Research Article

PHYTOCHEMICAL CHARACTERIZATION OF AN ENDEMIC PLANT USED AS FOODSTUFF IN TURKEY: *CENTAUREA URVILLEI* SUBSP. *STEPSOSA* AND ITS ANTIOXIDANT PROPERTIES

Sengul Uysal¹, Ramazan Ceylan¹, Gokhan Zengin^{1*}, Abdurrahman Aktumsek¹, Nilgun Zengin², Gokalp Ozmen Guler³, Yavuz Selim Cakmak⁴, Evren Yildiztugay¹, Sukru Karatas¹

¹Department of Biology, Science Faculty, Selcuk University, Konya, Turkey

²Department of Food Engineering, Agriculture Faculty, Selcuk University, Konya, Turkey

³Department of Biological Education, Ahmet Kelesoglu Education Faculty, Necmettin Erbakan University, Konya, Turkey

⁴Department of Molecular Biology and Biotechnology, Faculty of Science and Arts, Aksaray University, Aksaray, Turkey

*Corresponding Author Email: gokhanzengin@selcuk.edu.tr

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ABSTRACT

This study was focused on the analysis of the phenolic content, fatty acid composition, volatile compounds and antioxidant activity of methanol extract from *Centaurea urvillei* subsp. *stepposa*. The antioxidant capacity were detected as 43.35 mg TE/g, 52.18 mg TE/g and 98.78 mg TE/g for DPPH, FRAP and CUPRAC, respectively. Chlorogenic acid and apigenin were the major phenolic components in the extract. Linoleic acid (49.54 %) was the predominant fatty acid in the oil. 1, 3-cyclooctadiene (25.08 %) was major components in the essential oil. The study concluded that *Centaurea urvillei* subsp. *stepposa* can be used as a source of natural antioxidants for food and pharmacological industry.

Keywords: *Centaurea*; Phenolic; Antioxidant; Fatty acids; Essential oils; Turkey.

INTRODUCTION

The different uses (food, drug etc.) of natural products compounds that are derived from natural sources such as plants, precede recorded human history probably by thousands of years¹. Recent research continues to grow trend to antioxidants as healthful components of plant food². Free radicals, especially ROS (reactive oxygen species) such as superoxide anion, hydroxyl radical, peroxy radical are continuously generated by normal metabolism in the body and they are necessary to maintain biological homeostasis through various functions such as vasoregulation and various cellular signal transduction³. However, overproduction of ROS (called as oxidative stress) can also cause damage to the macromolecules (carbohydrate, protein and lipid) necessary for cell structure and function. Oxidative stress is involved in the process of aging and various chronic diseases such as atherosclerosis and eye disease. Antioxidants are involved in the defense mechanism of the organism against the pathologies associated with the oxidative stress⁴. As a result of this situation, synthetic antioxidants are used as effective for inhibiting oxidation in foods and other products; however, safety concerns restricted due to their toxicological effects on various species and suspected carcinogenic potential. Therefore, the search of natural and safe antioxidants, especially of plant origin, has greatly increased in recent years⁵. Plants are great source of antioxidant components as phenolic acids, flavonoids, coumarins, tannins, alkaloids, amines, vitamins and terpenoids, which are called as secondary metabolites. In many studies suggest that diet with foods rich in these antioxidants reduces the risk of some cancers, heart and degenerative diseases^{6,7}. The genus *Centaurea* L. (Asteraceae) comprises more than 400 species worldwide⁸. In Turkey, the genus is represented by 209 species, of which 135 are endemics. The genus is known as “Peygamber çiçeği, çoban kaldıran, kotan kıran, zerdali diken, acımkık, sarıbaş, deli göz diken, kötürüm” in

Anatolia⁹. Some *Centaurea* species are used as herbal remedies in Anatolian folk medicine to treat fever, diabetes and hemoroid and peptid ulcer¹⁰. Moreover, basal leaves of *C. triumfetti* and *C. urvillei* subsp. *stepposa* are used as foodstuff and eaten while fresh^{11,12}. The uses of its species in traditional medicine could be causes of the high number of studies on members of the genus *Centaurea*¹³⁻¹⁵. However, there is no report in the literature on the phytochemical profile and biological activities of *C. urvillei* subsp. *stepposa*. The aim of this work is to investigate the antioxidant properties of methanol extract obtained from *C. urvillei* subsp. *stepposa* by using *in vitro* methods. Moreover, this paper reports phytochemical components of *C. urvillei* subsp. *stepposa*, an endemic species to Turkey.

