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Isoflavones and Other Compounds from the Roots of *Iris marsica* I. Ricci E Colas. Collected from Majella National Park, Italy

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Abstract

In this study, a phytochemical analysis was performed, for the first time, on *Iris marsica* I. Ricci e Colas. In particular, the attention was focused on the constituents of the roots. Twenty-one compounds were isolated by column chromatography and were analyzed/identified by NMR spectroscopy and mass spectrometry. They all own chemotaxonomic, ethno-pharmacological and nutraceutical relevance which allowed us to provide a phytochemical rationale, for the correct botanical classification of this species, for the employment of its roots in folk medicine like for all the other species belonging to the *Iris* genus and, lastly, for their further uses as food with important healthy benefits. All of these parts were broadly discussed about within the text.

Keywords: *Iris marsica* I. Ricci e Colas.; Roots; Phytochemical analysis; Chemotaxonomy; Ethno-pharmacology; Nutraceutics

Introduction

Iris marsica I. Ricci e Colas. is a rhizomatous perennial herbaceous plant belonging to the *Iridaceae* family. It is also known with the common name of Marsican Iris with the epithet Marsican deriving from latin and referring to its growth area i.e., Marsican Mountains located in Abruzzo, central Italy. From the botanical point of view, this species is characterized by a stem generally high 30-40 cm, with two branches of different length. It possesses rounded spathes at the base of the branches, with a slighty scarious acute apex. The leaves are light green colored, slightly glaucous, falcate, acute, growing about 30 cm in length. The flowers have a very slight fragrance and may be colored in different shades of violet. Three of them are usually uphold by the stem and the two branches, and they bloom from May to June. It also possesses a thick rhizome, compressed, rather knobby, branchy, which grows at ground level [1] (Figure 1).

Morphologically, *I. marsica* is very similar to *I. germanica* but the latter possesses persistent leaves, stems with 4-6 flowers, spathes which are scarious for 1/3-2/3 of their length, wider flower lacinies, sub-globose red-brownish seeds while those of *I. marsica* are oval and light brown [2].

Figure 1: *Iris marsica* I. Ricci e Colas.

This species represents an important endemism to Central Italy where it only grows in the inner areas of Abruzzo, Latium, Marche and Umbria regions, on mountain dry pastures, between 1000 and 1800 masl [3]. The studied sample was collected in the area of Majella National Park which represents a hot spot for biodiversity in central Italy with the presence of several endemic and rare species with restricted distribution [4-8] and peculiar secondary metabolites contents [9-11]. Endemic species present a peculiar genotype that leads to important phenotypic peculiarities, which may be recognized in their metabolic patterns, and which could be related to the evolution and adaption of the endemic species to the colonized environments [12].

I. marsica is a rare species included in the Red Lists of Plants at European [13], national [14] and regional [15] level as a species at risk of extinction, in the annex I of the Berna Convention and in the annex IV of the Habitat Directive. Several *Iris* species own a well known employment in the European traditional medicine due to their extraordinary pharmacological properties. These range from the important anti-cancer, anti-inflammatory, anti-bacterial and anti-viral [16] to the interesting emetic, diuretic, stimulant and expectorant ones [17] not to consider the pain-killer effect for teething children [17] and the anti-malarial activity [18].

Also in the Eastern nations of the world, the medicinal use of Iris species was established. For instance, this is very true in Iran where *I. germanica* extract is still employed as diuretic and expectorant as well

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as to cure many diseases affecting liver, lungs and uteri and to treat hemorrhoids [19]. Yet this is also true in China where particular species of Iris are utilized to treat coughing and pharyngitis [20,21] and also to reduce inflammation and relieve respiratory disorders [22].

In literature, several works about phytochemistry of the *Iris* genus can be found, reporting the presence of several secondary metabolites such as steroids [23], terpenoids [24,25], flavonoids, xanthones, isoflavonoids [26,27] and anthocyanins [28]. On the other hand, a couple of studies have been performed in the past on phytochemistry of *I. marsica* evidencing the presence of particular flavonoids and xanthones [22,29].

This represents the main reason why this work was started in order to provide a much more general view on the total phytochemistry of this species. Moreover, the second aim of this work was to verify if also this species can be used in the ethno-pharmacological field just like all the other *Iris* species. These two elements will be verified according to the presence or not of specific secondary metabolites which may justify the employment of this species in these senses if they are endowed with very important medicinal or even nutraceutical properties.

Materials and Methods

Plant material

The plant material consisting of the only roots for the total weight of 450 g, was collected on the M. Pizzalto, in the municipality of Pescocostanzo – L'Aquila province (Central Italy) in August 2016 at an altitude of 1650 m a.s.l. The botanical classification was performed by the botanists of the same Park (Dr. Mirella di Cecco and Dr. Giampiero Ciaschetti) using available literature [1,2]. A sample of this collection is stored in our laboratory for further references under the voucher name IM0160408.

Chemicals

Throughout this study, the following reagents and solvents were utilized: ethanol 96% and distilled water as pure solvents or in mixture between them at the specific concentration of 80/20 v/v for the extraction procedure of the plant material; *n*-butanol, distilled water, methanol and chloroform as pure solvents or in mixture among them all at different concentrations to be used as eluting systems for the separation procedure via classical column chromatography; silica gel having 40-63 μm particle size to be used as stationary phase for the separation procedure via classical column chromatography; sulfuric acid 2N for the development of TLCs; deuterated solvents such as $CDCl_3$, CD_3OD and D_2O for the identification of the metabolites by NMR Spectroscopy; methanol with RS purity grade for the identification of the metabolites by Mass Spectrometry.

