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Isolation and Identification of Cholinesterase Inhibitors from the Bulbs of *Iris pseudacorus* L.

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Abstract: Most of the bulbous plants are known for their medicinal purposes in addition to their ornamental value. Turkey is one of the home country of many beautiful bulbous plants. In continuation of our extensive studies on finding new natural cholinesterase inhibitors from Turkish medicinal plants, *Iris* L. species were investigated for their *in vitro* cholinesterase inhibitory effects designed to assess cholinesterase inhibitor activities on both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) and antioxidant capacities with respect to their neuroprotective potential in this study.
The dichloromethane and methanol extracts prepared from the bulbs of 47 *Iris* taxa were screened by using modified Ellmann method and the highest butyrylcholinesterase inhibitory effect was found in the methanol extract of the bulbs of *Iris pseudacorus* L. (Sevim, 2018). The dichloromethane sub-extract, which is obtained bioactivity-guided fractionation of methanol extract of *I. pseudacorus* L., was exhibited significant butyrylcholinesterase inhibitory activity (73.65 ± 2.06 %). These active sub-extract was subjected to fractionation on column chromatography and obtained six fractions. Among the fractions, coded as N5 was shown the significant butyrylcholinesterase inhibitory activity (93.78 ± 1.49 %) compared with galanthamine (80.02 ± 0.12 %). Fractionation of N5 on flash chromatography the highest butyrylcholinesterase inhibitory activity of sub-fraction coded as DS-5 was determined as 94.00 ± 1.03 %. The responsible compound from the activity of this sub-fraction was detected as irisolidone glucopyranoside based on its mass data by using LC-ESI-Q/TOF-MS-MS technique.

Keywords: *Iris pseudacorus* L., Iridaceae, Activity, Anticholinesterase

1. Introduction

The genus *Iris* L. (Iridaceae) is a member of geophytes with attractive flowers. There are about 56 *Iris* taxa growing in Turkey, 24 of which are endemic (Güner, 2012). *Iris* species are an important plants as an ornamental due to their different colours and attractive flowers (Orhan et al., 2002, Atta-ur-Rahman et al., 2004, Wang et al., 2010); they have also evaluated in the preparation of products as an violet odour from their rhizomes in perfumery and cosmetic industries (Atta-ur-Rahman et al., 2004). Besides, *Iris* species were reported to be utilized for the treatment of different ailments in traditional medicine as an anticholinesterase, antioxidant, antimicrobial, antidiabetic, antiinflammatory, hepatoprotective, molluscisidal and cytotoxic effects depends on variety of secondary metabolites (Wang et al., 2010, Singab et al., 2016). Alzheimer's disease (AD) is one of the neurodegenerative disorder affecting the elder people (Howes et al., 2003). One of the main approaches has been for the control of AD is the inhibition of acetylcholinesterase and butrylcholinesterase for the elevation of acetylcholine level secreted from synaptic region in brain to restrain the cholinergic function related with memory loss. Currently, clinically available drugs are used for the treatment of AD. Continuing our researches in the field of anticholinesterase activity, we herein aimed to determine butyrylcholinesterase inhibitory activity of *Iris pseudacorus* L. showed the highest inhibitory activity *Iris* L. species (Sevim, 2018).

2. Materials and Method

2.1. Plant material

The rhizomes of *Iris pseudacorus* L. were collected from Hatay province and identified by Prof. Dr. Neriman Ozhatay and Prof. Dr. Adil Güner and preserved as *ex-situ* at Atatürk Horticultural Central Research Institute, Department of Ornamental Plant Breeding and Agronomy in Yalova, Turkey (population no. 3108) (Mathew, 1984, Güner, 2012).

2.2. Preparation of extracts

The washed with tap water, dried and powdered rhizomes (350 g) were extracted with methanol at room temperature by maceration. The combined methanolic extracts were evaporated *in vacuo* and obtained as 87.75 g extract (D18RM).

2.3. Cholinesterase inhibition assays

Extracts were investigated for their *in vitro* cholinesterase inhibitory activity at 200 µg/mL using ELISA microplate reader. AChE and BChE inhibitory activity was measured by slightly modified spectrophotometric method of Ellman et al. (Ellman et al., 1961). Electric eel AChE (Type-VI-S; EC 3.1.1.7, Sigma, St. Louis, MO, USA) and horse serum BChE (EC 3.1.1.8, Sigma, St. Louis, MO, USA) were the enzyme sources used, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma, St. Louis, MO, USA) were employed as the substrates of the reaction. 5,5'-Dithio-bis(2-nitrobenzoic)acid (DTNB; Sigma, St. Louis, MO, USA) was used for the measurement of the anticholinesterase activity. All reagents and conditions were same as described in our previous publication (Sevim et al., 2013). Galanthamine (Sigma, St. Louis, MO, USA), the anticholinesterase alkaloid-type of drug obtained from the bulbs of *Galanthus* sp., was used as the reference. The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software (Sunnyvale, CA, USA). Experiments were run in triplicate and the results were expressed as average values with S.E.M.

