

Assessment of Endemic *Cota fulvida* (Asteraceae) for Phytochemical Composition and Inhibitory Activities against Oxidation, α -Amylase, Lipoxygenase, Xanthine Oxidase and Tyrosinase Enzymes

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Abstract: In the present work, chemical compositions of essential oil and methanol extract of endemic *Cota fulvida* (Grierson) Holub were investigated as well as their antioxidant, antidiabetic, antiinflammatory and antimelanogenesis potent. The phytochemical analyses have been performed with GC-MS/FID and LC-MS/MS techniques. The essential oil was characterized with hexadecanoic acid (25.6 %), camphor (6.1 %), caryophyllene oxide (5.3 %), 1,8-cineole (4.9 %) and humulene epoxide (3.9 %). In the extract, phenolic acids, phenylpropanoid dimer and flavonoids were detected. The major constituents of the extracts were found to be 5-feruloylquinic acid, caftaric acid, 3,5-O-dicaffeoylquinic acid and quercetin rutinoside. The antioxidant activities of the oil and extract were evaluated through scavenging of free radicals, inhibition of linoleic acid peroxidation and superoxide anion radical (O²⁻) generated by xanthine - xanthine oxidase (XO) system. The extract showed free radical scavenging activity (IC₅₀ 0.131 mg/mL), Trolox equivalent antioxidant capacity (1.33 mM) and inhibited (Inh. 36.3 %) peroxidation of lipids. The oil and extract demonstrated significant hypoglycemic activity *via* inhibition of porcine pancreatic α -amylase. The antiinflammatory effects of the oil and extract *via* inhibition of 5-LOX enzyme were found as 53.7 % and 23.9 %, respectively. The extract demonstrated moderate inhibitory effect (23.3 %) on oxidation of L-DOPA *via* inhibition of tyrosinase enzyme.

Keywords: *Cota fulvida*; essential oil; extract; GC-MS/FID; LC-MS/MS; activity. © 2018 ACG Publications. All rights reserved.

1. Introduction

The genus *Cota* J. Gay is represented by 63 taxa in the world and is mainly distributed in Europe (excluding northern Europe), North Africa, Caucasia and Central Asia [1]. *Cota* was earlier classified as a section in the genus *Anthemis* L. in Flora of Turkey [2]. According to the last reports, the *Anthemis* section *Cota* has been accepted as a generic name, *Cota* [3,4]. In Turkey, the genus consists of 22 taxa,

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nine of which are endemic [5]. Recently, a new species *Cota hamzaoglui* Özbek & Vural has been described [1]. The rare endemic Turkish species *Cota fulvida* (Grierson) Holub (Syn. *Anthemis fulvida* Grierson) was rediscovered 113 year after its first collection.

The plants of Asteraceae family have been found to be the most commonly used families in the traditional medical treatments in Turkey. Ethnomedicinal aspects of potent of Asteraceae plants have recently been reported [7]. Many genera have been approved for applying in treatment of a number of diseases, *Tanacetum* [8], *Silybum* [9], *Matricaria* [10], *Achillea* [11], *Artemisia* [12] and *Anthemis* [13]. Several aspects on chemical and pharmacological potent of the genus *Anthemis* have recently been reported by Siasar-Karbasky et al. [14]. A previous phytochemical studies on *Anthemis* species resulted with polyphenols [2, 3], mono- and sesquiterpenes and fatty acids [15]. Biological activity investigations of *Anthemis* species encompasses antibacterial [16], antioxidant [17], cytotoxic [18], antiproliferative [19], antidiabetic [20], antiinflammatory [21] and lipoxygenase inhibition [22] potentials. A literature search revealed information about tyrosinase inhibition potent of extract from *Anthemis nobilis* [23].

Recently, mostly due to the potent side effects of modern synthetic drugs and increasing contraindications to their usage, a resurgent trend has emerged towards the use of medicinal plants [24]. Today there is increasing demand for cheap, safe and scientifically approved botanicals from domestic sources. However, there are still species have not been investigated for phytochemical and biological potentials. The plants of the genus *Cota* are among less-investigated species. To the best of our knowledge, there is no previous information about chemical composition and biological activity of *C. fulvida* species.

