



## Original Article

## Antioxidant $\alpha$ -amylase inhibitors flavonoids from *Iris germanica* rhizomes



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## ABSTRACT

A new isoflavonoid glycoside, iridin A (**9**), along with eight known isoflavonoids: irilone 4'-methyl ether (**1**), irilone (**2**), irisolidone (**3**), irigenin S (**4**), irigenin (**5**), irilone 4'-O- $\beta$ -D-glucopyranoside (**6**), iridin S (**7**), and iridin (**8**) were separated from *Iris  $\times$  germanica* L., Iridaceae, rhizomes. The structural elucidation of these flavonoids was achieved with the aid of extensive spectroscopic techniques and comparing with the published data. They were estimated for their  $\alpha$ -amylase and 1,1-diphenyl-2-picrylhydrazyl inhibitory capacities. Compounds **3**, **5**, and **9** showed  $\alpha$ -amylase inhibitory activities with % inhibition 70.8, 67.5, and 70.5, respectively compared to acarbose (a reference  $\alpha$ -amylase inhibitor). Moreover, **9** exhibited moderate antioxidant activity with IC<sub>50</sub> 8.91  $\mu$ M.

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## Introduction

Diabetes is a serious disease growing at alarming rates around the world. It constitutes the 6th leading cause of global mortality (WHO, 2016). Type-II diabetes mellitus (T2D) is a widespread disorder of fat and glucose metabolism, which is strongly linked with diets (Garg et al., 1994). Postprandial hyperglycemia (PPHG) is an independent risk factor for the development of diabetic complications (Kwon et al., 2005). It was reported that the best therapeutic path for type-II diabetes is to lower PPHG through retardation of the intake of glucose by repression of  $\alpha$ -amylases and  $\alpha$ -glucosidases, which are responsible for the oligo- and disaccharides breakdown into glucose (Kim et al., 2005; Hanhineva et al., 2010). Polyphenols and flavonoids function as chemo-preventive agents against reactive oxygen species (ROS) induced oxidative damage and inhibit carbohydrate hydrolyzing enzymes, contributing to the lowering of PPHG (Mai et al., 2007; Ibrahim et al., 2015). They are commonly found in nuts, vegetables, fruits, and tea (Arranz et al., 2010). Strong efforts have been made to seek for more safe and efficient antioxidants and  $\alpha$ -amylase inhibitors

from natural source to unveil functional foods for the prevention and control of diabetes (Tundis et al., 2010; Tarling et al., 2008). *Iris  $\times$  germanica* L., Iridaceae, leaves are rich in vitamins and ascorbic acid (Ibrahim et al., 2012). Its extract stimulates respiration, reduces the activity of the smooth muscle, possesses serotonin inhibitory activity, and induces a transient hypotension (Asghar et al., 2010; Kukula-Koch et al., 2015). Its root decoction is used as stimulants, antispasmodic, anti-inflammatory, aperients, diuretic, violently cathartic, and emmenagogue (Rahman et al., 2003a,b; Mohamed et al., 2013). Previous phytochemical studies of *I. germanica* revealed the isolation of different constituents, including flavonoids (Asghar et al., 2010; Ibrahim et al., 2012; Mohamed et al., 2013), triterpenes (Ito et al., 1995; Orhan et al., 2002), sterols, phenolics (Ibrahim et al., 2012), ceramides (Mohamed et al., 2013), and benzoquinones (Asghar et al., 2010). As part of an ongoing search for isolation of antioxidants and  $\alpha$ -amylases inhibitors from plant source, further investigation of the rhizomes of *I. germanica* identified a new isoflavonoid glycoside: iridin A (**9**) and eight known compounds (**1–8**). This study gives an account on the structural assignment of the isolated flavonoids using different spectroscopic analyses. Furthermore, the antioxidant and antidiabetic potentials of the isolated compounds were estimated using DPPH and  $\alpha$ -amylase inhibitory tests, respectively.