All the natural solvents and reagents having RPE analytical degree, if not otherwise specified, were purchased from "Carlo Erba Reagenti" or "Sigma Aldrich" as well as the deuterated solvents while silica gel was bought from "Fluka Analytical".

Instrumentation

NMR spectra were recorded on a Varian Mercury 300 MHz instrument or on a Bruker 400 MHz instrument. The chemical shifts were expressed using the TMS signal at 0 ppm (s) as reference for the spectra in $CDCl_3$. The internal solvent signal (m5) at 3.31 ppm was used as reference for the spectra recorded in CD_3OD, instead. Lastly, the HDO signal (s) at 4.78 ppm was set as reference for spectra in D_2O. MS spectra were, instead, performed on a Q-TOF MICRO spectrometer (Waters, Manchester, UK) equipped with an ESI source operating in the negative and/or positive ion mode. The flow rate of the sample infusion was 10 μl/min. with 50 acquisitions per spectrum. Data were analysed using the MassLynx software developed by Waters.

Extraction procedure

An aliquot of the whole plant material, weighing 420.0 g was inserted in a conical flask and fully covered with ethanol 96%. This was left closed for 48 hours in order to let primary and secondary metabolites come into solution. The solution, having a reddish coloration, was then filtered into one same round bottom flask and concentrated under reduced pressure at a temperature of about 50°C. A second extraction was performed for an exhaustive process but this time the solution utilized for this was a mixture of ethanol 96% and distilled water in ratio 80/20 v/v. This step was necessary in order to also let the most polar components come into solution. Also this solution with a yellowish coloration was filtered and concentrated under reduced pressure at a temperature of about 50°C but in the same round bottom flask of the previous solution, thus having one total extract solution. During the concentration of the first solution, pH was checked on normal litmus paper in order to verify that this was not too acid meaning under the value of 5.5 on the pH scale. This step was absolutely necessary since a pH value below 5.5 pH units could cause unpleasant secondary reactions on the metabolites such as the hydrolysis of the glycosidic and/or ester groups which would not happen at higher pH values (6-8 pH units), instead. Actually, this check gave a result of 6.5 on the pH scale. After concentration, the extract was lyophilized to obtain 25.9 g of orange-colored crude extract.

Isolation and identification of the metabolites

An aliquot of the total crude extract for the weight of 3.0 g was subjected to a first chromatographic separation procedure via classical column chromatography on silica gel. Its weight was 120.0 g (ratio 1:40) and the eluting system consisted of a solution of *n*-butanol and distilled water at concentration 82/18 (v/v). During the chromatographic run, the polarity of the eluting solution was raised in order to let the elution of the most polar compounds and so this became a solution of *n*-butanol, methanol and distilled water at concentration 70/10/30 (v/v/v). From this chromatographic separation, twelve compounds were identified: tristearoyl-*sn*-glycerol (**1**) and 1-margaroyl-2-lauroyl-3-palmitoyl-*sn*-glycerol (**2**) [30] as a mixture in ratio 2:1 from the assembly of fractions 1-5 for the total weight of 36.6 mg; nigricin 4'-*O*-β-D-glucopyranoside (**10**) [27] as almost pure compound from fraction 36 for the weight of 135.8 mg; glucose (**15**) and phenylalanine (**19**) [31] as a mixture in ratio 5:1 from the assembly of fractions 61-70 for the total weight of 81.4 mg; glucose (**15**), sucrose (**16**), raffinose (**17**), lactic acid (**18**), isoleucine (**20**) and valine (**21**) [31] in mixture (ratio not detectable) from the assembly of fractions 91-165 for the total weight of 217.2 mg.

Since not all compounds present were detected and identified, a second chromatographic separation was conducted on a specific assembly derived from the first chromatographic separation namely fractions 6-23 for the total weight of 308.9 mg. The correspective amount of silica gel was 12.0 g (ratio 1:40) and the eluting system was, this time, a mixture of chloroform and methanol at different concentrations. The initial one was 97/3 (v/v) but during the chromatographic run, this was changed in order to raise the polarity of the solution passing to 95/5 (v/v), 9/1 (v/v), 8/2 (v/v), 7/3 (v/v) and lastly, 6/4 (v/v). From this chromatographic separation seven compounds were identified: irigenin S (**8**) [25] in mixture with irisolidone (**5**) [17] in ratio 3:1 from fraction 3 for the weight of 6.8 mg; nigricin (**6**) [32], irigenin (**7**)

[33] and 1,2-distearoyl-*sn*-glycerol (**3**) [30] as a mixture in ratio 1:2:1 from fraction 5 for the total weight of 8.9 mg; nigricin (**6**) [32] again in mixture with α-irigermanal (**4**) [34] in ratio 1:2 from the assembly of fractions 15-20 for the total weight of 10.2 mg; irigenin S (**8**) [25] again in mixture with irisflogenin (**9**) [17] in ratio 1:1 from the assembly of fractions 50-52 for the total weight of 2.6 mg; irisolidone 7-*O*-β-D-glucopyranoside (**11**) [35] as almost pure compound from the assembly of fractions 72-75 for the weight of 11.9 mg; iridin (**12**) and irilone 4'-*O*-β-D-glucopyranoside (**13**) [25] in mixture in ratio 1.5:1 from the assembly of fractions 81-83 for the total weight of 13.4 mg; again iridin (**12**) [25] but this time in mixture with tectoridin (**14**) [36] in ratio 2:1 from the assembly of fractions 91-107 for the total weight of 2.7 mg.

