Bulgarian Chemical Communications, Volume 52, Special Issue D (pp. 168-173) 2020

Phenolic content and antioxidant capacity of *Inula britannica* from different habitats in Bulgaria

V. Ivanova[1], M. Todorova[1], M. Rangelov[1], I. Aneva[2], A. Trendafilova[1,]

[1]*Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl.9, 1113 Sofia, Bulgaria*
[2]*Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 2 Gagarin Str., 1113 Sofia, Bulgaria*

Received March 26, 2020; Revised April 25, 2020

The aim of this study was to evaluate the total phenolic and flavonoid contents and antioxidant capacity of the methanol extracts of 11 samples of *I. britannica* from different habitats and to identify the potent antioxidant compounds. The values of phenolics ranged from 85.35±7.64 to 141.01±4.97 mg GAE/g DE, while those of flavonoids – from 19.66±0.75 to 36.80±0.56 mg CE/g DE. Antioxidant capacity of the studied extracts measured by the DPPH method (0.229±0.015 to 0.620±0.001 mM TE/g DE) correlated well with the total phenolic and flavonoid content, while that determined by $ABTS^{\bullet+}$ assay (0.420±0.010 - 0.550±0.003 mM TE/g DE) showed a moderate correlation with total phenolic content only. HPLC analysis revealed that chlorogenic (5-CQA), 1,5- and 3,5-dicafeoylquinic (DCQA) acids were the major components in all samples, while the amounts of the other two positional isomers 4,5-, and 3,4-DCQA were significantly lower. Principal component analysis (PCA) was used to investigate the variations in the chemical content within *I. britannica* populations.

Keywords: *Inula britannica*; Asteraceae; phenolics; flavonoids; DPPH and ABTS assays; caffeoylquinic acids

INTRODUCTION

Inula britannica L. is widely distributed in Western Europe and Turkey, extending eastward to China through Iran and Pakistan [1, 2]. The plant is an important plant species used in Traditional Chinese Medicine (TCM) and Kampo Medicines as antibacterial, carminative, diuretic, laxative, stomach, tonic remedies, and for treating asthma, hepatitis and tumours [3]. *I. britannica* L. has shown to possess various biological activities – anti-inflammatory, antitumor, antibacterial, antitussive, antiproliferative, antioxidant, hepatoprotective, etc., which were attributed to the abundance of bioactive components mainly sesquiterpene lactones, phenolic acids, and flavonoids [3-5]. As a part of the ongoing project, we have recently studied *I. britannica* L. growing in Bulgaria and five sesquiterpene lactones, three triterpenoids, three flavonoids, and 1,5-dicaffeoylquinic acid were isolated and identified by spectral methods [6]. Considering the use of the species as herbal medicine, we have decided to expand the investigation on *I. britannica* from different populations in Bulgaria to investigate the qualitative and quantitative differences in the main constituents and their impact on biological activity. In this study, the results of the comparative study of the phenolic constituents and antioxidant capacity of *I. britannica* from 11 natural habitats in Bulgaria were described.

EXPERIMENTAL

Plant material

Aerial parts from wild growing *I. britannica* were collected in full flowering stage in July 2018 from 11 different natural habitats in Bulgaria: **1** – Berkovitsa (43°15'1.75"N 23° 9'30.78"E), **2** - Gavril Genovo village (43°23'54.35"N 23° 3'43.08"E), **3** – G. Brestniza village (42°16'49.0"N 22°37'33.4"E), **4** - Vetren village (44°08'24.6"N 27°01'49.7"E), **5** - Gabrovo village (41°53'50.45"N 22°56'36.22"E), **6** - Aglen village (43°12'56.1"N 24°19'38.5"E), **7** - Chervenata stena, Rhodopes Mts (41°51'52.34"N 24°56'38.09"E), **8** - Kraishte village (41°54'23.93"N 23°35'11.98"E), **9** - Belimel village (43°25'46.47"N 22°57'36.06"E), **10** - Brezhani village (41°52'4.69"N 23°12'2.70"E), 1**1** - Slavyanka Mts. (41°24'31.54"N 23°33'51.34"E). The plant was identified by Dr. Ina Aneva (Institute of Biodiversity and Ecosystem Research, BAS, Sofia).