2.4. Bioactivity-guided fractionation

The dried methanolic extract (85 g) was diluted with distilled water-methanol mixture (50:450) and extracted with hexane (D18RM-H). After hexane extraction, the residue has extracted with dichloromethane (D18RM-D), ethylacetate (D18RM-E), *n*-butanol (D18RM-B) and water (D18RM-W).

2.5. Column chromotography

The active dichloromethane extract (D18RM-D) was subjected on column chromatography using silica gel 60 (63-200 µm, Merck) and dichloromethane-methanol to obtain six main fractions (N1-N6). Among them, sub-fraction (N5) was applied on flash chromatography (Combi flash EZ prep) on normal phase silica gel (Silica 12 g gold) collected five sub-fractions (DS-1-DS-5).

2.6. LC-ESI-Q/TOF-MS-MS analysis

The LC-UV equipment used was an Agilent 1260 system with vacuum degasser, binary pump, autosampler, thermostated column compartment and ultraviolet detector (Agilent Corporation, Palo Alto, CA, USA). The chromatographic separation was performed at 40°C on an Agilent Poroshell 120 SB-C18 column (4,6 mm x 150 mm x 2,7 µm) with a flow rate of 0.6 mL/min and the injection volume was 5 µl. The mobile phase consisted of 0.1 % formic acid (A) and acetonitrile (B), the gradient program was optimized as follows: 0-2 min, 5% B; 2-5 min, 20% B; 5-15 min, 50% B; 15-17 min, 50% B; 17-22 min 95% B; 22-26 min, 95% B, 26-32 min, 5% B. Samples were detected at 254 nm.

LC-ESI-Q/TOF-MS-MS analyses was performed by an Agilent series 1260 Infinity instrument coupled with an Agilent 6550 iFunnel Q/TOF mass spectrometer (Agilent Corporation, Palo Alto, CA, USA) equipped with an ESI ion source as interface. The mobile phase consisted of 0.1% formic acid-water and acetonitrile was used. The gradient program and detection wavelength were the same with LC-UV detection system. The mass spectra were acquired across the range of m/z 100-1000 (for MS) and m/z 50-500 (for MS-MS) in positive mode. The operating parameters of mass spectrometer were as follows: drying gas flow rate 14 L/min; drying gas temperature, 290°C, nebulizer, 40 psi; capillary voltage, 3500 V; fragment voltage, 400 V; skimmer voltage, 65 V and Oct RFV, 750 V. The collision energy was set at 10, 20 and 40 V. All MS data were controlled by MassHunter software B.06.01 (Data Acquisition) and B.07.00 (Qualitative Analysis).

3. Results

3.1. Cholinesterase inhibition results

Although none of the extracts, sub-extracts and fractions had significant activity against AChE, methanol extract (D18RM) had shown moderate BChE inhibitory activity (53.06 ± 1.13 % at 200 µg/mL) against the standard alkaloid galanthamine. The BChE inhibitions of the sub-extracts obtained from methanolic extract by using liquid-liquid chromatography were given in Table 1.

Table 1. BChE inhibitory effects of the sub-extracts of *Iris pseudacorus*

Sub-extracts	Codes	BChE Inhibition (Inhibition % ± S.D*) 200 µg/mL
n-Hexane	D18RM-H	23.48 ± 1.97
Dichloromethane	D18RM-D	**73.65 ± 2.06**
Ethylacetate	D18RM-E	49.78 ± 2.85
n-Butanol	D18RM-B	19.71 ± 0.54
Water	D18RM-W	41.26 ± 2.67
Galanthamine		80.02 ± 0.12

* Values are expressed as mean ± S.D are three parallel assays

The highest BChE inhibitory activity had determined in dichloromethane sub-extract. Therefore, this sub-fraction (D18RM-D) was subjected to column chromatography to obtain 120 fractions, after the combination of similar fractions, 6 main fractions coded as N1-N6 were collected (Table 2).

Table 2. The main fractions of the dichloromethane (D18RM-D) sub-extract *Iris pseudacorus*

Solvent systems	Fractions
Dichloromethane (%100)	(N1) Fractions 1-5
Dichloromethane : Methanol (99:1)	(N2) Fractions 6-20
Dichloromethane : Methanol (90:10)	(N3) Fractions 21-50
Dichloromethane : Methanol (85:15)	(N4) Fractions 51-58
Dichloromethane : Methanol (80:20)	(N5) Fractions 59-73
Dichloromethane : Methanol (60:40) Methanol (%100)	(N6) Fractions 74-120

The BChE inhibitory activities of these main fractions were presented in Table 3.

Table 3. BChE inhibitory effects of the sub-extracts of *Iris pseudacorus*

Fractions	BChE Inhibition (Inhibition % ± S.D*) 200 µg/mL
D18RM (Methanol extract)	53.06 ± 1.13
D18RM-D (Dichloromethane sub-extract)	**73.65 ± 2.06**
N1	10.35 ± 0.01
N2	67.66 ± 0.48
N3	52.26 ± 2.64
N4	56.84 ± 1.52
N5	**93.78 ± 1.49**
N6	77.15 ± 3.12
Galanthamine	80.02 ± 0.12

* Values are expressed as mean ± S.D are three parallel assays

The main fraction coded as N-5 exerted the highest BChE inhibition (93.78 ± 1.49) which was comparable to that of the standard (galanthamine). After the fractionation of N-5 by using flash chromatography, five sub-fractions (DS-1, DS-2, DS-3, DS-4 and DS-5) were isolated and the sub-fraction DS-5 was shown the highest BChE inhibition (94.00 ± 1.03) which had a closer value to that of the main fraction N-5. The BChE inhibitory activities of these fractions alongwith the sub-fractions were given in Table 4.

