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Cancer Chemopreventive *in vitro* Activities of Isoflavones Isolated from *Iris germanica*

Abstract

Six known isoflavones were isolated from the rhizomes of *Iris germanica*, and were established by UV, MS and NMR techniques as irisolidone (**1**), irisolidone 7-*O*-α-D-glucoside (**1a**), irigenin (**2**), irilone (**3**), iriflogenin (**4**), and iriskashmirianin (**5**). These compounds were examined for their cancer chemopreventive potential. They were shown to be potent inhibitors of cytochrome P450 1A activity with IC_{50} values in the range 0.25 – 4.9 μM. The isoflavones **2**, **3** and **5** displayed moderate activity as inducers of NAD(P)H:quinone reductase (QR) in cultured mouse Hepa 1c1c7 cells, with CD values (concentration required to double the specific activity of QR) of 3.5 – 16.7 μM, whereas weak activity was observed with compounds **4** and **5** in the radical (DPPH) scavenging bioassay (IC_{50} values 89.6 and 120.3 μM, respectively). With respect to anti-tumor promoting potential based on anti-inflammatory mechanisms, none of the compounds demonstrated significant activity in the concentration range tested.

Key words
Iris germanica · Iridaceae · isoflavones · cancer chemoprevention · carcinogen metabolism

Abbreviations

APCI:	atmospheric pressure chemical ionization
CD:	concentration required for doubling of the specific activity of NAD(P)H:quinone reductase
Cox:	cyclooxygenase
Cyp:	cytochrome P450
DAD:	diode-array detector
DPPH:	1,1-diphenyl-2-picrylhydrazyl
HMBC:	heteronuclear multiple bond correlation
IC_{50}:	half-maximal inhibitory concentration
iNOS:	inducible nitric oxide synthase
NF:	naphthoflavone
QR:	NAD(P)H:quinone reductase
SC_{50}:	half-maximal scavenging concentration

Introduction

The use of underground parts of several species of *Iris* (Iridaceae) was well established in traditional European folk medicine for centuries. Peeled and dried rhizomes of *Iris germanica* L., *I. florentina* L. (syn. *I. germanica* var. *florentina* auct. vix. L.), or *I. pallida* Lam. (Iridaceae), collectively known as *Rhizoma iridis*, enjoyed popularity due to their emetic, cathartic, diuretic, stimulant, expectorant and errhine properties. In addition, powdered dry rhizomes of several *Iris* species, but in particular of *I. germanica*, were used as ingredients of toothpowders. As a specialty drug, *Rhizoma iridis pro infantibus*, being composed of small, selected rhizome pieces, provided pain relief as a masticatory for teething children [1], [2].

Cancer chemoprevention aims to halt or reverse the development and progression of cancer cells through use of non-toxic

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nutrients and/or pharmacological agents [3], [4]. Thus, the identification, mechanistic investigation, validation and utilization of dietary components, natural products or their synthetic analogous as potential cancer chemopreventive agents has become an important issue in current public health-related research [5], [6]. To this end, we have fractionated an extract of *I. germanica* rhizomes in order to determine the cancer chemopreventive potential of *Iris* constituents in a series of *in vitro* bioassays relevant for the inhibition of carcinogenesis *in vivo*.

Material and Methods

General experimental procedures

TLCs were run on polyamide (DC 11, Machery-Nagel, Düren, Germany) using solvents A (toluene-petrol$_{100-140}$-MeCOEt-MeOH, 12 : 6:2 : 1) and B (toluene-dioxane-MeOH, 8 : 1:1), and on silica gel using solvent C (toluene-MeCOEt, 9 : 1). Chromatograms were viewed under UV light (366 nm) before and after spraying with 'Naturstoffreagenz A' (a 1% methanolic solution of diphenylboric acid-ethanolamine complex); CC: Sephadex LH-20 (Pharmacia, Peaback, NJ, USA) and silica gel 60 (40 – 63 μm, Merck, Darmstadt, Germany).