Extraction of plant material

Air-dried and powdered plant material (1 g) was initially defatted by extraction with chloroform (20 mL, 3 times) followed by extraction with CH_3OH (20 mL, in triplicate).

* To whom all correspondence should be sent:
E-mail: trendaf@orgchm.bas.bg

Each extraction was performed for 12 hours at room temperature. After filtration, the solvent was evaporated under vacuum and the resulting methanol extracts were used for HPLC analysis, determination of total phenolic and flavonoid content, as well as for antioxidant activity assays.

Determination of total phenolic content

Total phenolic content (TPC) was measured using Folin–Ciocalteu method [7]. The concentration was calculated using gallic acid as a standard and the results were expressed as milligrams (mg) gallic acid equivalents (GAE) per 1 g of dry extract (mg GAE/g DE).

Determination of total flavonoid content

The total flavonoid content (TFC) was measured using a previously developed colorimetric assay [8]. The concentration was calculated using a calibration curve of (+)-catechin (in the range of 2 μg/ml to 80 μg/ml). The result was expressed as milligrams of catechin equivalent per gram of dry plant extract (mg CE/g DE).

Determination of DPPH radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity assay was performed according to the procedure described by Thaipong *et al*. [9]. The antioxidant activity was expressed as mM Trolox equivalents per gram dry extract (mM TE/g DE), using a calibration curve of Trolox dissolved in methanol at different concentrations (0.1- 0.5 mM).

Determination of $ABTS^{\bullet+}$ scavenging activity

The procedure was previously described by Re *et al.* [10]. Results were expressed as Trolox equivalents antioxidant capacity (mM Trolox equivalents per gram dry extract), using a calibration curve of different concentrations of Trolox in methanol (0.1-0.5 mM).

High-Performance Thin Layer Chromatography (HPTLC)

HPTLC analysis was done with pre-coated HPTLC glass plates (20 × 10 cm, Si G60 F_{254}, Merck) using a Camag HPTLC system (Switzerland). Toluene: ethyl acetate: formic acid: water (5:100:10:10 v/v/v/v) was used as a mobile phase. The chromatographic spots were visualized by UV light at 366 nm.

HPLC Analysis of mono- and dicaffeoyl esters of quinic acid

The HPLC equipment was a Waters HPLC system (Waters 2795) with a Waters binary pump, an auto-sampler, a column oven, and a Waters 2487 Dual wavelength absorbance detector. The LiChrospher 100 RP-18 column (5 μm, Merck) was used with a guard column that was filled with the same stationary phase. Solvent A (20% CH_3OH in H_2O) and solvent B (CH_3OH) were used as the mobile phase under gradient conditions (0 min, 0% A; 30 min, 0% A; 65 min, 20% A; 70 min, 0% A) to analyse the samples. The analysis was carried out at a flow rate of 0.8 mL/min. The detection wavelength was set at 327 nm and the sample injection volume was 10 μL. The peak identification was based on the retention time of the standard compounds (t_R) as follows: chlorogenic acid (7.1 min), 3,4-dicaffeoylquinic acid (49.5 min), 3,5-dicaffeoylquinic acid (53.0 min), 1,5-dicaffeoylquinic acid (56.4 min), and 4,5-dicaffeoylquinic acid (63.2 min). The correlation coefficients (R^2) were higher than 0.99 (five concentrations in three replicates each) and the relative standard deviations (% RSD) were < 5% confirming the linearity and repeatability of the method for each compound. All samples were run in triplicate and quantification was carried out using external standards. The content of each compound was calculated and expressed as mg/g of dry extract (DE).

Statistical analysis

All data were reported as means ± standard deviation (SD) using three independent measurements. Data were analysed using Student's t-test and differences were considered as significant at $p<0.05$. Analysis of variance with a confidence interval of 95% was performed using MS Excel software. Principal component analysis (PCA) was performed using the PAST 4.0 software.

