

Chemical Composition and Biological Activity of *Ixiolirion tataricum* (Pall.) Schult. & Schult.f. var. *tataricum*.

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Abstract: The role of plants in human life and the importance of plants in the food, drug and cosmetics industry have been increasing nowadays. This study investigates biological and chemical properties of root, branch, leaf, flower and aerial ethanol extracts of *Ixiolirion tataricum* (Pall.) Schult. & Schult. f. var. *tataricum* species, consumed as food in the population. The chemical composition of the species has been determined by the LC-MS/MS by specifying total phenolic and flavonoid contents. Antioxidant activity, cholinesterase, urease, tyrosinase, elastase and collagenase inhibitory activities have also been studied. According to the results of LC-MS/MS, quinic acid, protocatechuic acid, *p*-coumaric acid, and rutin have been detected. The branch ethanol extract contained higher amounts of quinic acid (12.101 µg analyte/g extract) than extracts of leaves, flowers, roots and aerial parts. On the other hand, the leaves ethanol extract exhibited high antioxidant activity in all tests (ABTS: IC₅₀= 57.47±0.86 µg/mL, CUPRAC: A_{0.5}= 66.76±1.86, DPPH: IC₅₀: 119.2±1.52). The species has the potential to be used in the food industry due to its high antioxidant capacity.

Keywords: *Ixiolirion tataricum*; LC-MS; antioxidant; enzyme activities; collagenase; elastase. © 2021 ACG Publications. All rights reserved.

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1. Introduction

The Amaryllidaceae family has 79 genus and about 1000 species composed of monocotyledon, flowering, bulbous plants [1]. *Ixiolirion* genus belonging to the Amaryllidaceae family has three perennial plant species spread in northeast Africa, middle and southwest Asia, and Egypt [2]. *Ixiolirion tataricum* (Pallas) Herbert subsp. *montanum* (Labill.) Takht. The species has been entitled the 'spring flower' in Anatolia. It has been used to strengthen memory, and it is used as an astringent in the population [3,4].

Plants of the Amaryllidaceae family produce unique alkaloids with many physiologic effects, including antitumoral, antiviral, cytotoxic, anticholinesterase, immunostimulatory, anti-inflammatory analgesic and DNA binding activities, and some of which are used in the treatment of Alzheimer disease [5]. So far, there has been no study investigating the chemical composition and biological activities of *I. tataricum* species except for its volatile compounds. Ghalandarnejad et al. (2014) reported that the volatile oil of the aerial parts of the species extracted by hydrodistillation afforded 35 compounds. Main components are 2-phenylethyl phenylacetate (16.8%), β -selinen (14.8%), bicyclovetivenol (4.8%), thymol (4.3%) and (*E*)-chalcone (4.3%) [6]. In a former study published in 1988, 30 compounds, including α -thujene, β -phellandrene, 3-carene, β -pinene, cumaldehyde, safranal, dodecyl alcohol, tetracosan, pentacosane, hexacosane, heptacosane and octacosane were reported as petroleum ether extract of *I. tataricum* [6,7].

Herein, leaves, branches, roots, flowers and all aerial parts of *I. tataricum* (Pall.) Schult. & Schult.f. var. *tataricum* were dried under the shadow to prepare their ethanol extracts. The total phenolic and flavonoid composition of the species was identified, and the amounts of secondary metabolites were determined by LC-MS/MS. In addition, the antioxidant activity by ABTS, CUPRAC, and DPPH assays, and acetylcholinesterase, butyrylcholinesterase, urease, tyrosinase, elastase and collagenase inhibitory activities were also reported herein.

2. Materials and Methods

2.1. Plant Material

Roots, leaves, branches, flowers and all aerial parts of the *Ixiolirion tataricum* (Pall.) Schult. & Schult.f. var. *tataricum* species were collected and identified by Mehmet Firat according to the Flora of Turkey, on April 2015 in Mardin [8]. Herbarium sample was kept in the herbarium of Van Yüzüncü Yıl University Science Faculty via M. Firat 32330 (VANF) herbarium number. The current valid name of the plant was also checked from the International Plant Names Index [9].

2.2. Extraction and LC-MS/MS Analyses

The roots, leaves, branches, flowers and aerial parts of the plants were dried under the shadow. Each studying sample was macerated with ethanol (3x8h). Briefly, 15 grams of each were weighted and extracted with 99% ethanol (1/5w/v). After filtration and solvent evaporation, crude extracts were obtained.