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## Materials and methods

### General experimental procedures

Electrothermal 9100 Digital Melting Point apparatus was used for measuring the melting points (Electrothermal Engineering Ltd., Essex, England). Ultraviolet spectra were assessed by a Hitachi 300 spectrophotometer. A LCQ DECA mass spectrometer was utilized to get the ESIMS (ThermoFinnigan, Bremen, Germany). HRESIMS was achieved using a LTQ Orbitrap (ThermoFinnigan, Bremen, Germany). Bruker Avance DRX 500 MHz spectrometer was used for measuring the NMR spectra. Compounds isolation was achieved using silica gel (0.063–0.200 mm), sephadex LH-20 (0.25–0.1 mm), and RP<sub>18</sub> (0.04–0.063 mm) (Merck, Darmstadt, Germany). Silica gel 60 F<sub>254</sub> pre-coated plates (0.2 mm, Merck, Darmstadt, Germany) were utilized for thin-layer chromatography. Six ml standard LiChrolut extraction tube (RP<sub>18</sub>, 40–63 μm, Merck, Darmstadt, Germany) was used for compounds purification. The spots on TLC were visualized by UV at λ<sub>max</sub> 255 and 366 nm, then exposed to NH<sub>3</sub> vapor after that spraying with *p*-anisaldehyde:H<sub>2</sub>SO<sub>4</sub> reagent and heating at 110 °C for 1–2 min. All chemicals materials were secured by Sigma Chemical Aldrich (St. Louis, MO, USA).

### Plant material

The rhizomes of *Iris × germanica* L., Iridaceae, were collected in May 2014 from the botanical garden at the Faculty of Agriculture, Assiut University. The plant was kindly authenticated by A. A. Fayed, Professor of Plant Taxonomy, Faculty of Science, Assiut University. A specimen under the registration number IG-5-2014 was kept in the Department of Natural Products herbarium, Faculty of Pharmacy, King Abdulaziz University, Saudi Arabia.

### Extraction and isolation

The powdered rhizomes (270 g) were extracted with MeOH (4 × 2.5 l). The MeOH extract was evaporated to yield a brown residue (12.9 g). The later was applied on a vacuum liquid chromatography (VLC) using hexane, CHCl<sub>3</sub>, and EtOAc to afford hexane (IG-1, 2.9 g), CHCl<sub>3</sub> (IG-2, 2.4 g), EtOAc (IG-3, 1.1 g), and aqueous

(IG-4, 4.8 g) fractions. Fraction IG-2 (2.4 g) was subjected to VLC using hexane:EtOAc as eluent to get seven subfractions: IG-2A to IG-2G. Subfraction IG-2D (240 mg) was separated on SiO<sub>2</sub> CC (40 g, 50 × 3 cm) using CHCl<sub>3</sub>:MeOH (98:2–90:10) to get compounds **1** (18 mg) and **2** (11.5 mg). Subfraction IG-2E (410 mg) was subjected to SiO<sub>2</sub> CC (70 g, 50 × 3 cm) eluting with CHCl<sub>3</sub>:MeOH (94:6–90:10) to yield **3** (12 mg) and **4** (14 mg). SiO<sub>2</sub> CC of subfraction IG-2F (218 mg) (70 g, 50 × 2 cm) using CHCl<sub>3</sub>:MeOH (93:7–85:15) as an eluent gave compound **5** (5.2 mg). Sephadex LH-20 CC (150 g, 100 × 3 cm) of IG-3 (1.1 g) using MeOH:CHCl<sub>3</sub> (9:1) afforded five subfractions: IG-3A to IG-3E. RP-18 CC of subfraction IG-3A (118 mg) (100 g, 50 × 2 cm) eluting with H<sub>2</sub>O:MeOH gradient gave **7** (17 mg) and **9** (6.9 mg). Subfraction IG-3B (130 mg) was applied on SiO<sub>2</sub> CC (20 g, 50 × 2 cm) using a CHCl<sub>3</sub>:MeOH (90:10–80:20) to give **8** (14.1 mg). SiO<sub>2</sub> CC of sub-fraction IG-3D (115.4 mg) (50 g, 50 × 2 cm), eluting with CHCl<sub>3</sub>:MeOH gave impure **6**, which was purified on LiChrolut EN/RP-18 solid phase extraction tube using H<sub>2</sub>O:acetonitrile gradient to yield **6** (6.7 mg).