NMR and MS data of the identified compounds

tristearoyl-*sn*-glycerol (**1**): ^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.12 (1H, m, H-2 (glycerol)), 4.29 (2H, dd, *J* = 11.8/3.9 Hz, H-1b and H-3b (glycerol)), 4.14 (2H, dd, *J* = 11.8/3.9 Hz, H-1a and H-3a (glycerol)), 2.35 (6H, t, *J* = 7.5 Hz, $\underline{CH_2}$-CO_2^-), 1.57 (6H, m, $\underline{CH_2}$-CH_2-CO_2^-), 1.25 (84H, m, n-$\underline{CH_2}$), 0.86 (9H, m, CH_3). ESI-MS for $C_{57}H_{110}O_6$: *m/z* = 912.65 $[M+Na]^+$ (Calcd. 914.4695 for $C_{57}H_{110}NaO_6^+$).

1-margaroyl-2-lauroyl-3-palmitoyl-*sn*-glycerol (**2**): ^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.10 (1H, m, H-2 (glycerol)), 4.25 (2H, dd, *J* = 11.5/3.7 Hz, H-1b and H-3b (glycerol)), 4.12 (2H, dd, *J* = 11.5/3.7 Hz, H-1a and H-3a (glycerol)), 2.32 (6H, br t, $\underline{CH_2}$-CO_2^-), 1.57 (6H, m, $\underline{CH_2}$-CH_2-CO_2^-), 1.29 (66H, m, n-$\underline{CH_2}$), 0.88 (9H, m, CH_3). ESI-MS for $C_{46}H_{88}O_6$: *m/z* = 759.27 $[M+Na]^+$ (Calcd. 760.1771 for $C_{46}H_{88}NaO_6^+$).

1,2-distearoyl-*sn*-glycerol: ^{1}H NMR (300 MHz, $CDCl_3$) ^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.11 (1H, m, H-2 (glycerol)), 4.25 (1H, dd, *J* = 11.8/4.2 Hz, H-1b (glycerol)), 4.12 (1H, dd, *J* = 11.8/4.2 Hz, H-1a (glycerol)), 3.81 (1H, m, H-3a (glycerol)), 3.68 (1H, m, H-3b (glycerol)), 2.35 (4H, br t, $\underline{CH_2}$-CO_2^-), 1.57 (4H, m, $\underline{CH_2}$-CH_2-CO_2^-), 1.29 (56H, m, n-$\underline{CH_2}$), 0.88 (6H, m, CH_3). ESI-MS for $C_{39}H_{76}O_5$: *m/z* = 647.16 $[M+Na]^+$ (Calcd. 648.0075 for $C_{39}H_{76}NaO_5^+$).

α-irigermanal (**4**): ^{1}H NMR (300 MHz, $CDCl_3$) δ: 10.18 (1H, br s, H-1), 5.34 (1H, m, H-20), 4.96 (1H, t, *J* = 7.0 Hz, H-14), 3.61 (2H, t, *J* = 6.2 Hz, H-3), 3.31 (1H, d, *J* = 10.1 Hz, H-6), 2.55 (3H, br s, H-25), 2.40 – 2.29 (m, 1H), 1.84 (3H, s, H-29), 1.53 (3H, s, H-28), 1.16 (3H, s, H-26), 1.09 (3H, s, H-24), 0.87 (3H, s, 1H-27), 0.82 (3H, d, *J* = 6.7 Hz, H-31), 0.61 (3H, s, H-30), 2.2-1.0 remaining overlapped signals. ESI-MS for $C_{31}H_{53}O_3$: *m/z* = 495.41 $[M+Na]^+$ (Calcd. 495.7325 for $C_{31}H_{53}NaO_3^+$).

irisolidone (**5**): ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.87 (1H, s, H-2), 7.45 (2H, d, *J* = 8.5 Hz, H-2' and H-6'), 6.98 (2H, d, *J* = 8.5 Hz, H-3' and H-5'), 6.52 (1H, br s, H-8), 4.03 (3H, s, 6-OCH_3), 3.84 (3H, s, 4'-OCH_3). ESI-MS for $C_{17}H_{14}O_6$: *m/z* = 315.10 $[M+H]^+$ (calcd. 315.2974 for $C_{17}H_{15}O_6^+$); *m/z* = 337.09 $[M+Na]^+$ (calcd. 337.2792 for $C_{17}H_{14}NaO_6^+$); *m/z* = 353.06 $[M+K]^+$ (calcd. 353.3878 for $C_{17}H_{14}KO_6^+$); *m/z* = 651.19 $[2M+Na]^+$ (calcd. 651.5687 for $C_{34}H_{28}NaO_{12}^+$).

