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A Study on Total Phenolic and Flavonoid Content, Antioxidant, Toxicity, and Anthelmintic Activities of Methanol Extract of *Crocus cancellatus* subsp. *lycuis*

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Abstract

The present study was planned to assess total phenolic and flavonoid content and some biological properties (antioxidant, toxicity, and anthelmintic) of the methanol extract of *Crocus cancellatus* subsp. *lycuis* from Muğla province (Turkey) for the first time. It was found that the total phenolic (2.08±0.06 mg GAE/g) and total flavonoid (3.63±0.09 mg QE/g) contents of the aerial part extract were higher than the bulb extract. The aerial part extract (IC_{50} value: 0,86±0,03 mg/mL, IC_{50} value: 0,26±0,003 mg/mL, 0,77±0,007 mg TE/g, and 5,92±0,30 mg TE/g, respectively) was determined to exhibit the higher activity than that of bulb parts extract in the assays shortly named as DPPH, ABTS, FRAP, and CUPRAC. Both extracts were observed to have anthelmintic activity on brine shrimp. According to these results, *Crocus cancellatus* subsp. *lycuis* can be accepted as a good source for the pharmaceutical and food industries.

Keywords: Anthelmintic, Antioxidant, Brine shrimp, Crocus, Flavonoid, Phenolic

1. Introduction

Natural products are important in the increases of health and avoiding illness and also, they have been known to have several biological features such as antioxidant, anti-inflammatory, and anti-apoptotic activities [1]. Seconder metabolites which are natural products generated and used by organisms for defense or adaptation remain the most significant resource with potential therapeutic properties [2]. Known to be of worldwide medicinal importance, plants have served as an important source of nutrition and also for the development of new treatments for diseases. Seconder metabolites synthesized by plants play important role in the discovery of novel drugs [3,4,5]. The Crocus genus which is a member of the large family Iridaceae including a large number of flowering plants is a perennial geophyte. Most *Crocus* species are native to the Mediterranean floristic region and distributed eastwards into the Iran-Turanian region [6,7]. This plant was reported to be widely cultivated in Iran, Morocco, India, Italy, Greece, Italy, Spain, and Afghanistan [7].

The *Crocus* genus has been reported to have biological activities like antimicrobial, anti-fungal, and antiseptic. The pharmacological properties of species belonging to the genus *Crocus* were reviewed by Mykhailenko et al. (2019) and Mohtashami et al. (2021) [6,7]. Turkey is a rich country in terms of Crocus species with about 70 *Crocus* taxa 31 of them are endemic to Turkey [8,9] the present study intended to examine the antioxidant, cytotoxic and anthelmintic potential, and to determine the amount of the total phenolic and total flavonoid contents of the methanol extracts of *Crocus cancellatus* subsp. *lycuis* from Muğla province (Turkey) for the first time.

2. Materials and Methods

2.1 Material

Crocus cancellatus subsp. *lycuis* were collected from Muğla province (28º22'34” K-28º22'18” D, 660 m, Ula-Menteşe) in November 2020 and were identified by Dr. Olcay Ceylan (Muğla Sıtkı Koçman University, Türkiye) in addition, a voucher specimen (O.2120) was kept in his herbarium.

2.2 Chemicals

DPPH, ABTS, BHA, Trolox, FCR, Sodium carbonate, gallic acid, and quercetin were obtained from Sigma-Aldrich (USA).

2.3 Methods
2.3.1 Preparation of plant extracts

For the extraction procedure, the dried samples (aerial and bulb) in shadow at room temperature were extracted with methanol at 50ºC for 24 h using a water bath. After filtration, methanol used as solvent was evaporated with a rotary evaporator (Heindolph LABOROTA 4011). The crude extracts were deposited at -20ºC [10].

2.3.2 Determination of total phenolic and flavonoid content of extracts

The total phenolic and flavonoid contents of aerial and bulb methanol extract were determined according to the methods described by Singleton and Rossi (1965) and Aryal et al., (2019), respectively [11,12]. The results were calculated as equivalents of gallic acid (mg GAE/g extract) and quercetin (mg QE/g extract), respectively.