Taking into consideration the previous literature data on chemistry and activity of *Anthemis* species as well as lacking information about *C. fulvida* we aimed to investigate chemical composition and biological potential of this species. Therefore, the essential oil and methanol extract of *C. fulvida* were screened for antioxidant activity using different *in vitro* methods like 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, Trolox equivalent antioxidant capacity (TEAC) and β -carotene bleaching tests. The antioxidant effects of *C. fulvida* oil and extract on oxidative damage were also evaluated with enzymatic test using xanthine - xanthine oxidase system that generated superoxide anion radical (O_2^-). The test samples which interact with xanthine oxidase can affect the kinetics of xanthine oxidation to uric acid which causes hyperuricacidemia associated with gout [25]. Xanthine oxidase (XO) has a role in the generation of reactive oxygen species in various pathologies such as viral infection, inflammation, brain tumors or the process of ischemia/reperfusion. Thus, inhibitors of XO are expected to be therapeutically useful for the treatment or prophylaxis of these diseases. In literature, there is information about antidiabetic properties of *Anthemis nobilis* [26]. Glucoside chamaemeloside was shown to have *in vivo* hypoglycemic activity [27]. The investigation of inhibitory activity of *C. fulvida* against pancreatic α -amylase, which is known as key enzyme in digestion of dietary carbohydrate in organism, may give information on antihyperglycemic potent of the plant.

In scope of the present work, we attempted to investigate the potential of *C. fulvida* against tyrosinase enzyme *in vitro*. Tyrosinase is the key enzyme in production of melanin [28]. The central role of tyrosinase in dopamine neurotoxicity as well as contribution to the neurodegenerative Parkinson's disease was well documented [29]. Inhibitors of tyrosinase found application in cosmetic products for whitening and depigmentation after sunburn as well as for the treatment of hyperpigmentation. Nowadays, there is increasing demand for naturally derived inhibitors of tyrosinase due to diverse side effects of synthetic products. The tyrosinase inhibitory activity of *A. nobilis* methanol extracts (flower, stem and root) was reported by Park et al. [23]. All these reports encouraged us to investigate *C. fulvida* for antityrosinase activity. The present research work is the first contribution into the chemistry and biological activities of *C. fulvida*, endemic species from Turkey.

2. Materials and Methods

2.1. Reagents and Materials

Cota fulvida: phytochemical and biological profile

3,4-Dihydroxyl-L-phenylalanin, β -carotene, linoleic acid, Tween-20, butylated hydroxytoluene (BHT), gallic acid, (\pm)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ammonium acetate, kojic acid, acarbose, 3,4-dihydroxy-L-phenylalanine (L-DOPA) and nordihydroguaiaretic acid (NDGA), allopurinol, α -amylase from porcine pancreas (Type VI-B, EC 3.2.1.1), tyrosinase from mushroom (EC 1.14.18.1), xanthine oxidase from bovine milk (Grade IV), lipoxidase from *Glycine max* (Type I-B) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Starch soluble extra pure, iodine and potassium iodide were purchased from Merck (Darmstadt, Germany). Sodium phosphate, disodium phosphate, aluminum chloride, water and methanol were extra pure analytical grade. A C₈–C₄₀ *n*-alkane standard solution was purchased from Fluka (Buchs, Switzerland). All solvents were purchased from Sigma Aldrich (Germany) and were of analytical grade.

2.2. Plant Material

The aerial parts of *C. fulvida* were collected on Dedegöl Mountain, above Melikler pastures in Yenişarbademli (Isparta) province, on 24 August 2012, and dried under the shade. Botanical identification was performed by Dr. M. Arslan and Dr. M. U. Özbek. The voucher specimen is kept in Gazi University Herbarium under the herbarium code GAZI.

2.3. Essential Oil Isolation

The aerial parts of *C. fulvida* were subjected to hydrodistillation (3 h) to yield essential oil in Clevenger-type apparatus according to European Pharmacopeia [30]. The oil was dried over anhydrous sodium sulfate and stored in sealed vials in refrigerator (4°C), until GC-FID/MS analyses and biological activity testing. The oil was dissolved in *n*-hexane (10 %, v/v) to conduct chromatographic determination of the composition.

2.4. Extract Preparation

Aerial parts of *C. fulvida* were subjected to maceration in methanol with continuous shaking (3000 per min) at room temperature for 24 h. The obtained liquid extract was filtered and evaporated until dryness *in vacuo*. The dried extract was kept at 4°C before phytochemical analysis and biological activities tests.