(**6**): ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.86 (1H, s, H-2), 7.39 (2H, d, *J* = 8.2 Hz, H-2' and H-6'), 6.90 (2H, d, *J* = 8.2 Hz, H-3' and H-5'), 6.52 (1H, br s, H-8), 6.07 (2H, s, 6,7-(OCH_2O)), 4.09 (3H, s, 5-OCH_3). ESI-MS for $C_{17}H_{12}O_6$: *m/z* = 313.09 $[M+H]^+$ (calcd. 313.2815 for $C_{17}H_{13}O_6^+$); *m/z* = 335.06 $[M+Na]^+$ (calcd. 335.2633 for $C_{17}H_{12}NaO_6^+$); *m/z* = 351.05 $[M+K]^+$ (calcd. 351.1719 for $C_{17}H_{12}NaO_6^+$)

irigenin (**7**): ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.88 (1H, s, H-2), 6.70 (2H, d, *J* = 2.9 Hz, H-2' and H-6'), 6.52 (1H, s, H-8), 4.03 (3H, s, 6-OCH_3), 3.92 (3H, s, 5'-OCH_3), 3.90 (3H, s, 4'-OCH_3). ESI-MS for $C_{18}H_{16}O_8$: *m/z* = 383.10 $[M+Na]^+$ (calcd. 383.3046 for $C_{18}H_{16}NaO_8^+$)

irigenin S (**8**): ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.87 (1H, s, H-2), 6.86 (1H, br s, H-2'), 6.70 (1H, d, *J* = 3.2 Hz, H-6'), 6.53 (1H, s, H-8), 4.08 (3H, s, 7-OCH_3), 4.04 (3H, s, 5'-OCH_3), 3.93 (3H, s, 6-OCH_3), 3.90 (3H, s, 4'-OCH_3). ESI-MS for $C_{19}H_{18}O_8$: *m/z* = 413.29 $[M+K]^+$ (calcd. 413.4396 for $C_{19}H_{18}KO_8^+$)

irisflogenin (**9**): ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.90 (1H, s, H-2), 7.41 (1H, dd, *J* = 8.4/4.1 Hz, H-2'), 7.14 (1H, d, *J* = 4.1 Hz, H-6'), 6.88 (1H, d, *J* = 8.4 Hz, H-3'), 6.07 (2H, s, 6,7-(OCH_2O)), 3.91 (3H, s, 6-OCH_3). ESI-MS for $C_{17}H_{12}O_7$: *m/z* = 351.17 $[M+Na]^+$ (calcd. 351.2627 for $C_{17}H_{12}NaO_7^+$)

nigricin 4'-*O*-β-D-glucopyranoside (**10**): ^{1}H NMR (300 MHz, CD_3OD) δ: 8.04 (1H, s, H-2), 7.43 (2H, d, *J* = 8.5 Hz, H-2' and H-6'), 7.14 (2H, d, *J* = 8.5 Hz, H-3' and H-5'), 6.75 (1H, br s, H-8), 6.10 (2H, s, 6,7-(OCH_2O)), 4.95 (1H, d, *J* = 6.8 Hz, H-1''), 4.04 (3H, s, 5-OCH_3), 3.92-3.48 (overlapped sugar signals). ESI-MS for $C_{22}H_{20}O_{11}$: *m/z* = 474.68 $[M+H]^+$ (calcd. 475.4221 for $C_{22}H_{21}O_{11}^+$); *m/z* = 496.63 $[M+Na]^+$ (calcd. 497.4038 for $C_{22}H_{20}NaO_{11}^+$); *m/z* = 512.60 $[M+K]^+$ (calcd. 513.5125 for $C_{22}H_{20}KO_{11}^+$).

irisolidone 7-*O*-β-D-glucopyranoside (**11**): ^{1}H NMR (300 MHz, CD_3OD) δ: 8.21 (1H, s, H-2), 7.50 (2H, d, *J* = 8.4 Hz, H-2' and H-6'), 7.00 (2H, d, *J* = 8.4 Hz, H-3' and H-5'), 6.88 (1H, br s, H-8), 5.10 (1H, d, *J* = 7.3 Hz, H-1''), 3.89 (3H, s, 6-OCH_3), 3.83 (3H, s, 4'-OCH_3), 3.80-3.35 (overlapped sugar signals). ESI-MS for $C_{23}H_{24}O_{11}$: *m/z* = 499.16 $[M+Na]^+$ (calcd. 499.4198 for $C_{23}H_{24}NaO_{11}^+$).

iridin (**12**): ^{1}H NMR (300 MHz, CD_3OD) δ: 8.23 (1H, s, H-2), 7.00 (1H, br s, H-2'), 6.88 (2H, br s, H-6'), 6.73 (1H, s, H-8), 5.10 (1H, d, *J* = 7.1 Hz, H-1''), 3.89 (3H, s, 5'-OCH_3), 3.88 (3H, s, 6-OCH_3), 3.82 (3H, s, 4'-OCH_3), 3.74-3.41 (overlapped sugar signals). ESI-MS for $C_{24}H_{26}O_{13}$: *m/z* = 545.15 $[M+Na]^+$ (calcd. 545.4452 for $C_{24}H_{26}NaO_{13}^+$); *m/z* = 561.12 $[M+K]^+$ (calcd. 561.5537 for $C_{24}H_{26}KO_{13}^+$).

irilone 4'-*O*-β-D-glucopyranoside (**13**): ^{1}H NMR (300 MHz, CD_3OD) δ: 8.19 (1H, s, H-2), 7.51 (2H, d, *J* = 8.3 Hz, H-2' and H-6'), 7.17 (2H, d, *J* = 8.3 Hz, H-3' and H-5'), 7.07 (1H, s, H-8), 6.11 (2H, s, 6,7-(OCH_2O)), 5.10 (1H, d, *J* = 7.1 Hz, H-1''), 3.74-3.41 (overlapped sugar signals). ESI-MS for $C_{22}H_{20}O_{11}$: *m/z* = 483.12 $[M+Na]^+$ (calcd. 483.3774 for $C_{22}H_{20}NaO_{11}^+$); *m/z* = 499.19 $[M+K]^+$ (calcd. 499.4859 for $C_{22}H_{20}KO_{11}^+$).