Semi-preparative HPLC separations were achieved on a 10 μm Econosil RP-18 (250 × 22 mm) column (Alltech, Deerfield, IL, USA). Linear gradient elution was used starting from 40% to 100% MeCN in 1% aq. HCOOH over 30 min at a flow rate of 11.2 ml/min. The UV trace was recorded at 280 nm. Peak fractions were collected manually, concentrated on a rotavapor and then lyophilized. Partially purified extracts and isolated isoflavonoids were analyzed by analytical HPLC with a 5 μm C-18e LiChrospher column (250 × 4.6 mm; Merck, Darmstadt, Germany) using a linear solvent gradient from 10% to 95% MeCN in 1% aq. HCOOH over 30 min at 1.0 ml/min. On-line UV spectra of isoflavonoids were recorded with a diode-array detector (Merck Hitachi L-4500A, Darmstadt, Germany).

^{1}H-NMR spectra were recorded at 300 and 500 MHz on Varian instruments (Varian Gemini 300 and Varian Unity 500) and at 600 MHz on a Bruker DRX 600 spectrometer. ^{13}C-NMR spectra were taken at 75 MHz (Varian Gemini 300) and at 150 MHz (Bruker DRX 600). ^{1}H-^{13}C HMBC experiments were carried out on the Bruker DRX 600 instrument using standard Bruker pulse sequences. All NMR experiments were run in DMSO-d_6 at room temperature.

Atmospheric pressure chemical ionization (APCI) and electrospray mass spectra were recorded on a PE Sciex API *III*-plus triple quadrupole instrument (PE Sciex, Thornhill, Ontario, Canada). For APCI-MS, samples were dissolved in MeCN-H_2O (1 : 1, v/v) and introduced into the mass spectrometer by loop injection at a flow rate of 0.5 ml/min using MeCN-H_2O (1 : 1, v/v) as carrier solvent. The heated nebulizer interface was set at 400 °C. Ionization of the analyte vapour mixture was initiated by a corona discharge needle set at ca 8 kV and a discharge current of ca 3 $\mu\alpha$. The orifice plate voltage was + 55 V (positive ion mode). Electrospray MS was carried out by continuous flow injection at 5 μl/min and a needle voltage of + 4.5 kV.

Chemicals

All cell culture material was obtained from GIBCO BRL Life Technologies (Eggenstein, Germany). Fetal bovine serum was from Greiner Labortechnik GmbH, (Frickenhausen, Germany). Calcein AM, 3-cyano-7-ethoxycoumarin (CEC), and 3-cyano-7-hydroxycoumarin (CHC) were purchased from Molecular Probes (Mobitec, Göttingen, Germany). All other chemicals were purchased from Sigma Chemical Co. (Deisenhofen, Germany).

Plant material

Rhizomes of *Iris germanica* L. (Iridaceae) (identified by Dr. S. Schneckenburger) were collected in March, 2001, in the Botanischer Garten der TU Darmstadt. A voucher specimen (no 40 032) is deposited in the herbarium of the Institut für Botanik in Darmstadt.

Extraction and isolation

After cleaning and removal of side roots and bad spots, 240 g of fresh rhizomes of *I. germanica* were cut into pieces and extracted in a blender with 1.7 l of MeOH. The homogenized mixture was filtered, and the filtrate concentrated and passed through a column of Sephadex LH-20 (36 × 5 cm) in order to remove large amounts of resinous material. Flavonoid containing fractions were pooled and evaporated to yield a crude flavonoid mixture. A portion of this mixture was submitted to column chromatography on silica gel, using toluene-MeCOEt-MeOH mixtures of increasing polarity as eluents. Fractions were collected and monitored by TLC on silica gel and polyamide. Flavonoids detected in mixed fractions were purified to homogeneity by preparative TLC on silica gel (solvent: toluene-MeCOEt, 9 : 1). A final purification step was carried out by semi-preparative HPLC which yielded the known isoflavones, irisolidone (**1**, 2 mg), its 7-*O*-glucoside (**1a**, 1 mg), irigenin (**2**, 15 mg), irilone (**3**, 10 mg), iriflogenin (**4**, 4 mg) and iriskashmirianin (**5**, 1.5 mg). These materials were obtained as white-yellow powders after lypophilization. Their purity was 95+% by analytical HPLC and ^{1}H-NMR.