MATERIALS AND METHODS

Plant Material

Centaurea urvillei subsp. *stepposa* was collected from Konya (Bozkir village) in July, 2012. Taxonomic identification of the plant material was confirmed by the Botanist Dr. Evren Yildiztugay, from the Department of Biology, Selcuk University. The voucher specimen was deposited in KNYA herbarium at Department of Biology, Selcuk University (Voucher number: EY2440).

Preparation of the Methanolic Extract

The aerial plant materials were dried at the room temperature and powdered to a fine grain using a laboratory mill. 10 g powdered aerial plant was extracted with 250 ml methanol at the room temperature with stirring until extraction solvent become colorless. Extract was filtered with Whatman blue band filter paper and methanol was evaporated at 40°C in rotary evaporator.

Quantification Phenolic Compounds by RP-HPLC

Phenolic compounds were evaluated by reversed-phase high-performance liquid chromatography (RP-HPLC, Shimadzu Scientific Instruments, Tokyo, Japan). Detection and quantification were carried out with a LC-10ADvp pump, a Diode Array Detector, a CTO-10Avp column heater, SCL-10Avp system controller, DGU-14A degasser and SIL-10ADvp auto sampler (Shimadzu Scientific Instruments, Columbia, MD). Separations were conducted at 30°C on Agilent® Eclipse XDB C-18 reversed-phase column (250 mm x 4.6 mm length, 5 µm particle size). The eluates were detected at 278 nm. The mobile phases were A: 3.0 % acetic acid in distilled water and B: methanol. For analysis, the samples were dissolved in methanol, and 20 µl of this solution was injected into the column. The elution gradient applied at a flow rate of 0.8 ml/min was: 93 % A/7 % B for 0.1 minute, 72 % A/28 % B in 20 minutes, 75 % A/25 % B in 8 minutes, 70 % A/30 % B in 7 minutes and same gradient for 15 minutes, 67 % A/33 % B in 10 minutes, 58 % A/42 % B in 2 minutes, 50 % A/50 % B in 8 minutes, 30 % A/70 % B in 3 minutes, 20 % A/80 % B in 2 minutes 100 % B in 5 minutes until the end of the run. Phenolic composition of the extract was determined by a modified method of Caponio, Alloggio and Gomes (1999)¹⁶. Gallic acid, protocatechuic acid, (+)-catechin, *p*-hydroxybenzoic acid, chlorogenic acid, caffeic acid, (-)-epicatechin, syringic acid, vanilin, *p*-coumaric acid, ferulic acid, sinapinic acid, benzoic acid, *o*-coumaric acid, rutin, naringin, hesperidin, rosmarinic acid, eriodictyol, trans-cinnamic acid, quercetin, naringenin, luteolin, kaempferol and apigenin were used as standard. Identification and quantitative analysis were done by comparison with standards. The amount of each phenolic compound was expressed as mg per gram of the extract.

Total Phenolic Content

The total phenolic content was determined by employing the methods given in the literature (Slinkard and Singleton, 1977)¹⁷ with slight modification. Sample solution (0.25 mL) was mixed with diluted Folin-Ciocalteu reagent (1 mL, 1:9) and shaken vigorously. After 3 minutes, Na₂CO₃ solution (0.75 mL, 1 %) was added and the sample absorbance was read at 760 nm after 2 h incubation at room temperature. The total phenolic content was expressed as equivalents of gallic acid according to the equation obtained from the standard gallic acid graph.

Total Flavonoid Content

The total flavonoid content was determined using the Dowd method as adapted by Berk *et al.* (2011)¹⁸. Briefly, sample solution (1 mL) was mixed with the same volume of aluminiumtrichloride (2 %) in methanol. Similarly, a blank was prepared by adding sample solution (1 mL) to methanol (1 mL) without AlCl₃. The sample and blank absorbances were read at 415 nm after 10 minutes incubation at room temperature. The absorbance of the blank was subtracted from that of the sample. The total flavonoid content was expressed as equivalents of rutin according to the equation obtained from the standard rutin graph.