tectoridin (**14**): ^{1}H NMR (300 MHz, CD_3OD) δ: 8.09 (1H, s, H-2), 7.49 (2H, d, *J* = 8.1 Hz, H-2' and H-6'), 7.17 (2H, d, *J* = 8.1 Hz, H-3' and H-5'), 6.74 (1H, br s, H-8), 5.10 (1H, d, *J* = 7.1 Hz, H-1''), 3.86 (3H, s, 6-OCH_3), 3.74-3.42 (overlapped sugar signals). ESI-MS for $C_{22}H_{22}O_{11}$: *m/z* = 485.21 $[M+Na]^+$ (calcd. 405.3932 for $C_{22}H_{22}NaO_{11}^+$); *m/z* = 501.14 $[M+K]^+$ (calcd. 501.5018 for $C_{22}H_{22}KO_{11}^+$).

α-glucose (**15a**): ^{1}H NMR (300 MHz, D_2O) δ: 5.24 (1H, d, *J* = 3.2 Hz, H-1), 3.96 (1H, m, H-6a), 3.86 (1H, m, H-5), 3.75 (1H, m, H-6b), 3.70 (1H, m, H-3), 3.55 (1H, m, H-2), 3.41 (1H, m, H-4). ESI-MS for $C_6H_{12}O_6$: *m/z* = 203.21 $[M+Na]^+$ (calcd. 203.1456 for $C_6H_{12}NaO_6^+$); *m/z* = 179.07 $[M-H]^-$ (calcd. 179.1479 for $C_6H_{11}O_6^-$).

β-glucose (**15b**): ^{1}H NMR (300 MHz, D_2O) δ: 4.60 (1H, d, *J* = 8.0 Hz, H-1), 3.91 (1H, m, H-6a), 3.73 (1H, m, H-6b), 3.51 (1H, m, H-3), 3.49 (1H, m, H-5), 3.42 (1H, m, H-4), 3.25 (1H, m, H-2). ESI-MS for $C_6H_{12}O_6$: *m/z* = 203.21 $[M+Na]^+$ (calcd. 203.1456 for $C_6H_{12}NaO_6^+$); *m/z* = 179.07 $[M-H]^-$ (calcd. 179.1479 for $C_6H_{11}O_6^-$).

sucrose (**16**): ^{1}H NMR (300 MHz, D_2O) δ: 5.42 (1H, d, *J* = 3.2 Hz, H-1(Glc)), 4.22 (1H, d, *J* = 8.8 Hz, H-3'(Fru)), 4.06 (1H, m, H-4'(Fru)), 3.90 (1H, m, H-5'(Fru)), 3.85 (1H, m, H-5(Glc)), 3.82 (4H, m, H-6(Glc)

and H-6'(Fru)), 3.79 (1H, m, H-3(Glc)), 3.69 (2H, s, H-1'(Fru)), 3.59 (1H, m, H-2(Glc)), 3.48 (1H, m, H-4(Glc)). ESI-MS for $C_{12}H_{22}O_{11}$: *m/z* = 365.17 $[M+Na]^+$ (calcd. 365.2862 for $C_{12}H_{22}NaO_{11}^+$); *m/z* = 341.29 $[M-H]^-$ (calcd. 341.2885 for $C_{12}H_{21}O_{11}^-$).

raffinose (**17**): ^{1}H NMR (300 MHz, D_2O) δ: 5.45 (1H, d, *J* = 3.2 Hz, H-1(Glc)), 5.01 (1H, d, *J* = 3.8 Hz, H-1"(Gal)), 4.22 (1H, d, *J* = 8.8 Hz, H-3'(Fru)), 4.06 (1H, m, H-4'(Fru)), 4.04 (2H, m, H-6a(Glc) and H-4"(Gal)), 3.95 (1H, m, H-5"(Gal)), 3.90 (2H, m, H-5'(Fru) and H-3"(Gal)), 3.85 (1H, m, H-5(Glc)), 3.82 (2H, m, H-6'(Fru) and H-2"(Gal)), 3.79 (3H, m, H-3(Glc) and H-6"(Gal)), 3.70 (1H, m, H-6b(Glc), 3.69 (2H, s, H-1'(Fru)), 3.59 (1H, m, H-2(Glc)), 3.48 (1H, m, H-4(Glc)). ESI-MS for $C_{18}H_{32}O_{16}$: *m/z* = 527.42 $[M+Na]^+$ (calcd. 527.4268 for $C_{18}H_{32}NaO_{16}^+$); *m/z* = 503.28 $[M-H]^-$ (calcd. 503.4291 for $C_{18}H_{31}NaO_{11}^-$).

lactic acid (**18**): ^{1}H NMR (300 MHz, D_2O) δ: 4.13 (1H, m, H-α), 1.32 (3H, d, *J* = 4.6 Hz, CH_3). ESI-MS for $C_3H_6O_3$: *m/z* = 89.02 $[M-H]^-$ (calcd. 89.0700 for $C_3H_5O_3^-$).

phenylalanine (**19**): ^{1}H NMR (300 MHz, D_2O) δ: 7.37 (2H, m, H-3 and H-5), 7.32 (1H, m, H-4), 7.28 (2H, m, H-2 and H-6), H-α (1H) and H-β (2H) are overlapped with carbohydrate resonances. ESI-MS for $C_9H_{11}NO_2$: *m/z* = 164.10 $[M-H]^-$ (calcd. 164.1812 for $C_9H_{10}NO_2^-$).