2.3.3 Determination of antioxidant activity

For determination of the antioxidant activity of the extract, DPPH (2,2-diphenyl-1-picrylhydrazyl) [10], ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) [13] radical scavenging activity assays, FRAP (ferric reducing antioxidant power) assay [14] and CUPRAC (cupric ion reducing antioxidant capacity) assay [15] were carried out. IC_{50} (half max inhibitory concentration) values were estimated for DPPH and ABTS assays. The results of FRAP and CUPRAC assays were expressed as equivalents of Trolox (mg TE/g extract).

2.3.4 Brine shrimp lethality bioassay

The brine shrimp lethality assay described by Krishnaraju et al. (2005) was carried out with some modifications for evaluation of the cytotoxic potential of the extracts. 10 mg of *Artemia salina* eggs were added to an aquarium with maintaining aeration containing 500 ml of artificial seawater (38 g/L of sea salt) at 28°C and kept for 48 h. 0.5 mL of each extract at different concentrations (0.01-1 mg/mL) were added to 4.5 mL of brine solution including 10 nauplii. Following 24 h, surviving larvae were counted under the light [16].

2.3.5 Determination of anthelmintic activity

The anthelmintic activities of the extracts were performed using a nearly 1-2 cm size of *Tubifex tubifex* (Annelida) according to the method described by Dutta et al. (2012) [17]. In brief, 20 ml of extract at different concentrations (2.5-20 mg/mL) dissolved in distilled water was transferred to a petri dish and then 6 worms were added. Albendazole as a reference standard (20 mg/mL) and distilled water were positive and negative controls, respectively. The worms were evaluated in terms of motility. When worms without motility were vigorously shaken, the time of paralysis and death were recorded if any movement and if no movement were perceived, respectively.

2.3.6 Data analysis

The results from the assay performed in triplicate were expressed as mean±SE (Standard Error). The IC_{50} (half maximal inhibitory concentration) values for DPPH and ABTS assay, and also LC_{50} value (half maximal lethality concentration) for brine shrimp lethality assay were calculated using the Statistical Package for the Social Sciences (SPSS) software version 22.0 for Windows. Analysis of Variance (ANOVA) and Tukey ($P<0.05$) test was performed to determine the difference in the groups.

3. Results and Discussion
3.1 Total phenolic and total flavonoid contents of the extracts

Phenolic compounds are important in human health due to their biological properties such as antioxidant activity, which helps to eliminate oxidative stress that can be associated with various diseases such as cancer, neurodegenerative, and others [18]. In the present study, the total phenolic and total flavonoid contents were found to be high in an aerial part extract with values of 2,08±0,06 mg GAE/g and 3,63±0,09 mg QE/g, respectively (Table 1).

Table 1. Total phenolic and total flavonoid contents of *Crocus cancellatus* subsp. *lycuis* extracts

Plant Extracts	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg QE/g)
Aerial Part	2,08±0,06[a]	3,63±0,09[a]
Bulb Part	0.59±0.02[b]	0.19±0.007[b]

The different letters in a column are significantly different ($p<0.05$)