RESULTS AND DISCUSSION

The total phenolic (TPC) and flavonoid (TFC) contents of the methanol extracts obtained from the aerial parts of eleven *I. britannica* native populations were analysed using spectrophotometric methods and were expressed as mg GAE/g DE and mg CE/g DE, respectively (Table 1). The values of phenolics varied from 85.35±7.64 mg GAE/g DE (sample **6**) to 141.01±4.97 mg GAE/g DE (sample **1**). The performed one-way ANOVA analysis indicated that the total phenolic contents in all 11 studied populations were significantly different at the 95%

confidence level. However, TPC in samples **2**, **4**, **5**, **7** and **10**, as well as those in samples **3, 7** and **9,** did not differ significantly from each other (p>0.05, t-test). Therefore, two main groups are formed according to the amount of phenolics: the first group (samples **1**, **2**, **4**, **5**, **10** and **11**) with TPC more than 110 mg GAE/g DE, and the second one (samples **3**, **6, 8** and **9**), in which TPC was less than 110 mg GAE/g DE. Sample **7** occupied an intermediate position.

Samples **1** and **11** contained approximately the same amount of flavonoids (35.72±0.42 and 36.80±0.56 mg CE/g DE, respectively) and were the richest in this type of compounds (Table 1). Sample **3** was the poorest in flavonoids and their amount was 19.66±0.75 mg CE/g DE only. Statistical analysis (one-way ANOVA) indicated that there were also significant differences (p<0.05) among populations. Nevertheless, TFC in samples **5**, **6**, **7** and **8** (> 30 mg CE/g DE), as well as in samples **2**, **4**, **9** and **10** (< 30 mg CE/g DE) were not significantly different from each other (p>0.05, t-test). Among the studied populations, the samples **1** and **11** were found to be the richest in both classes of compounds - phenolics and flavonoids.

The amounts of TPC and TFC in the 11 *I. britannica* samples were found to be higher from that in the flower methanol extract of *I. britannica* from another Bulgarian population (79.41 mg GAE/g DE and 19.94 mg CE/g DE, respectively) [6] and lower from that reported for the water extract of *I. britannica* var. *chinensis* (318.10 and 335.87 mg CE/g DE, respectively) of South Korean origin [11]. In another recent study, the water extract of *I. britannica* herb from South Korea was found to be richer in TPC than the ethanol extract (50.8 *vs* 42.1 mg GAE/g solid) [12]. On the contrary, flavonoids dominated in the ethanol extract (225.7 mg QE/g solid), while their quantity in the water extract was 51.6 mg QE/g solid only [12].

Table 1. Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant capacity (DPPH and ABTS assay) from *I. britannica* from different habitats in Bulgaria

Sample	TPC* [mg GAE/gDE]	TFC* [mg CE/gDE]	Antioxidant capacity* [mM TE/g DE]	
			DPPH	ABTS
1	141.01±4.97	35.72±0.42^{a}	0.620±0.010^{a}	0.550±0.003^{a}
2	115.84±3.03^{a}	25.47±0.81^{b}	0.365±0.005^{b}	0.501±0.004^{a,b}
3	97.93±2.79^{b}	19.66±0.75	0.229±0.015^{c}	0.420±0.013^{c}
4	118.95±5.44^{a,c}	24.34±0.41^{b,c}	0.350±0.007^{b,d}	0.430±0.004^{c,d}
5	120.02±6.14^{a,c,d}	30.66±0.44^{d}	0.430±0.009	0.430±0.009^{c,d,e}
6	85.35±7.64	32.89±0.22^{a,e}	0.340±0.033^{b,d,e}	0.440±0.011^{c,d,f}
7	101.73±10.40^{a,b,e,f}	33.05±1.29^{a,d,e,f}	0.380±0.024^{b,d,e,f}	0.420±0.021^{c,d,g}
8	104.47±4.89$^{b,e,f.g}$	31.73±1.21^{d,e,f}	0.354±0.031$^{b.d,e,f}$	0.462±0.010^{b}
9	100.80±4.78^{b,e,f,g}	25.12±3.42^{b,c,d,e,g}	0.240±0.021^{c}	0.430±0.007^{c,d,e,f,g}
10	114.14±2.07^{a,c,d,f}	27.11±1.02^{b,c,g}	0.300±0.010	0.540±0.008^{a,b}
11	126.24±5.89^{d}	36.80±0.56^{a}	0.600±0.020^{a}	0.490±0.007^{b}