The phytochemical quantitation of the studied extracts was performed by a previously developed and validated method [10]. The method validation parameters contains; inter-day and intra-day precision (repeatability), accuracy (recovery), linearity, limits of detection and quantification (LOD/LOQ), and relative standard uncertainty (U% at 95 % confidence level ($k = 2$)). Furthermore, to increase the credibility of the results by compensating for the matrix effects and analyte losses during the sample preparation and analyses, quercetin D3, rutin D3, and ferulic acid D3 were used as the deuterated internal standards for flavonoids, flavonoid glycosides and non-flavonoid compounds, respectively.

The LC-MS/MS system used for LC-MS/MS analysis of the plant extracts was composed of Shimadzu Neexera model UHPLC device and Shimadzu LC-MS 8040 model triple quadrupole mass spectrometer device. The liquid chromatography system was composed of an LC-30 AD model gradient pump, DGU-20A3R model degasser, CTO-10ASvp model column oven and SIL-30AC model auto-sampler. Chromatographic separation was performed with the Inertsil ODS-4 model C18 (100 mm×2,1

mm, 2 μ m) column. The extract was diluted to 250 mg/L, and after filtration by the 0.2 μ m injector nozzle, the samples were located into the device [10]. The mass spectrometric detection was carried out using a Shimadzu LCMS-8040 model tandem mass spectrometer equipped with an electrospray ionization (ESI) source operating in both negative and positive ionization modes. LC-ESI-MS/MS data were acquired and processed by LabSolutions software (Shimadzu). The MRM (multiple reaction monitoring) mode was used for the quantification of the phytochemicals. The MRM method was optimized to selectively detect and quantify phytochemical compounds based on the screening of specified precursor phytochemical-to-fragment ion transitions. The collision energies (CE) were optimized in order to generate optimal phytochemical fragmentation and maximal transmission of the desired product ions. The MS operating conditions were applied as: drying gas (N₂) flow, 15 L/min; nebulizing gas (N₂) flow, 3 L/min; DL temperature, 250 °C; heat block temperature, 400 °C, and interface temperature, 350 °C [10].

2.3. Total Flavonoid-Phenolic Content

Total phenolic contents of the extracts were determined as pyrocatechol equivalent by using Folin-Ciocalteu reactive [11]. After preparing 100 ppm pyrocatechol solution, 0, 1, 2, 3, 4, 5, 6, 7 ve 8 μ L were taken, and they were added distillate water to complete their volumes to 184 μ L. Ethanol extracts of the plant samples were prepared as 1000 ppm concentrate solutions. 4 μ L was taken from sample solutions with one-milligram extract and completed to 184 μ L with distillate water. 4 μ L Folin-Ciocalteu reactive (FCR) was added to pyrocatechol solutions and sample solutions, after 3 minutes 12 μ L of 2% Na₂CO₃ solution was also added. The final solutions were kept at room temperature for 2 hours, and the absorbance of the solution was measured at 760 nm. Total phenolic contents of the extracts were determined by pyrocatechol equivalents from the standard pyrocatechol graphic.

Total flavonoid contents of the extracts were determined as quercetin equivalent with the aluminium nitrate method [12]. After preparation of 1000 ppm quercetin solution, 0, 1, 2, 3, 4, 5, 6, 7 ve 8 μ L were taken and completed to 192 μ L with 80% ethanol. Four microliters of potassium acetate of 1M was added, and 1 minute later, four microliters of 10% aluminium nitrate were also added. After 40 minutes of incubation, the absorbances of solutions was measured at 415 nm by a microplate reader. The absorbance of solutions prepared from ethanol extract of samples at a fixed concentration (1000 ppm) was also read by the same method. Total flavonoid contents of the extracts were determined as quercetin equivalents using the standard quercetin graphic.

$$\text{Absorbance} = 0.0396 + 0.0217 \text{ pyrocatechol } (\mu\text{g}) \quad (r^2 = 0.9956)$$

$$\text{Absorbance} = 0.0374 + 0.0456 \text{ quercetin } (\mu\text{g}) \quad (r^2 = 0.9965)$$

2.4. Antioxidant Activity

Antioxidant activities of sample extracts were determined by DPPH free radical scavenging, ABTS cation radical scavenging and CUPRAC (cupric-reducing antioxidant capacity).