2.5. Gas Chromatographic Analysis

The oil was analyzed with GC-FID and GC/MS techniques simultaneously. Details of chromatographic analysis conditions are given in S1 (Supporting information).

2.6. Identification and Quantification of Volatile Compounds

Identification of the volatile constituents was given in Table 2. Identification methods are given in S2 (Supporting information)

2.7. LC- MS/MS Analysis

LC-MS/MS analysis was carried out using an Absciex 3200 Q trap MS/MS detector. The experiment's conditions are given in S3 (Supporting information).

2.8. Free Radical Scavenging Activity (DPPH assay)

The free radical scavenging ability of the essential oil and the extract of *C. fulvida* were evaluated according to bleaching of purple colored methanol solution of DPPH stable radical using a method of Brand-Williams [31] with slight modifications. Details of the experiments are given in S4

(Supporting information). The free radical scavenging activity of the samples was calculated on base of triplicate experiments and expressed as percentage of inhibition calculated according to equation (1):

$$\% \text{ Inh} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100, \quad (1)$$

where, $\text{Abs}_{\text{control}}$ is the absorbance of the control (containing all reagents except the test compound), $\text{Abs}_{\text{sample}}$ is the absorbance of the sample with added DPPH. The IC_{50} values were obtained by plotting the DPPH scavenging percentage of each sample against the sample concentration. Data were analyzed using the SigmaPlot software (Version 12.0).

2.9. Trolox Equivalent Antioxidant Capacity (TEAC assay)

ABTS⁺ free radical cation scavenging activity of the essential oil and extract were tested according to the procedure described by Re et al. [32] with slight modifications. Details of the experiments are given in S5 (Supporting information). ABTS⁺ scavenging activity of the samples was expressed as Trolox equivalent antioxidant capacity and calculated using linear equation obtained for Trolox ($y = 33.644x + 2.6523$, $r^2 = 0.9942$).

2.10. β -Carotene / Linoleic Acid Peroxidation Inhibition Assay

Inhibition of lipid peroxidation by *C. fulvida* essential oil and methanol extract was measured according to method of Marco [33] with slight modifications. β -Carotene / linoleic acid peroxidation inhibition assay uses a linoleic acid in an emulsified form (with Tween-20) as the model lipid substrate. Briefly, the method lies in between methods employing only model substrates (e.g. DPPH) and those using real lipids. Details of the experiments are given in S6 (Supporting information). The rate of β -carotene bleaching was monitored by measuring the absorbance at 15 min periods at 470 nm using an ELISA microplate reader (Biotek Powerwave XS) [33, 34]. The experiment was performed in triplicate and the results were expressed as average of inhibition percentage values calculated according to equation (2):

$$\% \text{ AA} = \left[1 - \frac{(\text{Abs}_{0\text{sample}} - \text{Abs}_{120\text{sample}})}{\text{Abs}_{0\text{control}} - \text{Abs}_{120\text{control}}} \right] \times 100, \quad (2)$$

where, AA is antioxidant activity, $\text{Abs}_{0\text{sample}}$ and $\text{Abs}_{120\text{sample}}$ are the absorbance of the sample at 0 min and 120 min, $\text{Abs}_{0\text{control}}$ and $\text{Abs}_{120\text{control}}$ are the absorbance values of the control at 0 min and 120 min.

2.11. Microtiter Assay for Determination of Xanthine Oxidase Inhibition

The xanthine oxidase inhibition assay was carried out according to procedure reported by Chen [35] with slight modifications. The stock solutions of the essential oil and the extract of *C. fulvida* (5 mg/mL) were prepared in methanol, then diluted with phosphate buffer (0.1 M, pH=7.5) up to 1 mg/mL. Details of the experiments are given in S7 (Supporting information). Absorbance at 295 nm was recorded with spectrophotometer. The percentage inhibition was calculated according to equation (3):

$$\% \text{ Inh} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{control blank}}) - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{sample blank}})}{\text{Abs}_{\text{control}} - \text{Abs}_{\text{control blank}}} \right] \times 100, \quad (3)$$

where $\text{Abs}_{\text{control}}$ and $\text{Abs}_{\text{control blank}}$ are the absorbance values of the control and its blank, $\text{Abs}_{\text{sample}}$ and $\text{Abs}_{\text{sample blank}}$ are the absorbance values of the sample and its blank.