Another portion of the crude flavonoid mixture was hydrolyzed by boiling it, after addition of some drops of 25% sulphuric acid, for 30 minutes. Isoflavonoid aglycones were recovered from the hydrolyzate by extraction into ethyl acetate and compared with the original flavonoid mixture and flavonoid standards.

Isoflavonoids isolated

5,7-Dihydroxy-6,4′-dimethoxyisoflavone (Irisolidone, **1**). HPLC: R_t = 21.4 min, DAD-UV λ_{max} = 265 nm; APCI-MS, *m/z* = 315 $[M+H]^+$ (100). ^{1}H-NMR (DMSO-d_6, 300 MHz): δ = 13.0 (br s, OH-5), 8.34 (1H, s, H-2), 7.48 (2H, d, *J* = 8.5 Hz, H-2′ and H-6′), 6.99 (2H, d, *J* = 8.5 Hz, H-3′ and H-5′), 6.45 (1H, s, H-8), 3.78 and 3.73 (each 3H, 2 × OCH_3).

5,7-Dihydroxy-6,4′-dimethoxyisoflavone-7-O-α-D-*glucopyranoside* (Irisolidone-7-*O*-α-D-glucoside, **1a**). HPLC: R_t = 15.3 min, DAD-UV λ_{max} = 265 nm; Electrospray-MS: *m/z* = 499 $[M+Na]^+$ (100), 477 $[M+H]^+$ (53), 315 $[Aglycone+H]^+$ (16); ^{1}H-NMR (DMSO-d_6, 300 MHz): δ = 13.9 (br s, OH-5), 8.48 (1H, s, H-2), 7.51 (2H, d, *J* = 8.8 Hz, H-2′ and H-6′), 7.00 (2H, d, *J* = 8.8 Hz, H-3′ and H-5′), 6.89 (1H, s, H-8), 5.45 (1H, d, *J* = 3.8 Hz, H-1 gluc), 3.78 and 3.76 (each 3H, 2 × OCH_3). ^{13}C-NMR (DMSO-d_6, 75 MHz): δ = 180.4 (C-4), 159.0 (C-4′), 156.5 (C-7), 154.8 (C-2), 152.7 and 152.3 (C-5 and C-9), 132.3

(C-6), 130.0 (C-2′ and C-6′), 122.6 (C-3), 121.6 (C-1′), 113.6 (C-3′ and C-5′), 106.3 (C-10), 100.0 (C-1 gluc), 94.0 (C-8), 77.2 (C-5 gluc), 76.6 (C-3 gluc), 73.1 (C-2 gluc), 69.6 (C-4 gluc), 60.6 (C-6 gluc), 60.3 and 55.2 (2 × O<u>C</u>H$_3$).

5,7,3′-Trihydroxy-6,4′,5′-trimethoxyisoflavone (Irigenin, **2**). HPLC: R_t = 17.1 min, DAD-UV: λ_{max} = 265 nm; APCI-MS: *m/z* = 361 $[M+H]^+$ (100); ^{1}H-NMR (DMSO-d_6, 600 MHz): δ = 13.03 (br s, OH-5), 10.8 and 9.27 (both br s, OH-7 and OH-3′), 8.38 (1H, s, H-2), 6.71 and 6.66 (1H each, d, *J* = 1.9 Hz, H-2′ and H-6′), 6.51 (1H, s, H-8), 3.79, 3.75 and 3.69 (3H each, s, 3 × OCH$_3$).

5,4′-Dihydroxy-6,7-methylenedioxyisoflavone (Irilone, **3**). HPLC: R_t = 19.7 min, DAD-UV: λ_{max} = 271 nm; UV (MeOH) λ_{max} = 273 nm; APCI-MS: *m/z* = 299 $[M+H]^+$ (100); ^{1}H-NMR (DMSO-d_6, 600 MHz): δ = 12.92 (br s, OH-5), 9.62 (br s, OH-4′), 8.43 (1H, s, H-2), 7.39 (2H, d, *J* = 8.5 Hz, H-2′ and H-6′), 6.89 (1H, s, H-8), 6.82 (2H, d, *J* = 8.5 Hz, H-3′ and H-5′), 6.18 (2H, s, O-C<u>H</u>$_2$-O).