2.4.1. DPPH (1,1-Diphenyl-2-Picrylhydrazil) Free Radical Scavenging Method

Free radical scavenging activities of extracts were determined by using 1,1-diphenyl-2-picrylhydrazil free radical [13]. Stock solutions were prepared by dissolving 10 mg of the ethanol extracts of the samples in 10 ml of ethanol. These stock solutions were diluted by taking 2,5,10 and 20 μ L and completed to 40 μ L by ethanol, then 160 μ L 0.1 mM DPPH solution was added to these four solutions. Absorbances of prepared solutions was measured at 517 nm after incubating the process at room temperature in the dark for 30 minutes. Inhibition (%) values were calculated from these absorbance values. The absorbance values of the samples were compared with the control [14].

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2.4.2. ABTS Cation Radical Scavenging Activity

ABTS cation radical scavenging activities of extracts were determined by using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) [15].

Stock solutions were prepared by dissolving 10 mg of the ethanol extracts of the samples in 10 ml of ethanol. These stock solutions were diluted by taking 2,5,10 and 20 μL and completed to 40 μL by ethanol, then 160 μL 7 mM ABTS cation radical solution was added to these four solutions. Absorbances of prepared solutions was measured at 734 nm after waiting for 6 minutes in the dark. The absorbance values of the samples were compared with the control.

2.4.3. CUPRAC (Cupric-reducing Antioxidant Capacity) Method

In the CUPRAC method, in the presence of antioxidant compounds in the samples, the Cu(II)-Neocuproin (Nc) complex is reduced to coloured Cu(I)-Nc chelate and the absorbance of this chelate is measured at 450 nm. Cu (II), neocuproin and NH_4OAc buffer were added to the prepared samples and standards as the final concentrations 10, 25, 50, 100 $\mu\text{g}/\text{mL}$. One hour later, absorbances were measured at 450 nm [16]. The absorbance values of the samples were compared with the control. Three parallel studies were performed in all three methods. BHT and $\alpha\text{-Toc}$ were used as positive standards.

2.5. Anticholinesterase Activity

Acetylcholinesterase (AChE) and butyrylcholinesterase inhibitory activities were measured based on the spectrophotometric method reported by Ellman et al. [17]. Acetylcholinesterase from electric fish and butyrylcholinesterase from horse serum were used as enzymes. Acetylcholine iodide and butyrylcholin iodide were acted as substrates, while yellow 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was used to measure activity. To each well of 96-well microplates 130 μL 0,1 M pH=8 phosphate buffer, 10 μL sample, and 20 μL AChE or BChE enzymes were added and incubated at 25 $^\circ\text{C}$ for 10 minutes. Finally, 20 μL DTNB solution and 20 μL acetylcholine iodide or butyrylcholin iodide were added, respectively. The yellow 5-thio-2-nitrobenzoic acid formed as a result of the reaction of thiocholin, released by the enzymatic hydrolysis of acetylcholine iodide or butyrylcholin iodide, and DTNB was monitored spectrophotometrically at 412 nm wavelength. Ethanol was used as control, and galanthamine was used positive standard to compare the activity [18].

2.6. Urease Inhibitory Activity

For the urease inhibition activity, urease was used as an enzyme. Ten microliters of sample solution prepared at 4000 ppm concentration in ethanol, 25 μL of enzyme solution in buffer, and 50 μL of urea as substrate were added to the microplate wells. The first absorbance was first was read at 630 nm. Then this solution was incubated at 30 $^\circ\text{C}$ for 15 minutes. After 15 minutes, 45 μL phenol reagent and 0 μL alkaline reagent were added. It was incubated at 30 $^\circ\text{C}$ again, and after 20 minutes, the microplate was placed in an ELISA reader, and the final absorbance was read at 630 nm wavelength. Thiourea was used as a positive standard [19,20].

2.7. Anti-aging Inhibitory Activity

Tyrosinase, elastase and collagenase inhibitory activity methods were used to determine the anti-ageing potential of the extracts. The technique improved by Hearing and Jimenez [21] was used with slight changes for the tyrosinase inhibitory activity. Kojic acid was used as a positive standard. For the elastase inhibitory activity, the reported method by Kraunsoe et al. (1996) [22] was applied to the extracts with slight changes. Oleanolic acid was used as a positive standard. For the collagenase inhibitory activity, however, the protocol improved by Thring et al. (2009) [23] was applied to extracts with slight modifications. Epicatechin gallate was used as a positive standard herein [24,25].