2.12. Microtiter Assay for Determination of α -Amylase Inhibition

The activity of α -amylase was measured using iodine/potassium iodide (IKI) method [36] with slight modifications. The substrate solution (0.05 %) was prepared as follow: soluble potato starch (10

Cota fulvida: phytochemical and biological profile

mg) was dissolved in 20 mL ultrapure water then boiled for 10 min and cooled to room temperature before use. Details of the experiments are given in S8 (Supporting information). The sample and blank absorbance values were read at 630 nm. The percentage inhibition of the α -amylase activity (Inh %) was calculated according to equation (3).

2.13. Microtiter Assay for Determination of Lipoxxygenase Inhibition

Inhibition of lipoxxygenase activity was measured with spectrophotometric method reported by Albano et al. [37] with slight modifications. The increase in absorbance at 234 nm was recorded for 6 min. The percentage inhibition was calculated according to equation (3). Details of the experiments are given in S9 (Supporting information).

2.15. Microtiter Assay for Determination of Tyrosinase Inhibition

An inhibitory activity of *C. fulvida* essential oil and methanol extract against tyrosinase was measured using the modified 96-well microplate method reported by Masuda [38]. The oil and the extract were dissolved in DMSO (10 %) and then diluted with buffer (pH 6.8) to get concentration 1 mg/mL. Details of the experiments are given in S10 (Supporting information). The percentage inhibition of the tyrosinase activity (Inh %) was calculated according to equation (4):

$$\text{Inh\%} = \{[(A - B) - (C - D)] / (A - B)\} \times 100 \quad (4)$$

2.16. Statistical Analysis of Data

Data obtained from antioxidant and enzyme inhibition experiments were expressed as mean standard error (\pm SEM). IC₅₀ values were estimated using a nonlinear regression algorithm.

3. Results and Discussion

In literature, it could be found highlighting promising phytochemical properties and biological activities of diverse *Anthemis* species. However, there is no information about phytochemistry and biological potential of endemic species *C. fulvida*. The main goal of the present work was to evaluate chemical composition and biological properties of endemic *C. fulvida* volatile and non-volatile metabolites.

3.1 Essential Oil Composition

In the present work, the essential oil of *Cota fulvida* has been hydrodistilled and investigated for chemical profile for the first time. The hydrodistillation of the herb of *C. fulvida* resulted with yellowish essential oil (0.1 % yield) with specific odor.

Gas-chromatographic analysis of the oil resulted with 70 compounds, which belong to diverse phytochemical groups, namely, monoterpene hydrocarbon, oxygenated monoterpene, sesquiterpene hydrocarbon, oxygenated sesquiterpene, fatty acids, aliphatic aldehydes and alkanes. The list of detected compounds with their relative retention indices, relative percentages and method of identification is given in Table 1 in order of their elution on the HP-Innowax FSC column. Gas-chromatographic profile of *C. fulvida* essential oil is presented on Figure S11 (Supporting information).

In general, the essential oil of *C. fulvida* was characterized with high abundance of the fatty acids (>30.0 %). Hexadecanoic acid (25.6 %) was found as predominate fatty acid in the oil. It is noteworthy to mark that the monoterpenes as hydrocarbons (12.6 %) and oxygenated (15.9 %) constituents were found as the second important group after fatty acids in the essential oil. Camphor (6.1 %), 1,8-cineole (4.9 %) and α -pinene (3.0 %) were the major monoterpenes in the oil. The sesquiterpenes comprised almost all of oxygenated constituents (19.0 %) with caryophyllene oxide (5.3 %), humulene epoxide

(3.9 %) and spathulenol (2.4 %) as major constituents. Distribution of the main compound groups detected in the essential oil of *Cota fulvida* is presented on Figure S12 (see supporting information).