5,4′-Dihydroxy-3′-methoxy-6,7-methylenedioxyisoflavone (Iriflogenin **4**). HPLC: R_t = 19.9 min, DAD-UV: λ_{max} = 273 nm; UV (MeOH) λ_{max} = 273 nm; APCI-MS: *m/z* = 329 $[M+H]^+$ (100); ^{1}H-NMR (DMSO-d_6, 600 MHz): δ = 12.95 (br s, OH-5), 9.18 (br s, OH-4′), 8.47 (1H, s, H-2), 7.15 (1H, d, *J* = 2.1 Hz, H-2′), 7.00 (1H, dd, *J* = 8.2, 2.1 Hz, H-6′), 6.90 (1H, s, H-8), 6.83 (1H, d, *J* = 8.2 Hz, H-5′), 6.18 (2H, s, O-C<u>H</u>$_2$-O), 3.80 (3H, s, 3′-OCH$_3$); ^{13}C-NMR (DMSO-d_6, 150 MHz): δ = 180.9 (C-4), 154.7 (C-2), 154.0 (C-7), 152.9 (C-9), 147.3 (C-3′), 146.8 (C-4′), 141.4 (C-5), 129.6 (C-6), 122.2 (C-3), 121.8 (C-6′), 121.3 (C-1′), 115.3 (C-5′), 113.3 (C-2′), 107.4 (C-10), 102.9 (O-<u>C</u>H$_2$-O), 89.5 (C-8), 55.7 (3′-O<u>C</u>H$_3$).

4′-Hydroxy-5,3′-dimethoxy-6,7-methylenedioxyisoflavone (Iriskashmirianin **5**). HPLC: R_t = 17.3 min, DAD-UV: λ_{max} = 265 nm; APCI-MS: *m/z* = 343 $[M+H]^+$ (100); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ = 9.12 (br s, OH-4′), 8.24 (1H, s, H-2), 7.11 (1H, d, *J* = 2.0 Hz, H-2′), 7.02 (1H, s, H-8), 6.93 (1H, dd, *J* = 8.3, 2.0 Hz, H-6′), 6.80 (1H, d, *J* = 8.1 Hz, H-5′), 6.19 (2H, s, O-C<u>H</u>$_2$-O), 3.90 (3H, s, 5-OCH$_3$), 3.79 (3H, s, 3′-OCH$_3$).

In vitro cancer chemopreventive assays

Inhibition of Cyp1A activity was measured according to Crespi et al. [7] with minor modification, using cell homogenates of H4IIE rat hepatoma cells (American Type Culture Collection, Manassas, VA, USA) cultured for 38 h with 10 μM β-naphthoflavone (β-NF) in MEME containing 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate and 250 ng/ml amphotericin B, supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere as a source of Cyp1A [8]. Activity was measured in 96-well plates by the rate of dealkylation of 3-cyano-7-ethoxycoumarin (CEC, 5 μM in PBS, 100 μl per well) to 3-cyano-7-hydroxycoumarin for 40 min at 37 °C in a Cytofluor 4000 fluorescence reader (PE Applied Biosystems, Wiesbaden, Germany, excitation wavelength Ex 408/20 nm, emission wavelength Em 460/40 nm). The protein content of cell homogenates was determined according to Smith [9] using bicinchoninic acid (BCA) reagent and bovine serum albumin as a standard (Sigma, Deisenhofen, Germany). Activity of β-NF-induced controls: 16.9 ± 4.5 nmol/min/mg of protein (n = 4). α-Naphthoflavone, a known Cyp1A inhibitor, was employed as a positive control; its IC_{50} value was 0.016 ± 0.005 μM (n = 4).