Total Antioxidant Capacity by Phosphomolybdenum method

The total antioxidant activity of the samples was evaluated by phosphomolybdenum method according to Berk *et al.* (2011)¹⁸ with slight modification. Sample solution (0.3 mL) was combined with 3 mL of reagent solution (0.6 M sulfuric

acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The sample absorbance was read at 695 nm after 90 minutes incubation at 95°C. The total antioxidant capacity was expressed as equivalents of trolox as determined by the equation obtained from the standard trolox graph.

Free Radical Scavenging Activity (DPPH)

The effect of the sample on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical was estimated according to Sarikurkcü (2011)¹⁹. Sample solution (1 mL) was added to a 4 mL of a 0.004 % methanol solution of DPPH. The sample absorbance was read at 517 nm after 30 minutes incubation at room temperature in dark. The DPPH radical scavenging activity was expressed as equivalents of trolox, according to the equation obtained from the standard trolox graph.

Cupric Ion Reducing (CUPRAC) Method

The cupric ion reducing activity (CUPRAC) was determined according to the method of Apak *et al.* (2006)²⁰. Sample solution (0.5 mL) was added to premixed reaction mixture containing CuCl₂ (1 mL, 10 mM), neocuproine (1 mL, 7.5 mM) and NH₄Ac buffer (1 mL, 1 M, pH 7.0). Similarly, a blank was prepared by adding sample solution (0.5 mL) to premixed reaction mixture (3 mL) without CuCl₂. Then, the sample and blank absorbances were read at 450 nm after 30 minutes incubation at room temperature. The absorbance of the blank was subtracted from that of the sample. CUPRAC activity was expressed as equivalents of trolox according to the equation obtained from the standard trolox graph.

Ferric Reducing Antioxidant Power (FRAP) Method

The FRAP assay was carried out as described by Aktumsek *et al.* (2013b)²¹ with slight modification. Sample solution (0.1 mL) was added to premixed FRAP reagent (2 mL) containing acetate buffer (0.3 M, pH 3.6), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) (10 mM) in 40 mM HCl and ferric chloride (20 mM) in a ratio of 10:1:1 (v/v/v). Then, the sample absorbance was read at 593 nm after 30 minutes incubation at room temperature. FRAP activity was expressed as equivalents of trolox according to the equation obtained from the standard trolox graph.

Fatty Acid Analysis

The oil extraction of dried and powdered aerial plants (10 g) was carried out at 60°C for 6 h by Soxhlet extractor using petroleum ether as a solvent. The solvent was evaporated by rotary evaporator. The obtained oil was esterified to determine fatty acid composition. The fatty acids in the total lipid were esterified into methyl esters by saponification with 0.5 N methanolic NaOH and transesterified with 14 % BF₃ (v/v) in methanol²². FAMES were analyzed on a HP (Hewlett-Packard) Agilent 6890 N model gas chromatograph (GC), equipped with a flame ionization detector (FID) and fitted to a HP-88 capillary column (100 m length, 0.25 mm i.d. and 0.2 µm thickness). Injector and detector temperatures were set at 240 and 250°C, respectively. The oven was held at 160°C for 2 minutes. Thereafter the temperature was increased up to 185°C at rate of 4°C/min then increased up to 200°C at rate of 1°C/min and held at 200°C for 46.75 minutes. Total run time was 70 minutes. Helium was used as carrier gas (1 mL/min). Identification of fatty acids was carried out by comparing sample FAME peak relative retention times with those obtained for Alltech and Accu standards. Results were expressed as FID response area in relative percentages. Each reported result is given in the

average value of three GC analyses. The results are offered as means $\pm$ SD.