Table 1. Chemical compositions of *Cota fulvida* herb essential oil

No	RRI ^(a)	RRI ^(b)	Compound	% ^(c)	ID
1	1032	1032 [39]	α -Pinene	3.0	d,e,f
2	1076	1076 [39]	Camphene	0.4	d,e,f
3	1118	1118 [39]	β -Pinene	2.1	d,e,f
4	1188	1177 [40]	α -Terpinene	t	d,e,f
5	1203	1203 [39]	Limonene	1.6	d,e,f
6	1213	1212 [40]	1,8-Cineole	4.9	d,e,f
7	1244	1242 [25]	Amyl furan	0.5	d,e,f
8	1255	1256 [41]	γ -Terpinene	0.4	d,e,f
9	1280	1278 [15]	<i>p</i> -Cymene	0.2	d,e,f
10	1290	1283 [42]	Terpinolene	t	d,e,f
11	1348	1347 [40]	6-Methyl-5-hepten-2-one	0.3	e,f
12	1400	1400 [40]	Nonanal	0.7	d,e,f
13	1452	1454 [40]	1-Octen-3-ol	0.3	d,e,f
14	1474	1474 [39]	<i>trans</i> -Sabinene hydrate	0.1	d,e,f
15	1479	1458 [25]	(<i>E,Z</i>)-2,4-Heptadienal	0.2	d,e,f
16	1507	1475 [25]	(<i>E,E</i>)-2,4-Heptadienal	0.4	d,e,f
17	1509		Dihydroedulane	0.5	e,f
18	1532	1532 [40]	Camphor	6.1	d,e,f
19	1548	1547 [25]	(<i>E</i>)-2-Nonenal	t	d,e,f
20	1553	1553 [40]	Linalool	1.0	d,e,f
21	1556	1556 [40]	<i>cis</i> -Sabinene hydrate	0.2	d,e,f
22	1586	1586 [40]	Pinocarvone	0.5	d,e,f
23	1611	1611 [40]	Terpinen-4-ol	2.0	d,e,f
24	1638	1638 [40]	β -Cyclocitral	0.6	d,e,f
25	1648	1648 [40]	Myrtenal	0.3	d,e,f
26	1670	1670 [40]	<i>trans</i> -Pinocarveol	1.0	d,e,f
27	1678	1678 [40]	<i>cis-p</i> -Mentha-2,8-dien-1-ol	t	d,e,f
28	1682	1682 [40]	δ -Terpineol	t	d,e,f
29	1683	1683 [40]	<i>trans</i> -Verbenol	0.3	d,e,f
30	1687	1687 [39]	α -Humulene	0.4	d,e,f
31	1706	1706 [40]	α -Terpineol	0.7	d,e,f
32	1719	1719 [40]	Borneol	0.9	d,e,f
33	1738	1740 [40]	<i>p</i> -Mentha-1,5-dien-8-ol	t	d,e,f
34	1798	1809 [40]	Methyl salicylate	0.5	d,e,f
35	1804	1804 [40]	Myrtenol	0.4	d,e,f
36	1811		<i>trans-p</i> -Mentha-1(7),8-dien-2-ol	t	d,e,f
37	1827	1826 [25]	(<i>E,E</i>)-2,4-Decadienal	0.8	d,e,f
38	1830	1830 [40]	Tridecanal	0.5	d,e,f
39	1838	1838 [40]	(<i>E</i>)- β -Damascenone	t	e,f
40	1845	1845 [40]	<i>trans</i> -Carveol	0.3	d,e,f
41	1868	1868 [40]	(<i>E</i>)-Geranyl acetone	1.3	d,e,f
42	1896		<i>cis-p</i> -Mentha-1(7),8-diene-2-ol	0.2	e,f
43	1933	1933 [40]	Tetradecanal	0.3	d,e,f
44	1958	1958 [40]	(<i>E</i>)- β -Ionone	1.5	d,e,f
45	1962		Birkenyl acetate	0.2	d,e,f
46	1985		<i>trans</i> - β -Ionone-5,6-epoxide	0.5	e,f
47	2008	2008 [43]	Caryophyllene oxide	5.3	d,e,f
48	2026	2024 [40]	Humulene epoxide II	3.9	e,f
49	2028		Caryophylla-2(12),6(13)-5-one	t	d,e,f
50	2081		Humulene epoxide-III	0.3	e,f
51	2084	2089 [40]	Octanoic acid	0.2	d,e,f
52	2144	2136 [44]	Spathulenol	2.4	d,e,f