*Values are means ± SD (n=3). Means in the columns with the same letter are not significantly different from each other (p>0.05) (t-test)

Further, DPPH and ABTS assays were used to estimate free radical scavenging properties of the studied extracts and the obtained results were expressed as mM Trolox equivalents per gram of dry extracts (mM TE/g DE (Table 1). The antioxidant capacity of the studied extracts measured by the DPPH method ranged from 0.229±0.015 to 0.620±0.001 mM TE/g DE. Samples **1** and **11** were the most active DPPH$^{\bullet}$ scavengers, while samples **3** and **9** showed the lowest values. A good correlation was observed between antioxidant capacity assessed with the DPPH test and TPC and TFC in the studied extracts (Pearson, r = 0.721 and 0.786, respectively). The antioxidant capacity determined by ABTS$^{\bullet+}$ assay showed values between 0.420±0.010 and

0.550±0.003 mM TE/g DE. The highest $ABTS^{\bullet+}$ scavenging activity was found for samples **1** and **10**, while the other samples exhibited activity in comparable values. The antioxidant capacity assessed with the ABTS test showed a moderate correlation with TPC (r = 0.652) and a weak correlation with TFC (r = 0.334). The different antioxidant activity levels obtained from both assays is probably due to the difference in the ability of antioxidant compounds in the extracts to quench ABTS and DPPH free radicals in *in vitro* systems. DPPH and ABTS activities of the studied *I. britannica* methanol extracts were comparable with those found for another Bulgarian sample (0.376 and 0. 403 mM TE/g DE) [6].

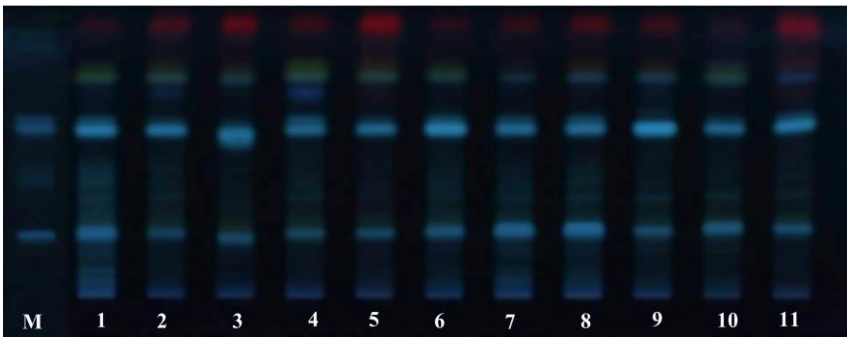
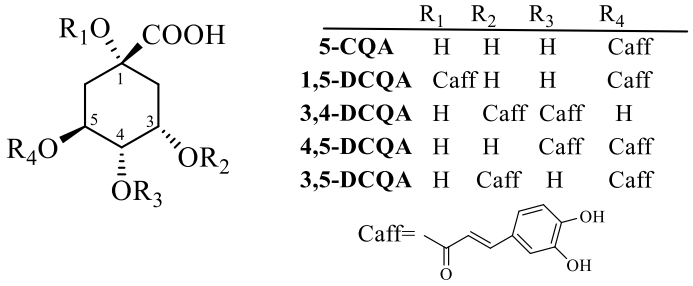