Volatile Component Analysis

The air-dried and ground plant material (200 g) was submitted for 5 h to water-distillation using a British-type Clevenger apparatus (ILDAM Ltd., Ankara-Turkey). The obtained essential oil was dried over anhydrous sodium sulphate and after filtration, stored at +4°C until tested and analyzed. The essential oil of sample was analyzed by GC-FID and GC/MS techniques. The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system coupled to an Agilent 7890A GC (Agilent Technologies Inc., Santa Clara, CA). HP-Innowax FSC column (60 x 0.25 mm, 0.25 μ m film thickness) was used with helium (purity 99.99 %) as carrier gas (1.2 mL/minute). The GC oven temperature was kept at 60°C for 10 minutes and programmed to 220°C at a rate of 4°C/minute, and kept constant at 220°C for 10 minutes and then programmed to 240°C at a rate of 1°C/minute. The split ratio was used at 40:1. The injector temperature was at 250°C mass spectra were recorded at 70 eV. Mass range was from 35 to 450 m/z. GC-FID analysis was carried out by simultaneous auto-injection using Agilent 7693A series auto sampler; 1 μ L injections were affected. The GC analysis was carried out using an Agilent 7890A GC system. In order to obtain the same elution order with GC/MS, simultaneous triplicate injections were done by using the same column and same operational conditions. The FID temperature was 300°C. The identification of constituents was achieved on the basis of retention index determined by co-injection with reference to a homologous

series of *n*-alkanes (C₈-C₃₀), under same experimental conditions. Further identification was carried out by comparison of their mass spectra with those from NIST 05 and Wiley 8th version and home-made MS library built up from pure substances and components of known essential oils, as well as by comparison of their retention indices with literature values²³.

RESULTS AND DISCUSSION

Total Bioactive Components

Phenolic compounds are the largest group of bioactive components in the plant; these compounds possess many biological properties including antioxidant, anti-inflammatory and anti-hyperglycemic^{24,25}. The total phenolic and flavonoid content of the methanol extract were evaluated by the Folin-Ciocalteu and AlCl₃ assays, respectively. The total phenolic and flavonoid content were found to be 33.11 mgGAEs/g and 24.74 mgREs/g extract, respectively (Figure 1). Zengin *et al.* (2011)²⁶ reported the total phenolic content of *C. urvillei* subsp. *hayekiana* was 17.12 mgGAEs/g extract in the assay. Total phenolic content of *C. urvillei* subsp. *stepposa* was observed to be higher than that of the *C. urvillei* subsp. *hayekiana*. In another study, the total phenolic content of *C. urvillei* subsp. *urvillei* was detected as 103.44 mgGAEs/g extract²⁷. From the result, it can be concluded that *C. urvillei* subsp. *stepposa* has lower phenolic content than *C. urvillei* subsp. *urvillei*. Many authors reported that differences were observed in phenolic content of plant species belonging to the same genus depending on several factors including temperature, soil content and altitude²⁸.

Table 1: Phenolic components in the methanol extract from *Centaurea urvillei* subsp. *stepposa*

No	Phenolic components	Concentration (μ g/g extract)	
		<i>C. urvillei</i> subsp. <i>stepposa</i>	
1	Protocatechuic acid	180 $\pm$ 10*	
2	(+)-Catechin	420 $\pm$ 8	
3	<i>p</i> -Hydroxybenzoic acid	nd	
4	Chlorogenic acid	2650 $\pm$ 60	
5	Caffeic acid	100 $\pm$ 2	
6	(-)-Epicatechin	nd	
7	Ferulic acid	170 $\pm$ 7	
8	Benzoic acid	nd	
9	Rutin	nd	
10	Rosmarinic acid	nd	
11	Apigenin	1300 $\pm$ 10	

*Values expressed are means $\pm$ S.D., nd, no detected

Table 2: Composition of essential oil of *Centaurea urvillei* subsp. *stepposa*

	Compounds	RT ^a	KI ^b	Composition (%)
1	Alpha-cubebene	31.171	1553	1.19
2	1-pentadecene	32.560	1574	3.41
3	Caryophyllene	34.762	1615	2.45
4	1,3-cyclooctadiene	42.207	1878	25.08
5	Caryophyllene oxide	45.820	2020	13.20
6	Cyclohexene, 6-(2-butenyl)-1,5,5 trimethyl	46.440	2045	2.74
7	3-cyclohexen-1-carboxaldehyde, 3,4 dimethyl	47.215	2077	1.39
8	2-Pentadecanone, 6,10,14-trimethyl	48.558	2134	1.03
9	Spathulenol	48.957	2151	6.13
10	Eicosane	52.457	2298	4.63
11	Ledene oxide(II)	55.090	2393	1.52
12	Pentacosane	58.545	2497	0.87
13	9,15-Occadecadienoic acid	58.877	2506	1.56
14	Phthalic acid	61.215	2563	3.57
15	Phenylethyl salicylate	77.940	2903	4.18
16	n-Hexadecanoic acid	78.949	2922	24.24
	Total identified			97.19