### Spectral data

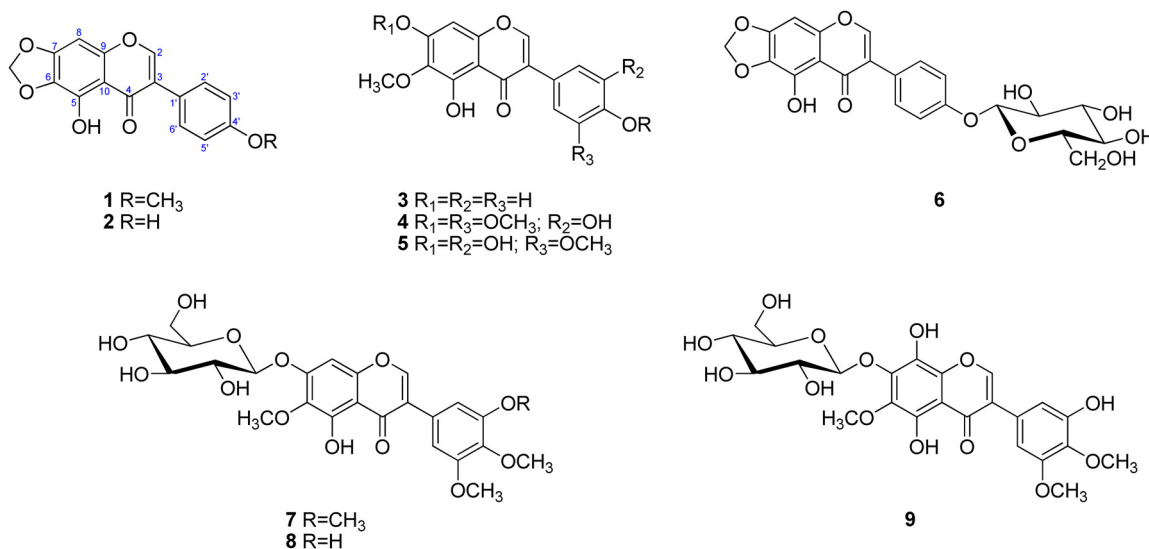
Iridin A (**9**): Yellow amorphous powder (6.9 mg); mp 211–213 °C (dec.); UV (MeOH) λ<sub>max</sub> (log ε): 207, 267, 330 nm; IR (KBr) γ<sub>max</sub>: 3397, 2956, 1694, 1605, 1509 cm<sup>-1</sup>; NMR data (DMSO-*d*<sub>6</sub>, 500 and 125 MHz) see Table 1; HRESIMS *m/z* 539.1391 (calcd for C<sub>24</sub>H<sub>27</sub>O<sub>14</sub>, [M+H]<sup>+</sup> 539.1395).

### α-Amylase inhibitory activity

The assay was carried out using α-amylase by EnzCheck® Ultra Amylase Assay Kit (E33651) as previously outlined (Mohamed, 2008; Sayed et al., 2008; Ibrahim et al., 2015).

### DPPH assay

The free radical scavenging capacities (FRS) of compounds **1–9** (Conc. 5, 10, 20, 40, 80, and 100 μM in HPLC MeOH) was evaluated using DPPH assay as outlined previously (Mohamed, 2014, 2016; Mohamed et al., 2014).



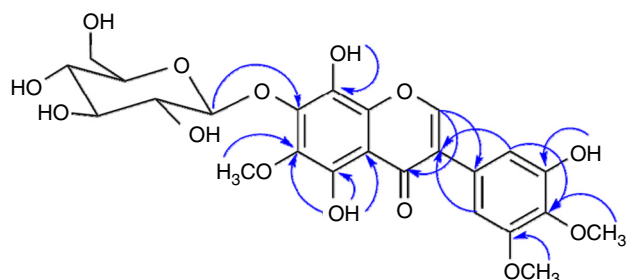
**Table 1**  
NMR spectral data of compound **9** (DMSO-*d*<sub>6</sub>, 500 and 125 MHz).