The following equation was used to calculate the inhibitory activities of acetylcholinesterase (AChE), butyrylcholinesterase (BChE), urease, tyrosinase, elastase, and collagenase. In all enzyme inhibitory assays, percentage (%) values were calculated at 100 $\mu\text{g}/\text{mL}$ of three parallel measurements

$$\text{Enzyme inhibition (\%)} = 100 - (\text{OD test well} / \text{OD control}) \times 100$$

3. Results and Discussion

Root, leaf, branch, flower and aerial parts of *I. tataricum* species extract's phenolic composition was determined by LC-MS/MS. According to the LC-MS/MS results, quinic acid, protocatechuic acid, *p*-coumaric acid and rutin content of this species were determined. According to the LC-MS/MS results, the branches ethanol extract contains the highest amount of quinic acid than the all aerial parts, roots, leaves and flowers (12.101, 5.809, 3.642, 3.421, 7.452 μg analyte/g extract, respectively) extracts. Additionally, while protocatechuic acid was not found in this species root and branch extracts, the leaf, flower and aerial parts extracts (0.229, 0.223, 0.14 μg analyte/g extract, respectively), existed. *p*-Coumaric acid was only detected and quantified in leaves and flowers (0.064, 0.189 μg , respectively). Rutin was only determined in flowers extract (0.299 μg analyte/g extract) (Table 1).

Total phenolic and flavonoid contents of the ethanol extracts of the species were determined to be equivalent to pyrocatechol and quercetin, respectively (Table 2). The results determined that phenolic and flavonoid contents of the leaves ethanol extract were higher than the other parts of the plant.

According to the antioxidant activity of ethanol extracts via three complementary assays. The leaves extract exhibited very high antioxidant activity (ABTS: $\text{IC}_{50} = 57.47 \pm 0.86$, CUPRAC: $A_{0.5} = 66.76 \pm 1.86$, DPPH: $\text{IC}_{50} = 119.20 \pm 1.52$ $\mu\text{g}/\text{mL}$) when compared to the other part extracts. In the studied enzyme inhibition experiments, it has been determined that *I. tataricum* showed low enzyme inhibition activity (Table 2).

Only two studies exist about the chemical and biological activity studies of *I. tataricum* species in the literature. These two studies are about only the essential oil content of the species. Likewise, there are no studies in the literature except ethnobotanical and morphological studies of other species of the *Ixiolirion* genus [3,26].

Table 1. Identification and quantification of phenolic compounds of ethanol extracts of *I. tataricum* by LC-MS/MS

No	Analytes	Parent ion(m/z) ^a	MS ² (Collision Energy) ^b	Quantification (μg analyte/g extract) ^c				
				IT-B	IT-M	IT-R	IT-L	IT-F
1	Quinic acid	190.8	93.0	12,101	5,809	3,642	3,421	7,452
24	<i>p</i> -Coumaric acid	163.0	93.0	N.D ^d	N.D	N.D	0,064	0,189
33	Rutin	608.9	301.0	N.D.	N.D.	N.D.	N.D.	0,299
35	Hesperidin	611.2	449.0	N.D	N.D	N.D	N.D	0,29
48	Naringenin	270.9	119.0	N.D	N.D	N.D	0,019	N.D
50	Luteolin	284.8	151.0/175.0	N.D	0,055	N.D	0,027	0,005
51	Genistein	269.0	135.0	N.D	N.D	N.D	N.D	N.D
52	Kaempferol	285.0	239.0	N.D	0,013	0,007	0,012	0,013
53	Apigenin	268.8	151.0/149.0	0,004	0,007	0,002	0,018	0,004

^aParent ion (*m/z*): Molecular ions of the standard compounds (mass to charge ratio), ^bMS²(CE): MRM fragments for the related molecular ions (CE refers to related collision energies of the fragment ions), ^cValues in $\mu\text{g}/\text{g}$ (w/w) of plant ethanol extract, ^dN.D: not detected.