isoleucine (**20**): ^{1}H NMR (300 MHz, D_2O) δ: 1.95 (1H, m, H-β), 1.52 (1H, m, H-γ'), 1.23 (1H, m, H-γ), 1.08 (3H, m, δ'-CH_3), 0.93 (3H, m, δ-CH_3), H-α (1H) is overlapped with carbohydrate resonances. ESI-MS for $C_6H_{13}NO_2$: *m/z* = 130.33 $[M-H]^-$ (calcd. 130.1650 for $C_6H_{12}NO_2^-$).

valine (**21**): ^{1}H NMR (300 MHz, D_2O) δ: 2.15 (1H, m, H-β), 1.04 (3H, d, *J* = 8.3 Hz, γ′-CH_3), 0.97 (3H, d, *J* = 8.3 Hz, δ-CH_3), H-α (1H) is overlapped with carbohydrate resonances. ESI-MS for $C_5H_{11}NO_2$: *m/z* = 116.07 $[M-H]^-$ (calcd. 116.1464 for $C_5H_{10}NO_2^-$).

Results and Discussion

The phytochemical analysis performed on a sample of the ethanolic dried extract of *I. marsica* roots allowed the isolation and the identification of twenty compounds in total. In detail, these were: tristearoyl-*sn*-glycerol (**1**), 1-margaroyl-2-lauroyl-3-palmitoyl-*sn*-glycerol (**2**), 1,2-distearoyl-*sn*-glycerol (**3**), α-irigermanal (**4**), irisolidone (**5**), nigricin (**6**), irigenin (**7**), irigenin S (**8**), irisflogenin (**9**), nigricin 4'-*O*-β-D-glucopyranoside (**10**), irisolidone 7-*O*-β-D-glucopyranoside (**11**), iridin (**12**), irilone 4'-*O*-β-D-glucopyranoside (**13**), tectoridin (**14**), glucose (**15**), sucrose (**16**), raffinose (**17**), lactic acid (**18**), phenylalanine (**19**), isoleucine (**20**) and valine (**21**) (Figure 2).

These compounds belong to six different classes of natural compounds such as glycerides (compounds **1-3**), triterpenoids (compound **4**), isoflavones (compounds **5-14**), sugars (compounds **15-17**), organic acids (compound **18**), amino acids (compounds **19-21**).

To the best of our knowledge, all these compounds represent new phytochemicals for the species and present a great relevance under three aspects: chemotaxonomy, pharmacology, nutrition. Triglycerides tristearoyl-*sn*-glycerol (**1**) and 1-margaroyl-2-lauroyl-3-palmitoyl-*sn*-glycerol (**2**) and diglyceride 1,2-distearoyl-*sn*-glycerol (**3**) are constituted by saturated fatty acids chains at different number of carbon atoms. Only compound (**2**) presents one chain with an odd number of carbons while all the other chains have an even number of carbons. All these compounds present several nutraceutical properties in spite of what it's known and said about compounds constituted by saturated fatty acids. Indeed, these fatty acids are responsible of these activities. In compounds (**1**) and (**3**), stearic acid represents the only fatty acid present respectively, in triplicate and in duplicate, in their structures. It exerts hypocholesterolemic effects [37] and does not increase the total risk of cardiovascular diseases [38]. Even better it seems to present some beneficial effects on several measures of thrombosis [38]. For these reasons, tristearoyl-*sn*-glycerol (**1**) and 1,2-distearoyl-*sn*-glycerol