Cota fulvida: phytochemical and biological profile

No	RRI ^{a)}	RRI ^{b)}	Compound	% ^{c)}	ID
53	2179		3,4-Dimethyl-5-pentylidene-2(5H)-furanone	0.8	e,f
54	2187	2187 [45]	T-Cadinol	0.6	d,e,f
55	2192	2196 [40]	Nonanoic acid	0.3	d,e,f
56	2206		Alismol (= 6,10(14)Guaiadien-4- β -ol)	0.2	e,f
57	2234		1-Pentadecanol	0.2	d,e,f
58	2298	2296 [40]	Decanoic acid	0.5	d,e,f
59	2300	2300 [46]	Tricosane	1.9	d,e,f
60	2316		Caryophylla-2(12),6(13)-dien-5 β -ol	0.7	d,e,f
61	2324	2324 [39]	Caryophylla-2(12),6(13)-dien-5 α -ol	2.0	d,e,f
62	2326		Eudesma-4(15),7-dien-1- β -ol	0.2	e,f
63	2336		Farnesyl acetone	1.1	d,e,f
64	2392	2392 [39]	Caryophylla-2(12),6-dien-5 β -ol	2.1	d,e,f
65	2500	2500 [39]	Pentacosane	0.9	d,e,f
66	2503	2503 [40]	Dodecanoic acid	0.7	d,e,f
67	2617	2617 [40]	Tridecanoic acid	0.3	d,e,f
68	2622	2606 [47]	Phytol	0.7	d,e,f
69	2670	2670 [40]	Tetradecanoic acid	4.0	d,e,f
70	2931	2931 [39]	Hexadecanoic acid	25.6	d,e,f
Total				91.0	

^{a)} RRI: Relative Retention Indices calculated against *n*-alkanes (C₈-C₄₀) on HP-Innowax column; ^{b)} RRI values obtained on polar column and reported in literature; ^{c)} % calculated from FID data; ^{d)} Identification based on retention index of genuine compounds on the HP-Innowax column; ^{e)} Identification on the basis of computer matching of the mass spectra from Basler Library; ^{f)} Tentative identified on the basis of computer matching of the mass spectra from Adams, MassFinder, Wiley and NIST libraries; *t*: Trace (< 0.1 %).

It was interesting to compare the chemical profile of *C. fulvida* essential oil with those reported earlier for different *Anthemis* species. Actually, there are several reports in the literature dealing with the essential oils of diverse *Anthemis* species. The fatty acids and especially hexadecanoic acid have earlier been observed to be the major constituents in previously studied essential oils obtained from aerial parts of *A. dipsacea* Bornm. (13.5%), *A. pseudocotula* Boiss. (9.5%) [48], *A. altissima* L. (39.6 %) [49], *A. ruthenica* M. Bieb. (9.9 %) and *A. arvensis* L. (21.2%) [50]. Camphor was reported as main volatile constituent in *A. cretica* subsp. *leucanthemoides* (Boiss.) Grierson (19.4 %) [51], *A. tenuisecta* Ball. (17.5 %) [52], *A. triumfetti* (L.) DC. (15.0 %) [53], *A. hyalina* DC. (11.6 %) [54] and *A. pseudocotula* Boiss. (9.4 %) [55]. 1,8-Cineole was mentioned as major constituent in the oils of *A. pseudocotula* (39.4 %) [55], *A. xylopoda* O. Schwarz (16.7 %) [56], *A. widemanniana* (8.9 %) [57] and *A. segetalis* Ten (6.1 %) [58]. Observation of the main constituents detected in different *Anthemis* species, it can be concluded that the oil of *C. fulvida* was found to be similar to many *Anthemis* species.

Table 2. Results of LC-MS/MS analysis of *Cota fulvida* methanol extract.

Rt	[M-H] ⁻	Fragments	Compound	Ref.
7.1	197	181, 167, 153	Syringic acid	[59]
9.5	469	323, 179, 161	Phenylpropanoid dimer + deoxyhexose	
10.3	353	353, 191	5-Feruloylquinic acid	[60]
11.7	339	179, 161	Caffeic acid derivative	
12.2	311	179, 161	Caftaric acid	[61]
13.0	609	301	Quercetin rutinoside	[62]
14.1	463	300, 271	Quercetin glucoside	[63]
15.1	515	353, 299, 203, 173,	3,4- <i>O</i> -Dicafeoylquinic acid	[63, 64]
15.7	515	353, 335, 191, 179,	3,5- <i>O</i> -Dicafeoylquinic acid	[63, 64]
16.5	615	515, 453, 353, 191	1,5-Dicafeoyl-3-succinoylquinic acid	[61]
18.9	473	311, 179, 161, 149	Chicoric acids	[61]
21.6	285	133	Luteolin	[63]