Position	$\delta_{\text{H}}$ [mult., J (Hz)]	$\delta_{\text{C}}$ (mult.)
2	8.49 s	154.8 (C)
3	–	124.7 (C)
4	–	180.2 (C)
5	–	148.6 (C)
6	–	135.1 (C)
7	–	150.2 (C)
8	–	126.5 (C)
9	–	151.9 (C)
10	–	106.1 (C)
1'	–	123.0 (C)
2'	6.89 d (1.5)	110.8 (CH)
3'	–	151.0 (C)
4'	–	136.9 (C)
5'	–	153.8 (C)
6'	6.74 d (1.5)	104.7 (CH)
1''	5.08 d (7.9)	100.8 (CH)
2''	–	73.7 (CH)
3''	–	77.9 (CH)
4''	5.21–3.65 m	70.1 (CH)
5''	–	76.8 (CH)
6''	–	61.0 (CH)
6-OCH <sub>3</sub>	3.82 s	58.7 (CH <sub>3</sub> )
4'-OCH <sub>3</sub>	3.72 s	60.1 (CH <sub>3</sub> )
5'-OCH <sub>3</sub>	3.84 s	55.9 (CH <sub>3</sub> )
5-OH	12.95 s	–
8-OH	9.21 s	–
3'-OH	9.30 s	–

## Results and discussion

The MeOH extract of *I. germanica* rhizomes was fractionated on VLC using *n*-hexane, CHCl<sub>3</sub>, and EtOAc. The CHCl<sub>3</sub> and EtOAc fractions were subjected to sephadex LH-20, silica gel, and RP-18 CC to afford one new (**9**) and eight known compounds (**1–8**).

Compound **9** was isolated as yellow amorphous powder and gave positive flavonoids tests (Mabry et al., 1970). It showed a pseudomolecular ion peak at *m/z* 539.1391 [M+H]<sup>+</sup> (calcd for C<sub>24</sub>H<sub>27</sub>O<sub>14</sub>, 539.1395) in HRESIMS spectrum, compatible with the molecular formula C<sub>24</sub>H<sub>26</sub>O<sub>14</sub>. The ESIMS spectrum showed a significant fragment ion peak at *m/z* 377 [M+H–Glu]<sup>+</sup>. Its UV absorption bands at 207, 267, and 330 nm characterized the isoflavone nature of **9** (Mabry et al., 1970; Harborne, 1994). The IR spectrum showed absorption bands for hydroxyl,  $\alpha,\beta$ -unsaturated carbonyl, aromatic functionalities at 3397, 1694, and 1605 and 1509 cm<sup>-1</sup>, respectively (Silverstein and Webster, 1998). The <sup>13</sup>C, DEPT, and HSQC NMR spectra revealed twenty four carbon signals. The <sup>1</sup>H NMR spectrum of **9** showed four singlet signals at  $\delta_{\text{H}}$  8.49, 9.30, 9.21, and 12.95, which were assigned to H-2 of isoflavone, 8-OH, 3'-OH, and 5-chelated OH group (Harborne, 1988; El-Shanawany et al., 2013). The two *meta*-coupled aromatic protons  $\delta_{\text{H}}$  6.89 (d, *J* = 1.5 Hz, H-2') and 6.74 (d, *J* = 1.5 Hz, H-6') indicated the presence of a tetra-substituted ring B (Table 1). They correlated to the carbons, resonating at  $\delta_{\text{C}}$  110.8 and 104.7, respectively

**Fig. 1.** Some key HMBC correlations of **9**.**Table 2**  
Results of  $\alpha$ -amylase and DPPH inhibitory activities of compounds **1–9**.