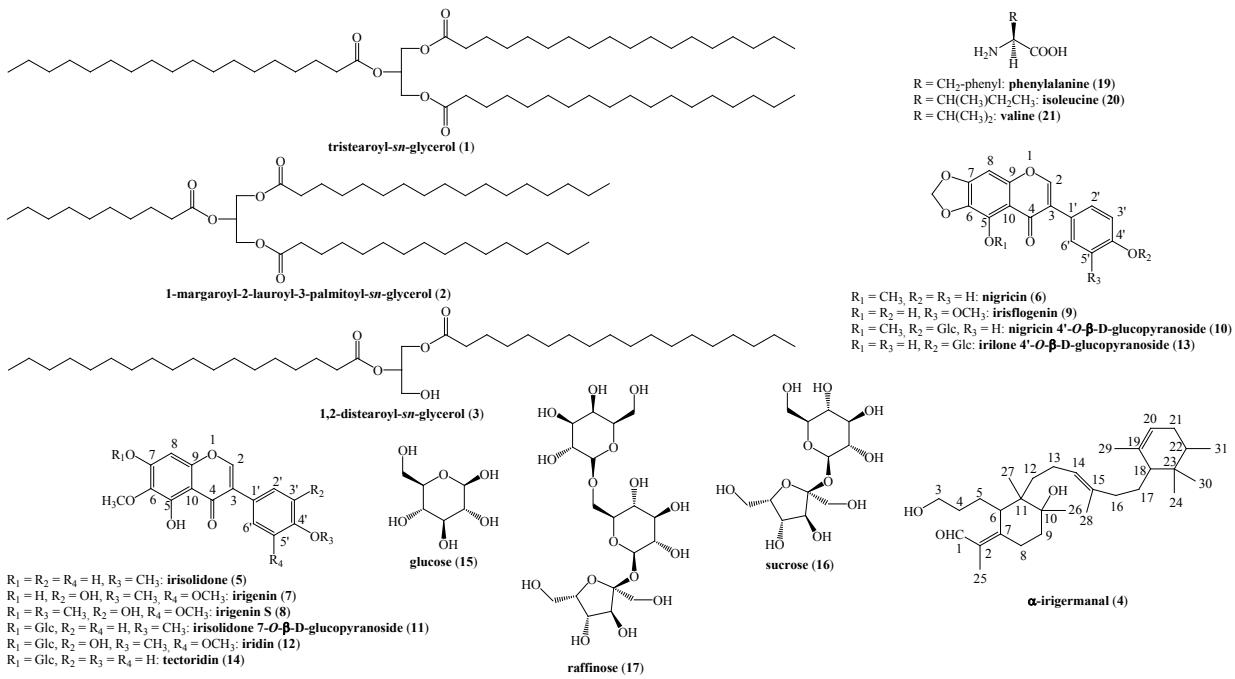

Figure 2: Chemical structures of the compounds identified in *I. marsica* roots.

(**3**) which are, indeed, supposed to have very similar properties, might represent beneficial compounds. The same discussion can be made for 1-margaroyl-2-lauroyl-3-palmitoyl-*sn*-glycerol (**2**), too. In fact, margaric acid, lauric acid and palmitic acid which constitute the structure of this compound, have important nutraceutical properties as well. In particular, margaric acid exerts a potent protective action against type-2 diabetes [39]. At its own time, lauric acid has antibacterial [40], antiviral [41] and strong cytotoxic [42] activities and, moreover, is able to decrease the ratio between total cholesterol and HDL cholesterol on which it, actually, acts [43]. Lastly, palmitic acid displays antioxidant and selective cytotoxic properties [44]. In the end, also this compound seems to be a beneficial one.

α-irigermanal (**4**) is the only terpenoid in general evidenced in the sample during this study. By the way, it is not a new phytochemical for the genus even if it seems to be specific to one only species, *I. germanica* L., where it has been reported several times before [34,45,46]. Thus, this is the first time that this compound was identified in another *Iris* species. From the pharmacological point of view, α-irigermanal (**4**) exerts a good anti-tumor activity [47]. It is also noteworthy to underline that (**4**) is the precursor of α-irone, via the α-dihydroirone intermediate [34,48], which is one of the violet scent-carrying 6-methylionone formed upon storage in *I. germanica* roots. On this base, also the roots of the *I. marsica* may be a potential source of aromatic principles for the preparation of perfumes and cosmetics with the fragrance of sweet violet.

Also irisolidone (**5**) is not a novelty phytochemical for the genus and just like the previous compound, represents a quite rare compound. In fact, its presence seems to be restricted to two specific *Iris* species i.e., *I. germanica* L. without differentiating the geographical provenience of the plant [17,49] and *I. kashmiriana* Baker [50]. With this phytochemical study, it was shown that there is a third species in which it could be found and this is *I. marsica* I. Ricci and Colas. This situation might make irisolidone (**5**) a important chemotaxonomic marker at specific level. Moreover, it possesses many interesting biological activities and, in particular, these are the antioxidant, the antibacterial, the antiviral, the hepatoprotective, the neuroprotective and the antitumor ones [17,51-55]. Lastly, it has a specific property in the nutraceutical field because it is able to lower the levels of total cholesterol in the serum [56] thus proving to be a compound with double beneficial effects on health.

Nigricin (**6**), also known as irisolone, represents a well spread compound in the *Iris* genus, instead. In fact, it has been evidenced before in several species such as *I. imbricata* Lindl. [57], *I. germanica* L. [58], *I. nigricans* Dinsm. [32], *I. pallida* Lam. [33] and *I. susiana* L. [59]. From the pharmacological point of view, this compound does not exert as many properties as all the other isoflavones but these are very relevant and peculiar. In particular, it can be used to treat osteoporosis, distal renal tubular acidosis [60] and inflammations [61] but also as a slight antioxidant [62].

Also irigenin (**7**) does not represent a new compound for the genus where it has been reported several times like for example in *I. germanica* L., *I. dichotoma* Pall., *I. tectorum* Maxim. [63], *I. kashmiriana* Baker [64], *I. pallida* Lam. [33] and *I. florentina* L. [65]. Under the pharmacological profile, this compound is able to exert a strong anti-inflammatory action acting on prostaglandin E2 [66] and a real inhibitory effect on cytochrome P450 against the outbreak of tumor forms in different parts of the human body [17,64].

Irigenin S (**8**) represents, chemically, a derivative of irigenin (**7**) with one more methoxy group in the position 7 of the structure to replace the original hydroxy group. This compound is not very common among all the *Iris* species and in fact its presence seems to be limited to only *I. germanica* L. [25,49]. This is the first time, for what we know, that it is reported in another species. From the pharmacological point of view, irigenin S (**8**) showed a moderate antioxidant activity [49].

Unlike the previous compound, irisflogenin (**9**) or iriflogenin is a quite widespread isoflavone in the *Iris* genus. This has been evidenced in several species such as *I. potaninii* Maxim. [67], *I. germanica* L. [17], *I. florentina* L. [68] and *I. pallida* Lam. [33]. In the pharmacological field, this compound is well known to exhibit one only but very important effect which is linked to cancer both as chemopreventive [17] and as drug [69].