Fig. 1. HPTLC of a model mixture (**M**, R_f 0.23 (5-CQA), 0.42 (4,5-DCQA), 0.44 (3,4-DCQA), 0.58 (1,5-DCQA) and 0.62 (3,5-DCQA) and *I. britannica* methanol extracts (**1**-**11**)



	R_1	R_2	R_3	R_4
5-CQA	H	H	H	Caff
1,5-DCQA	Caff	H	H	Caff
3,4-DCQA	H	Caff	Caff	H
4,5-DCQA	H	H	Caff	Caff
3,5-DCQA	H	Caff	H	Caff

Fig. 2. Structures of mono- and dicaffeoyl esters of quinic acid

Table 2. Content of individual compounds in *I. britannica* methanol extracts [mg/g DE]

Sample	CQA*	3,4-DCQA*	3,5-DCQA*	1,5-DCQA*	4,5-DCQA*
1	23.72±0.82^{a}	3.96±0.14^{a}	18.68±0.27	62.76±1.31	8.37±0.24
2	18.02±0.25^{b}	4.75±0.17	11.52±0.16	48.51±1.01^{a}	5.63±0.17^{a}
3	20.20±0.69^{b}	3.68±0.13^{a}	7.09±0.10^{a}	40.46±0.85^{b}	3.32±0.10^{b}
4	14.99±0.54^{c}	5.23±0.19	14.21±0.20	30.75±0.64	5.81±0.16^{a}
5	24.69±1.34^{a,d}	0.83±0.03	22.07±0.32	47.28±0.99^{a}	7.56±0.22
6	20.57±0.28^{b}	0.17±0.01	7.54±0.11^{a,b}	39.13±0.82^{b,c}	3.21±0.10^{b,c}
7	26.37±0.91^{d,e}	1.86±0.07^{b}	7.04±0.10^{a,c}	27.63±0.58	2.61±0.07^{d}
8	27.68±1.50^{e,f}	1.44±0.05^{c}	7.65±0.11^{b}	31.33±0.65	2.80±0.08^{d,e}
9	16.02±0.55^{b,c}	1.73±0.06^{b}	5.78±0.08	54.33±1.14^{d}	3.30±0.10^{b,c}
10	27.36±0.19^{f}	1.31±0.05^{c}	6.84±0.10^{a,c}	37.88±0.79^{b,c}	2.70±0.08^{d,e}
11	51.41±0.72	2.44±0.09	8.80±0.13	53.02±1.11^{d}	2.28±0.07

*Values are means ± SD (n=3). Means in the columns with the same letter are not significantly different from each other (p>0.05) (t-test)