Our results were showed lower values when compared to those reported by Loizzo et al (2016), who declared that total phenols and total flavonoids for *C. cancellatus* subsp. *damascenus* stigmas extract (Ethanol %70) were 146.8±2.5 mg chlorogenic acid equivalents per g extract and 57.5±1.2 mg quercetin equivalents per g extract, respectively [19]. The total phenolic contents of methanol, ethanol, and boiling water extracts of stigmas of C. sativus from İran, with values of 6.54, 6.35, and 5.70 mg GAE/g of dry weight (DW), respectively were found to be higher than those of our extracts. In addition, the total flavonoid content of these *C. sativus* stigmas extracts was reported as 5.88, 2.91, and 3.68 mg rutin equivalent/g DW, respectively [20]. On the other hand, Satybaldiyeva et al. (2015) showed that the total phenolic contents of the extracts from aerial and bulb parts of *C. alatavicus* (Kazakhstan) using different solvents (distilled water, 96% ethanol, 99% methanol, and 99% dichloromethane) were ranged from 13.63 to 72.29 mg GAE/g extract and that the ethanolic extract of aerial part contained the highest total phenolic content [21]. Under different brewing conditions, total polyphenol, and flavonoid contents in stamens and tepals of *C. sativus* from Italy were reported to be in the range of 83-186 mg GAE/L and 34-91 mg CE (catechin equivalents)/L, respectively [22]. It was shown that the total phenolic content of the extracts of the different parts of *C. pallasi* was in the range of 13.48-28.92 mg GAE/g, while the total flavonoid content was in the range of 0.50 - 31.44 mg RE (rutin equivalent)/g [23]. According to these results, the total phenolic and total flavonoid content of the *Crocus* species may vary depending on the plant species, the geographical region where it was collected, the parts of the plant used, and the extraction method.

3.2 Antioxidant activities of the extracts

The different assays (DPPH, ABTS, FRAP, and CUPRAC) were applied to evaluate the antioxidant activity of the extracts (Table 2).

Table 2. Antioxidant activity of *Crocus cancellatus* subsp. *lycuis* extracts

Plant Extracts	DPPH (IC_{50}, mg/mL)	ABTS (IC_{50}, mg/mL)	FRAP (mg TE/g)	CUPRAC (mg TE/g)
Aerial Part	0,86±0,03^{b}	0,26±0,003^{b}	0,77±0,007^{a}	5,92±0,30^{a}
Bulb Part	1,11±0,01^{c}	0,61±0,035^{c}	0,46±0,003^{b}	1,33±0,07^{b}
BHA	0,009±0,0002^{a}	0,008±0,004^{a}		

The different letters in a column are significantly different ($p<0.05$)

In the DPPH and ABTS radical scavenging assays, the antioxidant activity of both extracts was lower than the BHA as control. However, when compared to the bulb extract, the extract of the aerial parts was determined to exhibit high activity with the IC_{50} values of 0,86±0,03 and 0,26±0,003 mg/mL, respectively. In addition, the extract of the aerial parts (0,77±0,007 and 5,92±0,30 mg TE/g) showed higher antioxidant activity than the bulbs extract, in terms of FRAP and CUPRAC assays which were performed to determine the reducing power potentials of the extracts. Phenolic compounds based on plants have been known to show antioxidant activity [24]. The reason why the aerial part shows higher activity than the bulb part maybe it contains more phenolics.

Antioxidants are of great importance in neutralizing the harmful effects of free radicals on human health. Due to the possible negative effects of synthetic antioxidants for example BHA (butylated hydroxyl anisole) and BHT (butylated hydroxyl toluene) on health, the search for natural antioxidants instead them is of great interest. The various plant samples have been reported to be a source of antioxidants with a natural origin [25]. There are various outcomes in the literature displaying the antioxidant activities of the genus *Crocus* [6]. Contrary to the current study, the extract of the bulb of *C. cancellatus* herb. gained from Qarachugh mountain (Erbil) showed better antioxidant activity at all concentrations tested in the DPPH assay compared to the BHT served as control [26]. Stigma extract of C. ca*ncellatus* subsp. *damascenus* from Iran was reported to cause the DPPH and ABTS radical scavenging activity with IC50 values of 34.6 and 21.6 µg/mL, respectively, and exhibit a good reducing potency (53.9 mM Fe (II)/g) [6]. The DPPH activity of the extracts tested herein is less than the methanol extracts of aerial parts and bulbs of *C. alatavicus* from Almaty (Kazakhstan) because its IC50 values were shown as 510 and 853 µg/mL, respectively [21]. Also, several Crocus species for example *C. caspius* [27], *C. sativus* [28], and *C. pallasi* [29] were declared to exhibit antioxidant activity.