Induction of NAD(P)H:quinone reductase (QR) activity was determined in Hepa 1c1c7 mouse hepatoma cells (provided by Dr. J.P. Whitlock, Jr., Stanford University) as described earlier [8], [10]. Cells were grown in α-MEM containing 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate and 250 ng/ml amphotericin B supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere. Enzymatic activities of compound-treated cells were measured and compared to the activity of solvent-treated cells to calculate relative induction. The protein content was estimated by crystal violet staining of an identical set of test plates. Specific activities of untreated controls: 103.2 ± 6.7 nmol/min/mg protein (n = 5). β-NF with a CD value (concentration required to double the specific activity of QR) of 0.025 ± 0.007 μM (n = 5) was used as a positive control.

Radical-scavenging activity was determined photometrically by reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals at 515 nm using a microplate reader (Spectramax 340, Molecular Devices, Ismaning, Germany). Briefly, test compounds dissolved in DMSO were treated with a solution of 100 μM DPPH in ethanol for 30 min at 37 °C. Scavenging potential was compared with a solvent control (0% radical scavenging) and ascorbic acid (250 μM final concentration, 100% radical scavenging, used as a blank), and the half-maximal scavenging concentration SC_{50} was generated from the data obtained with 8 serial two-fold dilutions of test compounds tested in duplicates ([8], modified from [11]). The SC_{50} value obtained with (–)-epigallocatechin gallate from green tea used as a positive control substance was 4.0 ± 0.8 μM (n = 2).

Inhibition of cyclooxygenase 1 (Cox-1) activity using 0.2 U Cox-1-containing microsomal fractions derived from ram seminal vesicles (specific activity 0.2 – 1 U/mg protein) was measured at 37 °C by monitoring oxygen consumption using a Clark-type O_2-electrode (Hansatech Ltd., Kings Lynn, Great Britain) [8], [12]. Piroxicam, a nonsteroidal anti-inflammatory drug, was used as a control with an IC_{50} value of 0.35 ± 0.05 μM (n = 2).

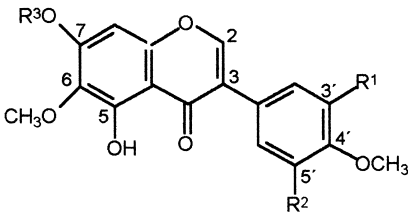
Inhibition of lipopolysaccharide-mediated inducible nitric oxide synthase (iNOS) induction in murine Raw 264.7 macrophages (American Type Culture Collection, Manassas, VA, USA) was determined *via* nitrite levels in culture supernatants by the Griess reaction and effects on cell growth estimated by sulforhodamin B (SRB) staining were measured as described previously [8], [13]. Nitrite levels in supernatants of unstimulated controls were 2.7 ± 0.3 nmol nitrite/mg protein, after lipopolysaccharide-stimulation 86.6 ± 7.7 nmol nitrite/mg protein (n = 4). Curcumin with an IC_{50} value of 13.7 ± 1.3 μM (n = 4) was used as a positive control.

Results and Discussion

Identification of Iris isoflavonoids

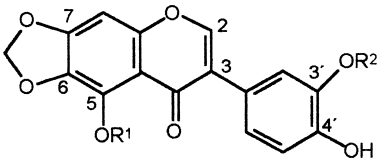
The crude flavonoid mixture, obtained from the rhizomes of *I. germanica*, was compared chromatographically with a hydrolyzed aliquot of the same mixture. This comparison indicated that the larger portion of the flavonoid material was present in glycosidic form. Because the hydrolysate did not contain additional aglycones, it was decided to focus on the aglycones of the crude flavonoid mixture.

The known isoflavonoids, irisolidone (**1**), irigenin (**2**), and irilone (**3**), were isolated from the crude flavonoid mixture by repetitive column chromatography (Fig. **1**). Their identification followed from mass and NMR spectroscopy. The ^{1}H- and ^{13}C-NMR spectra of compound (**1a**) contained both signals of irisolidone (**1**) and glucose. Because of an upfield shift of the H-8 singlet (δ 6.89 in **1a** *versus* δ 6.45 in **1**), the position of the glucosyl residue was determined to be O-7, while the size of the coupling constant of the anomeric proton (J = 3.8 Hz) led us to conclude that the glycosidic linkage was α (β-linkage exhibits couplings in the range 7 – 8 Hz). Glycoside **1a** was thus identified as irisolidone-7-*O*-α-D-glucopyranoside. In addition, traces of quercetin-7,3′-dimethyl ether (rhamnazin) and myricetin-7,3′,4′-trimethyl ether were detected by co-TLC with known flavonoids.