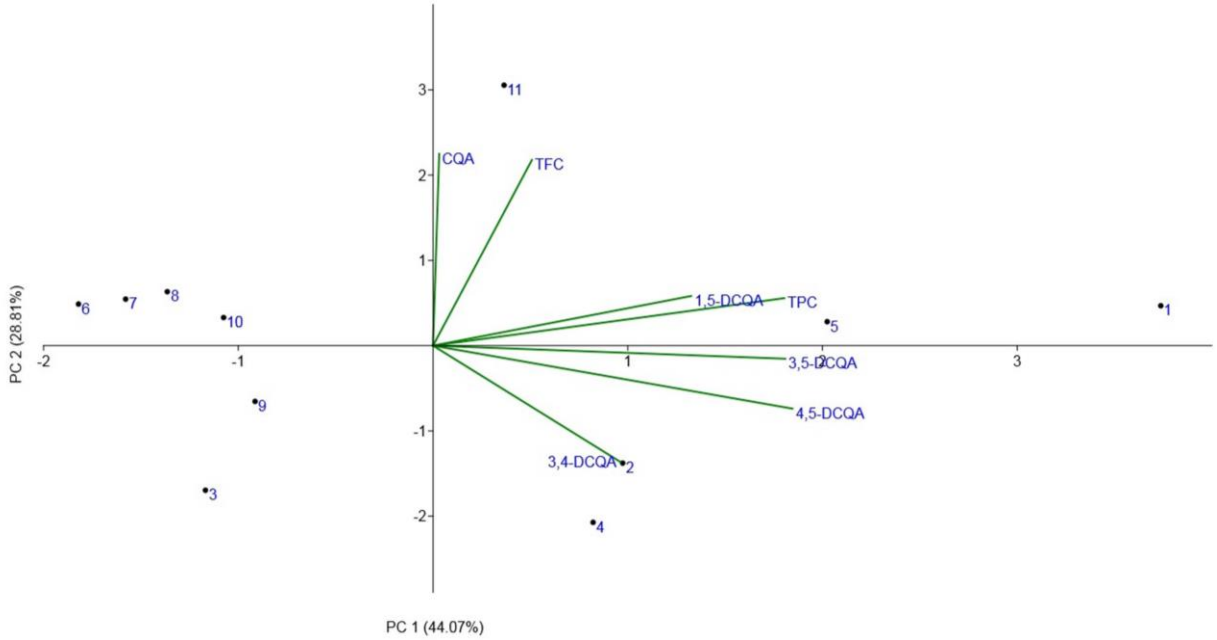


Fig. 3. Biplot (PCA) carried out on TPC, TFC and the content of individual acids of each sample of *I. britannica* (**1** – **11**)

It is difficult to compare the obtained results with those published for other *Inula* species, because of different methods of testing and presentation of the results.

Preliminary HPTLC analysis (Fig. 1) of the methanol extracts of *I. britannica* has shown that they contained chlorogenic (5-CQA) and dicaffeoyl esters of quinic acid (DCQA) (Fig. 2). They were recognized by the characteristic blue fluorescence at 366 nm in the presence of commercially available standards. As can be seen, chlorogenic (5-CQA) and 1,5-dicafeoylquinic (DCQA) acids were the major components in all samples, while the amount of the other three positional isomers 4,5- 3,5- and 3,4-DCQA was lower. This observation was further confirmed by HPLC analysis which was used for their quantitative determination. As can be seen from Table 2, the amount of 5-CQA varied from 14.99±0.54 (sample **4**) to 51.41±0.72 mg/g DE (sample **11**) and 1,5-DCQA (27.63±0.58-62.76±1.31 mg/g DE) was the most abundant dicaffeoyl ester of quinic acid followed by 3,5-DCQA (5.78±0.08 - 18.68±0.27 mg/g DE), 4,5-DCQA (2.28±0.07 - 8.37±0.24 mg/g DE) and 3,4-DCQA (0.17±0.01– 5.23±0.19 mg/g DE). Sample **1** was found to be the richest in 1,5- and 4,5-DCQA, sample **5** - in 3,5-DCQA, and sample **4** – in 3,4-DCQA.

Literature survey showed only several reports on the content of 5-CQA and DCQA isomers in *I. britannica*, but it was difficult to compare their quantity as the data referred to their isolation, LC/MS or TLC detection, but not their real quantity. Thus, 5-CQA has been detected in ray and disk florets of the plant from Hungary [13] and the herb from South Korea [12], while 1,5-DCQA was isolated from another Bulgarian population [6]. The average contents of 5-CQA in 33 samples of *I. britannica* flowers, stems and aerial parts from China were found to be 323, 146 and 302 μg/g DM, respectively [14]. Finally, principal component analysis (PCA) was applied to study variability between different populations of *I. britannica*. The PCA performed on TPC, TFC, the content of 5-CQA, 1,5-, 3,5-, 4,5- and 3,4-DCQA of each sample showed that the first two principal components (PC) accounted for 72.88 % of the total variations (Fig. 3). As can be seen, PC1 (44.07 %) had a strong positive correlation with all variables, while PC2 (28.81 %) was positively related to TPC, TFC, 5-CQA, and 1,5-DCQA and negatively related to 3,5- DCQA, 3,4-DCQA, and 4,5-DCQA. The samples **1** and **11** occupied the most distant positions because of the highest amounts of phenolics (TPC) and 1,5-DCQA in sample **1** and TFC and 5-CQA in sample **11**. Sample **5** was situated at the positive sites of PC1 and PC2 and was associated with 3,5-DCQA. Samples **2** and **4** were settled at the negative side of PC2 and were connected with 4,5- and 3,4-DCQA. In fact, these compounds were detected in the highest concentration in the respective samples (Table 2).