3.3 Toxicity of the extracts on brine shrimps

The brine shrimp lethality assay is one of the significant bioassays for the pre-screening of cytotoxicity of the plant extracts and is used for evaluating the preliminary screening of the anti-cancer activity of these extracts [30,31]. The present study revealed that the mortality percentage of shrimp nauplii increased depending on the increasing concentrations of both extracts (Table 3). According to Meyer et al. (1982), the aerial parts and bulb extracts with LC_{50} values of 0.014 and 0.012, respectively were found to be active, because the LC_{50} values were recorded as lower than 1000 µg/mL for each extract tested [32].

Table 3. Average mortality rates (%) of *Artemia salina* after 24 h of exposure to *Crocus cancellatus* subsp. *lycuis* extracts at different concentrations

Concentrations	Aerial Part 24 h Later (%)	Bulb Part 24 h Later (%)
0.01 mg/mL	52.77	55.55
0.05 mg/mL	61.11	63.88
0.1 mg/mL	72.22	72.22
0.5 mg/mL	85.55	88.88
1.0 mg/mL	100	100
Negative Control*	11.36	11.36
LC_{50} (mg/mL)	0,014	0,012
LC_{90} (mg/mL)	0,523	0,423

*Negative Control: Distilled water

Similarly in our study, the ethanolic aerial part extract of *C. alatavicus* was stated to cause a high cytotoxic activity on brine shrimp with an LC_{50} value of 15.75 µg/ml [21].

3.4 Anthelmintic activities of the extracts

Tubifex tubifex belonging to the same group of annelids as the intestinal roundworm parasites of humans was used in this study due to anatomical and physiological resemblances to those found in human beings. The anthelmintic activity was determined to increase with the increasing concentration of the extract tested (Table 4).

Table 4. Anthelmintic activity of *Crocus cancellatus* subsp. *lycuis* extracts

	Concentration (mg/mL)	P (Min)*	D (Min)**
Aerial Part	2,5	8	12
	5	4	6
	10	2	3
	20	1	2
Bulb Part	2,5	13	23
	5	5	10
	10	2	5
	20	1	3
Positive Control***	20	3	7
Negative Control****	-	-	-

*P=Time taken for paralysis. **D=Time taken for death of worms. ***Positive Control: Albendazole® ****Negative Control: distilled water

The aerial and bulb methanol extracts of the plant showed higher activity than the albendazole used as the control. The aerial extract exhibited a higher anthelmintic activity than the bulb extract. When compared in terms of paralysis and death times, at 20 mg/mL, the anthelmintic activity of both extracts was seen to be better than the standard. Helminth infections and anthelminthic resistance are among considerable health concerns for both animals and humans. Plants as an important resource in the research of new bioactive natural agents may be greatly significant for providing some advantages such as obtaining substances with anthelmintic properties which are safe for animal and human health. Investigations concerning plants that have anthelmintic properties continue to be important [17,33,34].

4. Conclusion

To the best of our knowledge, the properties of the methanol extracts of *C. cancellatus* subsp. *lycuis* from Muğla province (Turkey), which were investigated here, were determined for the first time in the present study. The aerial part extract of *C. cancellatus* subsp. *lycuis* was found to be richer than the bulb part extract in terms of total phenolic and flavonoid contents. In addition, the aerial extract exhibited higher antioxidant activity than the bulb extract. *C. cancellatus* subsp. lycuis extracts were observed to exhibit toxicity on brine shrimp, and anthelmintic activities. The results of the present study suggest that the *C. cancellatus* subsp. *lycuis* is capable of being evaluated as a natural source for different applications such as the pharmaceutical and food industry, due to its biological properties determined here. This study will allow its application to further studies for the isolation and identification of substances with bioactive properties from *C. cancellatus* subsp *lycuis*.

Author's Contributions

Mehlika Alper: Helped with data interpretation and statistical analysis and wrote the manuscript.

Mehmet Özgür Atay: Supported the antioxidant test

Olcay Ceylan: Helped wrote the manuscript

Ramazan Mammadov: Performed the project, researched the design of all experiments, and wrote the manuscript.

Ethics

There are no ethical issues after the publication of this manuscript.

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