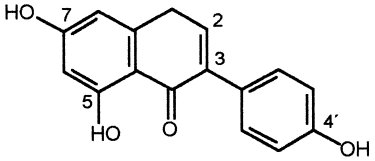


Irisolidone (**1**): $R^1 = R^2 = R^3 = H$
Irisolidone-7-O-glucoside (**1a**):
$R^1 = R^2 = H$, $R^3 = \alpha$-D-glucose
Irigenin (**2**): $R^1 = OH$, $R^2 = OCH_3$, $R^3 = H$

Fig. **1** The chemical structures of compounds **1 – 5**.



Irilone (**3**): $R^1 = R^2 = H$
Iriflogenin (**4**): $R^1 = H$, $R^2 = OCH_3$
Iriskashmirianin (**5**): $R^1 = R^2 = OCH_3$



Genistein (**6**)

Compound **4** was isolated from the crude irisolidone fraction by semi-preparative HPLC. The APCI mass spectrum of compound **4** showed a molecular ion $[M+H]^+$ peak at *m/z* 329, consistent with the molecular formula $C_{17}H_{12}O_7$. The ^{1}H-NMR spectrum showed two hydroxy signals (δ_H 12.95 and 9.18), a vinylic proton signal at δ_H 8.47 (typical of an isoflavone H-2), an aromatic proton singlet (δ_H 6.90), an AMX spin system, a two-proton methylene singlet (δ_H 6.19), and a methoxy signal at δ_H 3.80. From these data it was concluded that compound **4** is a 5,6,7,3′,4′-pentaoxygenated isoflavone. The positions of the aromatic proton (H-8), the methylenedioxy functionality (C-6/C-7) and the B-ring methoxy group (3′-OMe) were determined by ^{13}C- and ^{1}H-^{13}C HMBC experiments. Compound **4** was thus identified as 5,4′-dihydroxy-3′-methoxy-6,7-methylenedioxyflavone, trivially know as iriflogenin [14].

Compound **5** was also isolated from the crude irisolidone fraction by preparative HPLC. APCI mass spectrometry showed a molecular ion $[M+H]^+$ with *m/z* 343, suggesting compound (**5**) is a methyl ether of iriflogenin (**4**). This assumption was supported by ^{1}H-NMR spectroscopy which showed the presence of only one OH group (δ_H 9.12), an isoflavone H-2 (δ_H 8.24), an aromatic A-ring proton (δ_H 7.02), a methylenedioxy functionality (δ_H 6.19), an AMX spin system for the B-ring protons (δ_H 7.11 d, 6.93 dd and 6.80 d), and two OMe groups (δ_H 3.90 and 3.79). ^{1}H-^{13}C HMBC experiments showed that these methoxy substituents were located at C-5 and C-3′, respectively. Compound **5** was thus identified as 4′-hydroxy-5,3′-dimethoxy-6,7-methylenedioxyisoflavone. This isoflavone was previously isolated from *I. kashmiriana* (named 'iriskashmirianin') and from *I. nigricans*, *I. petrana*, and *I. atrofusca* (named 'nigricanin') [15], [16], [17], [18].

Chemotaxonomic significance

Most isoflavonoids known to date (870 aglycones in 1994) have been found in members of the Fabaceae, primarily in the subfamily Papilionoideae. Abundant occurrence is also observed in the Iridaceae, with 26 aglycones in the genus *Iris*, seven in *Belamcanda*, and two aglycones in *Patersonia*. Isoflavones constitute the largest group within the isoflavonoids, followed by the comparatively rare isoflavanones. A considerable number of *Iris* isoflavones (9 aglycones) exhibits methylenedioxy substitution as in isoflavone **4**. Among the 300 or so isoflavone structures Dewick listed in 1994 [19], a total of 27 isoflavones contained methylenedioxy moieties.