Compound	$\alpha$ -Amylase inhibition, %	Antioxidant activity IC <sub>50</sub> ( $\mu$ M)
<b>1</b>	44.2 $\pm$ 0.10	17.03 $\pm$ 0.29
<b>2</b>	51.7 $\pm$ 0.13	15.15 $\pm$ 0.21
<b>3</b>	70.8 $\pm$ 0.19	12.62 $\pm$ 0.15
<b>4</b>	56.9 $\pm$ 0.41	14.30 $\pm$ 0.17
<b>5</b>	67.5 $\pm$ 0.32	13.24 $\pm$ 0.09
<b>6</b>	32.8 $\pm$ 0.11	11.91 $\pm$ 0.12
<b>7</b>	34.1 $\pm$ 0.08	13.63 $\pm$ 0.23
<b>8</b>	38.7 $\pm$ 0.22	11.74 $\pm$ 0.11
<b>9</b>	70.5 $\pm$ 0.32	8.91 $\pm$ 0.22
Acarbose <sup>a</sup>	100	–
Propyl gallate <sup>b</sup>	–	6.72 $\pm$ 0.04

Note: Each value represents the mean  $\pm$  SD, *n* = 3.

<sup>a</sup> Reference standard for  $\alpha$ -amylase inhibitory activity.

<sup>b</sup> Reference standard for antioxidant activity.

in the HSQC spectrum. Moreover, three methoxy group signals at  $\delta_{\text{H}}$  3.82 (6-OCH<sub>3</sub>)/ $\delta_{\text{C}}$  58.7 (6-OCH<sub>3</sub>), 3.72 (4'-OCH<sub>3</sub>)/60.1 (4'-OCH<sub>3</sub>), and 3.84 (5'-OCH<sub>3</sub>)/55.9 (5'-OCH<sub>3</sub>) were observed. Their attachment at C-6, C-4', and C-5' was confirmed by observed HMBC cross peaks of 6-OCH<sub>3</sub> to C-6 ( $\delta_{\text{C}}$  135.1), 4'-OCH<sub>3</sub> to C-4' ( $\delta_{\text{C}}$  136.9), and 5'-OCH<sub>3</sub> to C-5' ( $\delta_{\text{C}}$  153.8) (Fig. 1). Furthermore, the <sup>1</sup>H NMR spectrum displayed anomeric proton signal at  $\delta_{\text{H}}$  5.08 (d, *J* = 7.9 Hz), indicating the presence of  $\beta$ -configured glucose moiety (Mohamed et al., 2013, 2015). Its placement at C-7 was secured by the HMBC correlation of H-1'' to C-7 ( $\delta_{\text{C}}$  150.2). Thus, **9** was assigned as 5,8,3'-trihydroxy-6,4',5'-trimethoxy-7-O- $\beta$ -D-glucopyranosyl isoflavone and named iridin A.

The known compounds were identified as irilone 4'-methyl ether (**1**) (Bilia et al., 1993), irilone (**2**) (Eckhard et al., 2003), irisolidone (**3**) (Kang et al., 2008), irigenin S (**4**) (Ibrahim et al., 2012), irigenin (**5**) (Akashi et al., 2005), irilone 4'-O- $\beta$ -D-glucopyranoside (**6**) (Mohamed et al., 2014), iridin S (**7**) (Mohamed et al., 2013), and iridin (**8**) (Arisawa and Morita, 1976) by inspection of the spectroscopic data and comparing of these data with literature as well as co-TLC with authentic samples.

Isoflavonoids are phenols with low molecular weight, which can give hydrogen atoms from their phenolic hydroxyl group(s) to the deleterious oxy radicals, forming the less-reactive phenoxyl radicals. Also, they have ability to chelate metal ions and exhibit potential antioxidant activity (Tikkanen et al., 1998). The antioxidant activity of **1–9** was assessed using DPPH assay. This assay is a widely used process for estimating the antioxidants capacity to scavenge the stable free radical produced from DPPH, which results changing in color from purple to yellow upon gaining a hydrogen radical (H<sup>•</sup>) to form the stable DPPH-H molecule (Lim et al., 2007). It is noteworthy that the best activity was exhibited by compound **9** (IC<sub>50</sub> 8.91  $\mu$ M) compared to propyl gallate (IC<sub>50</sub> 6.72  $\mu$ M). Other compounds showed moderate activities with IC<sub>50</sub> ranging from 11.74 to 17.03  $\mu$ M (Table 2). Additionally, the results of the  $\alpha$ -amylase inhibitory potential of the tested compounds revealed that **3**, **5**, and **9** displayed the highest activity with % inhibition 70.8, 67.5, and 70.5, respectively compared to acarbose (100%, reference  $\alpha$ -amylase inhibitor) (Table 2). The rest of compounds exhibited moderate to weak activities.