^aRetention time (as minutes), ^bKovats Index HP Innowax column in reference to n-alkanes

Table 3: Fatty acid composition of *Centaurea urvillei* subsp. *stepposa* (%)

Fatty acids	<i>Centaurea urvillei</i> subsp. <i>stepposa</i>
C8:0	0.03 ± 0.01 ^a
C10:0	0.03 ± 0.01
C11:0	0.02 ± 0.00
C12:0	0.62 ± 0.01
C13:0	0.03 ± 0.00
C14:0	0.39 ± 0.00
C15:0	0.21 ± 0.18
C16:0	12.21 ± 0.04
C17:0	0.27 ± 0.01
C18:0	3.25 ± 0.01
C20:0	1.48 ± 0.09
C21:0	0.07 ± 0.02
C22:0	0.21 ± 0.01
ΣSFA ^a	18.79 ± 0.21
C14:1 ω5	0.25 ± 0.00
C15:1 ω5	0.04 ± 0.03
C16:1 ω7	0.22 ± 0.01
C17:1 ω7	0.04 ± 0.01
C18:1 n9	21.85 ± 0.01
C18:1 n11	0.67 ± 0.00
C20:1 ω9	0.04 ± 0.00
C22:1 ω9	0.75 ± 0.02
ΣMUFA ^a	23.85 ± 0.02
C18:2 ω6	49.54 ± 0.16
C18:3 n6	2.33 ± 0.01
C18:3 n3	3.00 ± 0.01
C20:4 ω6	0.79 ± 0.00
C22:2 ω6	0.73 ± 0.01
C20:5 ω3	0.71 ± 0.01
C22:6 ω3	0.27 ± 0.03
ΣPUFA ^a	57.37 ± 0.23

^aValues expressed are means ± S.D., ^aSFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids

Figure 1: Antioxidant properties of the methanol extract from *Centaurea urvillei* subsp. *stepposa* (Values expressed are means ± S.D. of three parallel measurements)

Figure 2: Major phenolics of the methanol extract from *Centaurea urvillei* subsp. *stepposa* (a: Apigenin, b: Catechin, c: Chlorogenic acid)

Antioxidant Activity

The radical scavenging activity of the methanol extract was evaluated by DPPH assay. This assay based on the reduction DPPH solution (purple) in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H (yellow) by the reaction. The radical scavenging activity of the methanol extract was found to be 43.35 mgTEs/g extract (Figure 1). Reducing power assays are often used as an indicator of electron-donating activity, which are an important mechanism of antioxidant compounds, especially phenolics²⁹. Therefore, FRAP and CUPRAC methods were applied to evaluate reducing power potentials of the methanol extract. The FRAP assay depends on the reduction from the ferric ion Fe (III)/TPTZ complex to Fe (II)/TPTZ by antioxidants in extract. CUPRAC method is also based on reduction of Cu (I)-neocuproin to Cu (II)-neocuproin by antioxidants. FRAP and CUPRAC activities for the methanolic extract were determined as 52.18 and 98.78 mgTEs/g extract, respectively (Figure 1). As far as our literature survey could ascertain, DPPH radical scavenging and reducing power activity of several *Centaurea* species (*C. mucronifera*, *C. solstitialis*, *C. centaurium* and *C. huber-morathii*) were reported as IC₅₀ (% 50 of free radical inhibition of samples) or EC₅₀ value (the effective concentration at which the absorbance was 0.5)³⁰⁻³³. For these reason, direct comparisons between results of different studies is difficult or, in some cases, impossible. Total antioxidant activity of the methanol extract was determined by phosphomolybdenum assay. The phosphomolybdenum assay is based on reduction of Mo (VI) to Mo (V) and subsequent formation of a green phosphate/Mo (V) complex, which maximum absorption at 695 nm³⁴. The total antioxidant activity of the methanol extract was 335.98 mgTEs/g (Figure 1); these values are comparable to the values reported in the literature for other *Centaurea* species such as *C. cheirollopha* (234.77 mg ascorbic acid equivalents (AEs)/g)³⁵ and *C. antalyense* (248.74 mgAEs/g)²¹.