Iris species have been the subject of many phytochemical studies, and there are at least eight papers that deal with *I. germanica*. This is the first report, however, on the occurrence of iriflogenin (**4**) and irskashmirianin (**5**) in this species. On the other hand, we did not find several other isoflavone aglycones reported from *I. germanica* earlier: 5,7,4′-triOH-6-OMe isoflavone (tectorigenin), 5,7,3′-triOH-6,4′-diOMe-isoflavone (iristectorigenin A), 5,7,4′-triOH-6,3′-diOMe-isoflavone (iristectorigenin B), 4′-OH-5′-OMe-6,7-methylenedioxyisoflavone (irisolone) 5,3′,4′-triOH-6,7-methylenedioxyisoflavone (methyliriskumaonin) [20] 5,3′-diOH-4′,5′-diOMe-6,7-methylenedioyisoflavone (dichotomin) [21] and 5,3′,4′-triOH-6,7-methylenedioyisoflavone (irisflorentin) [20]. The latter work also reported the dihydroflavonol, taxifolin-7,3′-dimethyl ether, whereas in our material we found the corresponding flavonol, quercetin-7,3′-dimethyl ether (rhamnazin) and traces of myricetin-7,3′,4′-trimethyl ether. These findings suggest that the different cultivars of *I. germanica* have rather different biosynthetic potential.

Cancer chemoprevention *in vitro* activities

As a first indication of cancer chemopreventive potential, the isolated isoflavones were tested in a series of *in vitro* bioassays with relevance for inhibition of carcinogenesis at the initiation and promotion stage, and the activities were compared to those of genistein (**6**), a well-known isoflavone chemopreventive agent found in soy products [22].

One approach to achieve cancer chemoprevention is to inhibit the carcinogen activation process with inhibitors of so-called phase I enzymes such as cytochrome P450 enzymes. Metabolic activation of xenobiotic carcinogens is mediated by a limited number of human cytochrome P450 s, of which we selected cytochrome P450 1A (Cyp1A) [8], [9] for our investigations. Cyp1A contributes to the generation of ultimate carcinogens, e.g., from grilled meat. Irilone (**3**) and genistein (**6**), with free hydroxy groups in 5 and 4′-positions, were identified as the most potent inhibitors of Cyp1A activity with IC_{50} values of 0.2 μM and were only about 15-times less active than α-naphthoflavone used as a positive control. An additional methoxy group in 3′-position, as in iriflogenin (**4**) in comparison with irilone (**2**) lead to a nearly 6-fold reduction in Cyp1A inhibitory activity (IC_{50} value = 1.4 μM), whereas a 5,3′-dimethoxy-substitution, as seen in iriskashmirianin (**5**) completely abrogated Cyp1A inhibitory potential at concentrations below 5 μM (summarized in Table **1**).

We further monitored the induction of NAD(P)H:quinone reductase (QR) as a model phase 2 enzyme in Hepa 1c1c7 murine hepatoma cell culture [9], [10]. Phase 2 enzymes are involved in the elimination of electrophilic metabolites of carcinogens through conjugation with endogenous ligands like glucuronic acid and glutathione (transferase enzymes) to facilitate excretion. QR contributes to detoxification by 2-electron reduction of quinones to hydroquinones, which are then excreted in the form of their conjugation products. Irigenin (**2**), irilone (**3**), iriskashmirianin (**5**) and genistein (**6**) were found to induce QR specific activity in a dose-dependent manner. Iriskashmirianin (**5**) and irigenin (**2**) were identified as the most potent inducers with CD values of 3.5 and 7.8 μM, without demonstrating cytotoxic effects at concentrations up to 50 μM (Table **1**). As indicated by a reduction in QR activity at higher concentrations, irilone (**3**) and genistein (**6**) were cytotoxic at concentrations above 12.5 μM, and IC_{50} values of cell viability were determined to be 17.6 and 23.9 μM, respectively. Irisolidone (**1**), irisolidone 7-*O*-glucoside (**1a**) and iriflogenin (**4**) were not toxic at concentrations up to 50 μM, but also did not induce QR activity > 2-fold in this concentration range.