Li et al. (2007) stated that the isoflavonoids antioxidant effect increased cell membranes stability and protected them from damage which participates in increasing insulin sensitivity and inhibited free radical generation metabolic disorders in diabetes. Also, Mosihuzzman et al. (2013) reported that the isoflavonoids of *I. loczyi* and *I. unguicularis* inhibit  $\alpha$ -glucosidase and might have considerable potency to treat diabetes and its late complications (Mosihuzzman et al., 2013). Previous study by Prieto-Hontoria et al. (2009) indicated that isoflavonoids possessed hypoglycemic effect by inhibiting the intestinal  $\alpha$ -amylase activity and glucose

absorption rate. Moreover, they activated hepatic glycogen synthesis and accelerated glucose utilization in peripheral tissues (Hamden et al., 2011). At conclusion, the isolated isoflavonoids from *I. germanica* showed a capacity as inhibitors of DPPH and  $\alpha$ -amylase. The results clearly showed the effects of the tested isoflavonoids on suppression of the  $\alpha$ -amylase enzyme, which is responsible for the high glucose level in the blood. Also, they may reduce ROS production in T2D. Hence, the present work proposes fundamentals for the probable use of *I. germanica* as functional food. Consequently, an *in vivo* anti-diabetic research should be undertaken to recognize the other reasonable mechanism of action to control the hyperglycemia.

#### Structure–activity relationship

Comparing the antioxidant and  $\alpha$ -amylase inhibitory capacities of these groups of isoflavonoids, we were able to postulate the structure activity relationship of these compounds, which is in congruence with other reported researches (Arora et al., 1998; Xiao et al., 2011). The position and number of hydroxyls were found to be a significant determinant of the antioxidant efficacy. The C-4' hydroxyl is highly significant toward the antioxidant potentials and its substitution by methoxy and/or its loss reduces the abilities of these compounds. The introduction of a hydroxyl group at C-8 significantly increases the activity. The loss of the C-5 hydroxyl group diminishes the activity of isoflavonoids. Blocking the C-7 hydroxyl by glucose had no influence on the antioxidant activities, that way indicating that the existence of a C-7 hydroxyl has insignificant effect on the activities of these compounds. For  $\alpha$ -amylase inhibitory activity, hydroxylation at C-5 increased the inhibitory activities for  $\alpha$ -amylase. The glycosylation of isoflavonoids lowered their activities. Increase number of hydroxyls in rings A and B increases activity.

#### Conclusion

One new isoflavonoid glycoside, iridin A (**9**) and eight known isoflavonoids were separated from *I. germanica* rhizomes. Their structural elucidation was achieved with the aid of extensive spectroscopic techniques. Compounds **3**, **5**, and **9** showed  $\alpha$ -amylase inhibitory activities. Moreover, **9** exhibited moderate antioxidant activity.

#### Ethical disclosures

**Protection of human and animal subjects.** The authors declare that no experiments were performed on humans or animals for this study.

**Confidentiality of data.** The authors declare that no patient data appear in this article.

**Right to privacy and informed consent.** The authors declare that no patient data appear in this article.

#### Authors' contribution

AIMK collected and extracted of the plant material, also helped in the interpretation of the spectral data. GAM and SRMI contributed in carrying out the laboratory work, interpretation of the spectroscopic data, and writing the manuscript. AMA carried out the biological assays and interpreted the results. GAM and SRMI have revised and approved the submission.

#### Conflicts of interest

The authors declare no conflicts of interest.

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