Nigricin 4'-*O*-β-D-glucopyranoside (**10**), better known with the common name of germanaism B, is also not new in the genus. In particular, this compound is well reported in *I. germanica* L. where its presence has been massively evidenced in spite of the growth area of the plant [27,58,61]. Anyway, this compound has been also found in other *Iris* species such as *I. leptophylla* Lingelsh. ex H. Limpr. [70] and *I. pseudopumila* Tineo [71]. This compound has one only demonstrated pharmacological activity which is the cytotoxic one [72] and also with a discrete efficacy thus making it a very important isoflavone even if with this peculiarity.

On the other hand, irisolidone 7-*O*-β-D-glucopyranoside (**11**) seems to be a quite rare compound in the genus just like its aglycone compound. In fact, its presence has been evidenced only in particular species such as *I. germanica* L. [58] and *I. imbricata* Lindl. [57]. Yet, unlike its aglycone compound, this shows only two pharmacological effects which are the antitumor [17] and the radical scavenger ones [63].

Iridin (**12**) is, in practice, the main glycosidic isoflavone of the entire *Iris* genus so that it is considered to be its chemosystematic marker for excellence. Indeed, its presence within the genus is very common and comprises most of the existent nominal species like, for example, *I. germanica* L. [25], *I. unguicularis* Poir., *I. dichotoma* Pall., *I. florentina* L. [63], *I. kumasnensis* Wall. [73] and *I. milesii* Baker ex Foster [74]. This compound presents two main pharmacological properties i.e., antioxidant and α-amylase inhibitor thus preventing the insurgence of pancreatitis [48]. Nevertheless, iridin (**12**) has several nutraceutical activities. In fact, it exerts a very strong diuretic effect [63] and, moreover, it is a very efficient intestinal stimulant and a perfect cholagogue [75].

On the contrary, irilone 4'-*O*-β-D-glucopyranoside (**13**) represents another glycosidic isoflavone with a rare distribution in the *Iris* genus although it is not a new compound for it. In fact, it has been reported in several plants of *I. germanica* L. coming from different areas of the world [25,27,58], thus being a specific chemotaxonomic marker for this species. Outside this particular species, this compound has been evidenced only in *I. pseudopumila* Tineo [75] and now also in *I. marsica* I. Ricci and Colas. Irilone 4'-*O*-β-D-glucopyranoside (**13**) presents some outstanding pharmacological activities such as the antiproliferative [76], the antioxidant and mainly the anti-inflammatory ones [25].

Tectoridin (**14**) is not a new compound for the *Iris* genus, too. Its presence has been mainly detected in *I. tectorum* Maxim. [63] but recent and old studies have also reported it in other species such as *I. dichotoma* Pall. [63] and *I. germanica* L. [77]. From the pharmacological point of view, this compound shows several different medicinal properties i.e., antioxidant [36], anti-inflammatory, antiproliferative, antineoplastic [63], antiallergic, anti-platelet [78], hepato and neuroprotective [79,80]. Moreover, it's able to lower the level of glucose in the blood [36] presenting also a nutritional value.

The presence of compounds from (**4**) to (**14**) is important also under the nutraceutical aspect for what concerns their nature. In fact, consumption of isoflavonoids in general has been demontrated to really reduce the risk of cardiovascular diseases and of cancer outbreak [81].

Glucose (**15**), sucrose (**16**) and raffinose (**17**) are respectively a monosaccharidic, a disaccharidic and, lastly, a trisaccharidic sugar. The glucosidic unit is present in the structures of all these compounds, the fructosidic unit is present in both compounds (**16**) and (**17**) while the galactosidic unit is present only in compound (**17**). Anyway, all of them present similar nutraceutical properties. In particular, they are essential for several metabolomic processes and, in controlled quantity, are promoters of several health-beneficial effects. In fact, glucose (**15**) favors brain activity and can be used to treat hypoglycemia. Sucrose (**16**) displays, instead, a preservative and an antioxidant effect [82]. Lastly, raffinose (**17**) is able to reduce calories in excess.

Lactic acid (**18**) is also important in the nutraceutical field. Under the chemical aspect, it is one of the simplest organic acid with one carboxyl and one alcoholic functional group and these are responsible of its activities. In detail, these are mainly the disinfectant, keratolytic and preservative ones. Above all, the latter property makes it so essential because it's able to reduce the loss of nutritional value of foods in which it has been added.

Lastly, phenylalanine (**19**), isoleucine (**20**) and valine (**21**) are, probably, the most relevant compounds identified in this species for what concerns their nutraceutical effects. First of all, these are essential amino acids which means that they must be supplied to our organism through nutrition. As second thing, phenylalanine (**19**) is used to treat depression [83], attention deficit-hyperactivity disorder (ADHD) [84], and Parkinson's disease [85]. Moreover, isoleucine (**20**) is able to increase endurance and heal muscle tissues and valine (**21**) is fundamental for hematopoetic stem cell self-renewal [86].

Conclusions

From the chemotaxonomic point of view, in general, *Iris marsica* I. Ricci e Colas. seems to be very similar to *I. germanica* L. In addition to this, the presence of all the identified compounds in this sample provides a phytochemical rationale for the employment of also this species in the ethno-pharmacological field due to the several medicinal properties that these compound are able to exert. Lastly, all of these compounds have also nutraceutical value. So, we hope this species may be widely cultivated, in the future, for ethno-pharmacological, cosmetic and nutraceutical aims since it is a rare and protected species and cannot be collected on the wild.

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