Activation of carcinogens might lead to the generation of reactive radicals with potential to interact with and damage DNA. Consequently, we determined the test compounds' radical-scavenging potential using the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) [8], [10]. Only iriflogenin (**4**) and iriskashmirianin (**5**) were identified as weak DPPH-scavengers, with SC_{50} values of 89.6 and 120.3 μM, respectively, indicating an about 20 to 30-times lower activity than that of (–)-epigallocatechin gallate from green tea used as a positive control.

For the detection of anti-inflammatory and potential anti-tumor promoting potential, we tested the influence of compounds **1 – 6** on cyclooxygenase-1 (Cox-1) enzymatic activity. None of the compounds inhibited Cox-1 activity more than 50% at a concentration of 100 μM; therefore, IC_{50} values were not generated. Chronic inflammation and infections result in the induction of the inducible form of nitric oxide synthase (iNOS). Aberrant or over-production of nitric oxide (NO) has been implicated in the pathogenesis of cancer *via* reactive nitrogen oxide species (RNOS)-mediated reactions like nitrosative deamination of DNA bases, lipid peroxidation and DNA strand breaks [23]. Induction of iNOS activity and release of NO was tested in murine Raw 264.7 macrophages stimulated with bacterial lipopolysaccharides. All compounds were inactive up to a concentration of 50 μM.

In summary, the *Iris* isoflavones irigenin (**2**), irilone (**3**) and iriflogenin (**4**) were shown to be potent inhibitors of cytochrome P450 1A isoenzyme which is involved in the metabolic conversion of procarcinogens into carcinogens. Their Cyp1A inhibitory activities were comparable with that of the well-known cancer chemopreventive isoflavone, genistein (**6**). Iriskashmirianin (**5**), irigenin (**2**) and irilone (**3**) displayed moderate activity as inducers of quinone reductase, a carcinogen detoxifying enzyme, and iriflogenin (**4**) and its 5-*O*-methylated derivative (**5**) were identified as weak radical scavengers in the DPPH bioassay. The activity profile observed with irilone (**3**) was very similar to that of genistein (**6**), which is in agreement with their structural similarity.

Table **1** Summary of anti-tumor initiating activities of *Iris isoflavonoids* in comparison with genistein

Sample	*Inhibition of Cyp1A activity*	*Induction of QR activity in Hepa 1c1c7 cell culture*		*Scavenging of DPPH radicals*
	IC_{50} [µM] [a]	CD [µM] [b]	IC_{50} [µM] [a]	SC_{50} [µM] [c]
Irisolidone (**1**)	4.9 ± 0.5	> 50 (1.4)[e]	> 50	> 250 (8)[d]
Irisolidone-7-*O*-glucoside (**1a**)	> 5 (38)[d]	> 50 (1.2)	> 50	> 250 (1)
Irigenin (**2**)	1.2 ± 0.3	7.8 ± 0.1	> 50	> 250 (15)
Irilone (**3**)	0.3 ± 0.1	16.7 ± 2.3	17.6 ± 1.6	> 250 (2)
Iriflogenin (**4**)	1.4 ± 0.6	> 50 (1.9)	> 50	89.6 ± 4.4
Iriskashmirianin (**5**)	> 5 (0)	3.5 ± 1.5	> 50	120.3 ± 7.4
Genistein (**6**)	0.2 ± 0.1	17.1 ± 8.5	23.9 ± 5.9	> 250 (2)

[a] IC_{50}: half-maximal inhibitory concentration.
[b] CD: concentration required to double the specific activity of QR.
[c] SC_{50}: half-maximal radical scavenging concentration.
[d] Numbers in parentheses indicate the percentage of inhibition/scavenging at the indicated concentrations
[e] Values in parentheses indicate the maximal fold induction at a concentration of 50 μM.

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