Samples **3** and **9** were depleted in flavonoids (TFC) therefore they were located at the negative sites of PC1 and PC2. Similarly, samples **6** – **8** and **10** were settled at the positive side of PC2 because of the relatively low content of 4,5-DCQA.

CONCLUSION

The results of this study revealed significant variability in the contents of total phenolics, total flavonoids and individual compounds (chlorogenic, 1,5-, 3,5-, 4,5-, and 3,4- dicafeoylquinic acids) within the investigated populations of *I. britannica*, which reflected on their antioxidant capacity too. The samples containing the highest amounts of phenolics and flavonoids were found to be the best DPPH and ABTS radical scavengers. The obtained results could be used in the selection of the prospective populations of *I. britannica* providing a greater concentration of active components and, consequently, a higher biological activity.

Acknowledgments: *This work was supported by the Bulgarian Ministry of Education and Science under the National Research Programme "Young scientists and postdoctoral students" approved by DCM # 577/17.08.2018 and by the National Science Fund, Ministry of Education and Science, Bulgaria, Project DN 09/11.*

REFERENCES

1. S. I. Ali, M. Qaiser, R. Abid, Flora of Pakistan – Astereaceae, University of Karachi, Karachi Printing Press, Karachi, Pakistan, vol. 210, 1992, p. 71.
2. P. W. Ball, T. G. Tutin, Inula L, In: Flora Europaea, Vol. 4, T. G. Tutin, V. H. Heywood, N. A. Burges, D. M. Moore, D. H. Valentine, S. M. Walters, D. A. Webb (eds.), Cambridge Univ. Press, Cambridge, UK, 1976, p. 133.
3. A. L. Khan, J. Hussain, M. Hamayun, S. A. Gilani, S. Ahmad, G. Rehman, Y.-H. Kim, S.-M. Kang, I.-J. Lee, *Molecules*, **15**, 1562 (2010).
4. A. M. L. Seca, A. Grigore, D. C. G. A. Pinto, A. M. S. Silva, *J. Ethnopharmacol.*, **154**, 286 (2014).
5. A. M. L. Seca, D. C. G. A. Pinto, A. M. S. Silva, *Chemistry & Biodiversity*, **12**, 859 (2015).
6. V. Ivanova, A. Trendafilova, M. Todorova, K. Danova, D. Dimitrov, *Nat. Prod. Comm.*, **12**, 153 (2017).
7. K. M. Yoo, Ch. H. Lee, H. Lee, B. K. Moon, Ch. Y. Lee, *Food Chem*., **106**, 929 (2008).
8. J. Zhishen, T. Mengcheng, W. Jianming, *Food Chem.*, **64**, 555 (1999).
9. K. Thaipong, U. Boonprakob, K. Crosby, L. Cisneros-Zevallos, D. H. Byrne, *J. Food Comp. Anal.*, **19**, 669 (2006).
10. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, *Free Radic. Biol. Med.*, **26,** 1231 (1999).
11. N. H. Lee, J. I. Hong, J. Y. Kim, M. H. Chiang, *Korean J. Food Sci. Tech.*, **41**, 87 (2009).
12. W. Y. Bae, H. Y. Kim, K. S. Choi, K. H. Chang, Y. H. Hong, J. Eun, N. K Lee, H. D. Paik, *BMC Complementary and Alternative Medicine*, **19**, 253 (2019).
13. A. Péter, G. Dósa, *Acta Botanica Hungarica*, **44**, 129 (2002).
14. X. Shi, K. Zhang, N. Xue, L. Su, G. Ma, J. Qi, Y. Wu, Q. Wang, Q. Shi, *Food Chem.*, **141**, 4019 (2013).