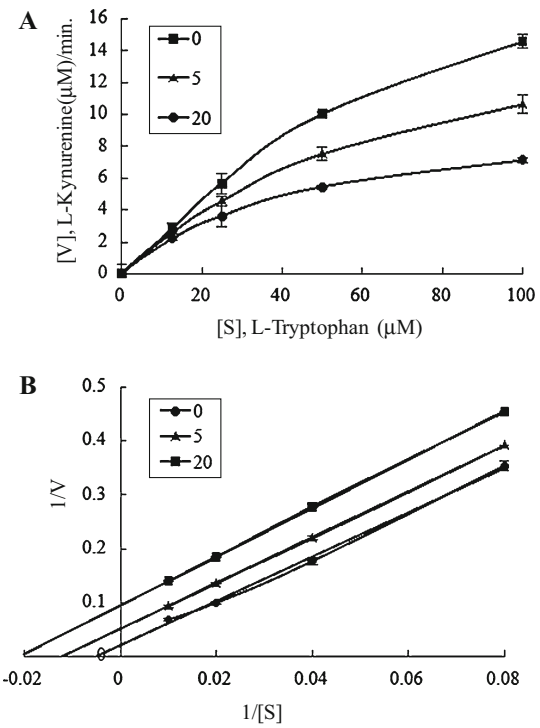


Fig. 4 The inhibition pattern of compound **11**. **a** Michaelis–Menten plots with data obtained in the presence (5 and 20 M, respectively) or absence of compound **11**. **b** Lineweaver–Burk plots generated after transformation of data used in (**a**)

Table 3 Inhibition constants of compound **10** and **11**

Compound	Inhibition pattern	*Ki* (μM)
10	Uncompetitive	6.715 ± 2.0
11	Uncompetitive	4.405 ± 1.1

with those of reported hopane-type triterpene unequivocally established 26β,27α,28α configuration of **1** (Kakuda et al. 2002). According to the COSY, HMBC, and NOE correlations, as well as comparisons of the 1D- and 2D-NMR spectroscopic data of compound **5**, the overall framework of compound **2** was determined and nameds cabranol. Compound **2** was isolated from nature for the first time, but it has been synthesized by hydrolysis of gilvanol, which contains an ozonide group (Itokawa et al. 1978).

To determine whether isolated triterpenes affect IDO activity, compounds **1**–**21** were tested at 100 μM. While most of the compounds showed minimal effects, compounds **2**, **10**, **11**, **12**, and **13** showed over 50 % inhibition at 100 μM. The activities of these compounds were examined further at lower concentrations to determine IC_{50} values. Compounds **10** and **11** showed the strongest inhibitory activities with IC_{50} values of 12.5 and 9.5 μM, respectively.

Compound **12** showed a moderate inhibitory effect, with an IC_{50} value of 18.7 μM, and compounds **2** and **13** showed weaker inhibitory effects, with IC_{50} values of 56.8 and 60.6 μM, respectively (Fig. 3; Table 2).

In the structure–activity relationships of isolated triterpenoids, ursane-type (compounds **3**, **4**, and **15–19**), swertane-type (compounds **1**, **20**, and **21**), and hopane-type (compounds **5–9**) triterpenoids exhibited no inhibitory activity against IDO. Among the oleanane-type triterpenoids (compounds **10–14**), compounds **10** and **11** exhibited significant inhibitory activity against IDO, as well as compound **12**, which showed moderate effects. Compounds **10–12** had similar structures, which contained one or two OH groups located at C-2/C-3, respectively. Three OH groups were present at C-1–3 in compound **13**, which decreased activity. This suggests that OH groups at C-2/C-3 are key functional elements (Dolušic 2011b).

To examine the inhibition patterns of compounds **10** and **11**, experiments for generating Lineweaver–Burk (double-reciprocal) plots were performed. Double-reciprocal plots obtained in the presence of compound **11** showed lines almost parallel to the line obtained in the absence of the inhibitor (Fig. 4). These parallel lines imply that compound **11** is an uncompetitive inhibitor of IDO. Compound **10** also showed a similar pattern (data not show), suggesting that the inhibitory effects of compound **10** and **11** have an identical underlying mechanism. The inhibition constants of compounds **10** and **11** (i.e., dissociation constants of respective inhibitor-IDO complexes) were calculated from the y intercepts of double-reciprocal plots (Table 3) (see "Materials and methods").

The definition of uncompetitive inhibition is binding of an inhibitor to an enzyme only when it is in complex with the substrate; i.e., the inhibitor binds only to the enzyme-substrate (ES) complex (Palmer 1991). Thus, compound **10** and **11** likely interact only with IDO, with which L-tryptophan had formed a complex. Further experiments are required to confirm this hypothesis and characterize the conformational change of IDO that may accompany ES complex formation.

In recent years, several studies have reported IDO inhibition of fungal metabolites (Oh et al. 1997; Jang et al. 2014). However, studies on natural IDO inhibitors are limited. To our knowledge, this is the first report on the IDO inhibitory activity of triterpenoids from *G. scabra*. We suggest that rhizomes and roots of *G. scabra* can be used as natural IDO inhibitors for the treatment of cancer and neurological disorders.

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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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