3.2. Methanol Extract Composition

In scope of the research, the total phenolics content and total flavonoids content of *C. fulvida* methanol extract were determined with Folin Ciocalteu reagent and $AlCl_3$, respectively, via spectrophotometric measurements. Subsequent phytochemical analysis via LC-MS/MS technique of the extract led us to identification of phenolics acids, flavonoids and phenylpropanoid dimer. The list of the constituents detected in *C. fulvida* methanol extract with MS detector is summarized in Table 2.

The composition of the extract was constituted by syringic, 5-feruloylquinic, caftaric, 3,4-O-dicafeoylquinic, 3,5-O-dicafeoylquinic, 1,5-dicafeoyl-3-succinoylquinic and chicoric and caffeic acid derivative; quercetin glycosides and luteolin. Chromatographic profile of *C. fulvida* methanol extract obtained with liquid chromatography is given in Figure S13 (Supporting information).

3.3. Antioxidant Activity

The essential oil and methanol extract of *C. fulvida* have been investigated for an antioxidant activity by using non-enzymatic and enzymatic systems employing model substrates: stable free radical DPPH[•] and cation radical ABTS^{•+} as well as real substrates: linoleic acid peroxides and superoxide anion radicals ($O_2^{\cdot-}$). Such approach allowed obtaining rather realistic results about antioxidant potent of the samples. According to antioxidant activity experiments, the methanol extract of *C. fulvida* demonstrated the highest antioxidant activity in all applied assays. Namely, the extract scavenged DPPH radicals with IC_{50} 0.131 mg/mL. It was more effective than BHT (IC_{50} 0.50 mg/mL) but less effective than ascorbic acid (IC_{50} 0.03 mg/mL) and gallic acid (IC_{50} 0.003 mg/mL). The essential oil did not demonstrated activity in this assay. The extract prevented bleaching of β -carotene by inhibition of linoleic acid peroxidation at 36.3 %, while the essential oil was inactive. In the TEAC assay, the methanol extract showed ABTS^{•+} bleaching activity equivalent to 1.33 mM of Trolox (water soluble tocopherol), while the oil demonstrated weak activity (0.44 mM of Trolox). In the xanthine/ xanthine oxidase system, the extract demonstrated the highest inhibitory effect (69.36 %). Therefore, as can be seen from the antioxidant activity determination results (Table 2), the essential oil and the extract possess different potentials in diverse oxidation systems.

In the extract of *C. fulvida* polyphenols have been presented by phenolic acids as well as flavonoids and their glycosides. The phenolic acids in the extract comprised of 3,4-O-dicafeoylquinic, 3,5-O-dicafeoylquinic, 1,5-dicafeoyl-3-succinoylquinic, caftaric and chicoric acids. In literature there is information that caffeoylquinic acid derivatives display more potent free radical-scavenging activity than the most commonly used antioxidants (such as vitamin C, vitamin E, and caffeic acid) [65].

On the other hand, the biological activity of phenolic compounds is known to be due to the activity of nonsubstituted hydroxyl groups. In flavonoid's structure, the presence of a C2–C3 double bond on the ring C, a dihydroxyl group (catechol-type) or three adjacent hydroxyl group (pyrogallol-type) on the ring B, and the presence of C-5, and C-7 hydroxyl group on the ring A are mentioned as requirements for antioxidant activity [66, 67]. Luteolin, quercetin rutinoside and glucoside detected in the extract of *C. fulvida* have all mentioned requirements. Therefore, noteworthy antioxidant activity of the extract may be due to those compounds. In conclusion, the methanol extract of *C. fulvida* may be considered as potential antioxidant agent in preventing of oxidative damage.

3.4 Inhibitory Effects of the Plant on Pancreatic α -Amylase Activity

The essential oil and methanol extract of *C. fulvida* were *in vitro* evaluated for hypoglycemic activity via inhibition of the pancreatic α -amylase. As can be seen in Table 2, both of the tested extracts demonstrated inhibitory activity. The oil inhibited the enzyme's activity up to 75.11 % at concentration 0.5 mg/mL. The extract demonstrated significant inhibition and prevented activity of α -amylase with IC_{50} 0.35 mg/mL. It seems to be that the plant of *C. fulvida* can be source of an effective hypoglycemic phytochemicals.