Phenolic Components

Phenolic constituents of the methanol extract were quantified by HPLC and results are shown in Table 1. The phenolic components of the methanol extract are reported for first time in this study. The major component present in the extract was quantified as chlorogenic acid (2650 µg/g extract) followed by apigenin (1300 µg/g extract) and catechin (420 µg/g extract), while the least abundant compound present in the extract was caffeic acid (100 µg/g extract) (Figure 2). Chlorogenic acid and apigenin may be responsible for the antioxidant properties of the methanol extract, as has been observed in other studies^{36,37}. Moreover, chlorogenic acid and apigenin possesses a wide range of biological effects, such as antimutagenic, antiviral, anticarcinogenic and enhancing cellular defense³⁸⁻⁴¹. In this direction, *C. urvillei* subsp. *stepposa* can be considered as a source of chlorogenic acid and apigenin.

Essential Oil Composition

Sixteen different components of *C. urvillei* subsp. *stepposa* essential oil were characterized by GC-MS and the results are presented in Table 2. 1, 3-cyclooctadiene (25.08 %) was the major essential oil constituent followed by hexadecanoic acid (24.27 %) and caryophyllene oxide (13.20 %). Hexadecanoic acid was reported as major compound in the essential oils of *Centaurea urvillei* subsp. *urvillei*, *C. calolepis*, *C. euxina*, *C. sibthorpii* and *C. amanicola*⁴²⁻⁴⁵. According to these results,

the essential oils of *C. urvillei* species were characterized with high contents of hexadecanoic acid. The result may be considerable interest for chemotaxonomic analysis. However, other components were identified as predominant compounds in essential oils from some *Centaurea* species. For example, Germacrene D (44.83 %) from *C. drabifolia* subsp. *Detonsa*⁴⁶ and *C. cineraria* subsp. *umbrosa*⁴⁷ and, β-eudesmol (26.5 %) from *C. cuneifolia*⁴⁵ were detected as the main volatile components. Changes in the composition of essential oil obtained from *Centaurea* species might have arisen from several different factors such as climatical, seasonal and geographical⁴⁸.

Fatty Acid Composition

The fatty acid composition of *Centaurea urvillei* subsp. *stepposa* was identified by GC-FID. The fatty acid profile is presented in Table 3. Twenty-eight fatty acids were identified, wherein C18:2 ω6 (linoleic acid), C 18:1 ω9 (oleic acid) and C 16:0 (palmitic acid) were defined as the major fatty acid in the oil. Similarly, linoleic acid was the most abundant in fatty acid composition of some *Centaurea* species such as *C. cheirollopha*, *C. pseudoscabiosa* subsp. *araratica*^{12,35}. Palmitic acid was the major saturated fatty acids (SFA) and other SFA were found in low amounts. Oleic acid was the main monounsaturated fatty acid followed by C 22:1 ω9 and C18:1 n11. The proportion of SFA, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were 18.79 %, 23.85 % and 57.37 %, respectively. PUFA was previously reported as the highest proportion fatty acid composition in the several *Centaurea* species^{3,49}. Linoleic and α-linolenic acid are called “essential fatty acids” because the human body is not able to produce them. In oil studied, levels of essential fatty acids were determined as approximately 51 % in percentage of all fatty acids. Therefore, the oil is considered to be a source of essential fatty acids.

CONCLUSION

The present work is shown for first time the antioxidant capacity, phenolic components, fatty acids and volatile components of *C. urvillei* subsp. *stepposa*. The extract showed moderate antioxidant properties. Chlorogenic acid and apigenin were the major phenolic components in the methanol extract. Linoleic acid was detected as the predominant fatty acid in the oil. 1, 3-cyclooctadiene and hexadecanoic acid were identified as the major components in essential oil. These findings suggest that *C. urvillei* subsp. *stepposa* is a valuable source of natural agents for human health and could be useful as additives for food, cosmetic or pharmaceutical applications

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