Table 4. BChE inhibitory effects of the sub-fractions isolated from the main fraction of N-5

Extract, sub-extract, main fraction and sub-fractions	BChE Inhibition (Inhibition % ± S.D*) 200 µg/mL
DS-1	20.87 ± 1.95
DS-2	19.29 ± 3.39
DS-3	22.47 ± 3.40
DS-4	20.85 ± 4.91
DS-5	**94.00 ± 1.03**
Galanthamine	80.02 ± 0.12

* Values are expressed as mean ± S.D are three parallel assays

4. Discussion

The responsible BChE inhibitory active compound of *Iris pseudacorus* L., which obtained from sub-fraction coded as DS-5, was analyzed by liquid chromatography-electron spray ionization-quadrupole/time-of-flight-mass spectrometry-mass spectrometry (LC-ESI-Q/TOF-MS-MS) and molecular ions at $[M+1]^+$ 477 and $[M+1]^+$ 315 were indicated (Spectrum 1 and 2). According to mass fragmentation pattern of $[M+1]^+$ 315; 300, 282 and 269 ions were obtained and the comparison of the data given in literature (Schütz et al., 2011, Xie et al., 2014, Bhat et al., 2014), this ion was determined as 'Irisolidone' (Fig. 1). On the other hand, the mass fragmentation pattern of $[M+1]^+$ 477 were given 315 and 163 ions belonged to irisolidone and glucose. These findings were also confirmed with the data given in previous studies (Schütz et al., 2011, Xie et al., 2014, Bhat et al., 2014) and established as "Irisolidone glucopyranoside".

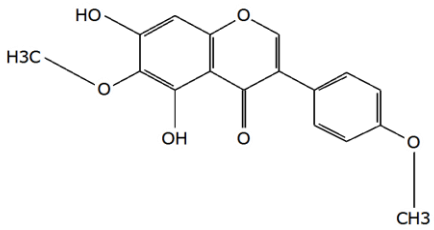
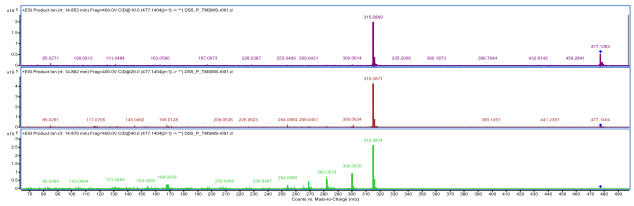
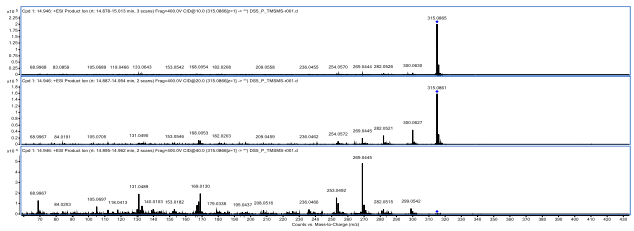


Figure 1. Irisolidone



Spectrum 1. Mass fragmentation of $[M+1]^+$ 477 (irisolidone glucopyranoside)



Spectrum 2. Mass fragmentation of $[M+1]^+$ 315 (irisolidone)

5. Conclusions

Discoveries of lead compounds for the development of new drug candidates from bioresources can help to promote incentives for conservation by providing an economic return to innovative use of those sources. Screening of natural sources has had an impressive tool of determining active agents. The key to successfully discovering therapeutic agents from bioresources is based on bioassay-directed isolation techniques. HTS tests and mechanism-based screening protocols as well as information of folkloric utilization of plants have led to the discovery of lead compounds as drug candidates.

In this study, *Iris* L. species belonged to ornamental geophytes growing in Turkey were investigated for their *in vitro* cholinesterase inhibitory effects and antioxidant capacities. The dichloromethane and methanol extracts prepared from the bulbs of 47 *Iris* species were screened by using modified Ellmann method and the highest butyrylcholinesterase inhibitory effect was found in the dichloromethane extract of the bulbs of *I. pseudacorus* L.

(Sevim, 2018). According to bioassay-guided fractionation procedure, the active dichloromethane extract was subjected to fractionation on column and flash chromatographies and the activities of the fractions were tested. The determined active fraction was analyzed by using LC-ESI-Q/TOF-MS-MS technique. The responsible compound from the activity of this fraction was detected as irisolidone glucopyranoside as an isoflavonoid derivatives based on their mass data by comparison with the mass fragmentation pattern of irisolidon glucopyranoside given in the literature (Schütz, 2011). Further studies should be performed to determine *in vivo* studies.

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