Undetected components, 2: Fumaric acid. 3: Aconitic acid. 4: Gallic acid. 5: Epigallocatechin. 6: Protocatechuic acid. 7: Catechin. 8: Gentisic acid. 9: Chlorogenic acid. 10: 10 Protocatechuic aldehyde. 11: Tannic acid. 12: Epigallocatechin gallate. 13: 1,5-Dicaffeoylquinic acid. 14: 4-OH Benzoic acid. 15: Epicatechin. 16: Vanilic acid. 17: Caffeic acid. 18: Syringic acid. 19: Vanillin. 20: Syringic aldehyde 21: Daidzin 22: Epicatechin gallate. 23: Piceid. 25: Ferulic acid-D3-IS. 26: Ferulic acid. 27: Sinapic acid. 28: Coumarin. 29: Salicylic acid. 30: Cynaroside. 31: Miquelianin. 32: Rutin-D3-IS. 34: isoquercitrin. 36: *o*- Coumaric acid. 37: Genistin. 38: Rosmarinic acid. 39: Ellagic acid. 40: Cosmosiin. 41: Quercitrin. 42: Astragalin. 43: Nicotiflorin. 44: Fisetin. 45: Daidzein. 46: Quercetin-D3-IS: 47: Quercetin. 49: Hesperetin. 54: Amentoflavone. 55: Chrysin. 56: Acacetin.

Table 2. Total phenolic-flavonoid content, antioxidant and biological activities of the *I. tataricum* extracts ^a

Samples ^b	Total phenolic (μg PEs/mg) ^d	Total flavonoids (μg QEs/mg) ^e	Antioxidant activity			Enzyme inhibitory activity					
			IC ₅₀ ($\mu\text{g/mL}$) DPPH assay	IC ₅₀ ($\mu\text{g/mL}$) ABTS assay	(A _{0.5}) ($\mu\text{g/mL}$) CUPRAC assay	Inhibition (%) at 100 $\mu\text{g/mL}$					
			AChE assay	BChE assay	Urease assay	Tyrosinase assay	Elastase assay	Collagenase assay			
IT-R	10.05±0.35	2.58±0.72	>1000	240.1±1.35	217.3±1.24	NA	NA	NA	NA	4.21±0.68	NA
IT-B	14.55±1.29	3.47±0.63	509.1±4.21	71.42±1.58	135.1±1.55	NA	NA	NA	NA	3.53±0.36	4.08±0.24
IT-L	52.47±1.96	16.84±1.31	119.2±1.52	57.47±0.86	66.76±1.86	NA	NA	NA	NA	NA	2.46±0.19
IT-F	18.25±1.06	4.54±0.44	166.6±1.39	62.50±1.19	87.71±0.95	NA	NA	NA	9.42±1.08	NA	6.81±1.23
IT-M	23.68±1.60	7.92±1.39	160.2±1.61	60.12±0.67	106.8±1.95	NA	NA	NA	1.31±0.82	NA	NA
BHT ^c	-	-	54.12±1.33	13.77±0.13	8.24±0.09	-	-	-	-	-	-
α -TOC ^c	-	-	16.43±0.24	9.88±0.34	18.27±0.67	-	-	-	-	-	-
Gаланthamine ^c	-	-	-	-	-	94.46±1.22	77.21±0.48	-	-	-	-
Thiourea ^c	-	-	-	-	-	-	-	99.73±0.13	-	-	-
Kojic acid ^c	-	-	-	-	-	-	-	-	74.26±1.25	-	-
Oleanolic acid ^c	-	-	-	-	-	-	-	-	-	60.28±0.50	-
Epicatechin gallate ^c	-	-	-	-	-	-	-	-	-	-	71.77±1.22

^a Values expressed are means \pm S.D. of three parallel measurements, and values were calculated according to the negative control.

^b The abbreviations for ethanol extracts (roots, branches, leaves, flowers and aerial parts) of the *I. tataricum* are expressed as IT-R, IT-B, IT-L, IT-F and IT-M, respectively.

^c Standard compound

^d PEs: pyrocatechol equivalents ($y=0.0396x + 0.0217$, $r^2=0.9956$), ^e QEs: quercetin equivalents ($y=0.0374x + 0.0456$ $r^2=0.9965$).

NA: Not active

AChE: acetylcholinesterase; BChE: butyrylcholinesterase;

3. Conclusion

The chemical composition of ethanol extracts of *I. tataricum* species has been determined by LC-MS/MS. The total phenolic and flavonoid amount, antioxidant capacity and biological activities of the samples of the species have been investigated for the first time in this study. It has been determined that leaf extract shows high antioxidant features so that the species can be considered in the food industry as a food preservative. However, further studies especially, cytotoxic and *in vivo* studies, should be performed to concise the commentary.

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