One of prominent inhibitors of α -amylase in herbal products are the flavonoids. The molecular structures that influence the inhibition of α -amylase by flavonoids are the following: the hydroxylation

Cota fulvida: phytochemical and biological profile

of flavonoids improved the inhibitory effect on α -amylase; the presence of an unsaturated 2,3-bond in conjugation with a 4-carbonyl group has been associated with stronger inhibition [67]. The methanol extract of *C. fulvida* contained flavonoid aglycone as well as flavonoid glycosides. Walle et al. reported that the glycosylation of flavonoids decreased the inhibitory effect on α -amylase depending on the conjugation site and the class of sugar moiety [68].

3.5. Inhibitory Effects of the Plant on Lipoxygenase Activity

Antiinflammatory effect of *C. fulvida* was tested in 5-LOX enzyme inhibition system. Actually, 5-lipoxygenase catalyzes the oxidation of arachidonic acid and produces 5(S)-hydroxyperoxyeicosatetraenoic acid (5-HETE) which undergoes dehydration, resulting in the formation of leukotriene. Enzymatic hydrolysis of leukotriene, as well as conjugation with other substances, leads to the formation of inflammatory mediators. In the present study, the oil and extract of *C. fulvida* were found to be able to inhibit 5-lipoxygenase, particularly the oil (Inh 53.67 % at 1 mg/mL), in comparison to the extract (Inh 23.91% at 5 mg/mL) which showed poor activity (Table 2).

3.6. Inhibitory Effects of the Plant on Mushroom Tyrosinase Activity

In this experiment, tyrosinase inhibitory effects of *C. fulvida* oil and extract on diphenolase activity of mushroom tyrosinase were evaluated. The extract demonstrated the highest inhibitory activity (45.46 %). However, the oil was found inactive when tested at a concentration 1 mg/mL (Table 3). In general, it can be assumed that *C. fulvida* have moderate antityrosinase effect.

Table 3. The biological activity of *C. fulvida* essential oil and extract[§]

Sample	DPPH, IC ₅₀	β -Carotene bleaching ^{a)} , %Inh	TEA C ^{a)} , mM	XOD, %Inh	TPC, GAE mg/mL	TFC, QE mg/mL	α -Amylase ^{b)} , %Inh	LOX ^{a)} , %Inh	Tyro ^{c)} , % Inh
Essential oil	N/A	N/A	0.44	1.87	-	-	75.11	53.67	N/A
Methanol extract	0.131	36.31	1.33	69.36	0.294	0.046	0.350	23.91	45.46
Gallic acid	0.003	-	-	-	-	-	-	-	-
Ascorbic acid	0.03	-	-	-	-	-	-	-	-
BHT	0.50	85.0	-	-	-	-	-	-	-
Acarbose	-	-	-	-	-	-	98	-	-
NDGA	-	-	-	-	-	-	-	86.0	-
Allopurinol	-	-	-	81.00	-	-	-	-	-
Kojic acid	-	-	-	-	-	-	-	-	84

BHT: butylated hydroxytoluene; N/A: not active; NDGA: nordihydroguaiaretic acid; [§] the deviation from the mean is <%10 of the mean value; ^{a)} Concentration of sample 5 mg/mL; ^{b)} Concentration of sample 0.5 mg/mL; ^{c)} Concentration of sample 1 mg/mL

As a conclusion, we herein disclose the first report on chemical profile of the volatile and non-volatile secondary metabolites obtained from endemic species *C. fulvida*. This species can be considered as source of valuable metabolites: the oil is rich with diverse mono- and sesquiterpenes, and fatty acids, while the extract contained phenolic acids, flavonoids and phenylpropanoids. In scope of the present study, the biological potential of volatile and non-volatile fractions of *C. fulvida* was evaluated for the first time. It seems, that *C. fulvida* can be considered as valuable source of bioactive components useful in combating various diseases such as cell damage, inflammation, skin disease, neurodegenerative problems as well as in the provision of cheap, safe and natural phytopharmaceuticals.

Disclosure statement

The authors declare that there are no conflicts of interest.

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