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Phenolic profiles and brine shrimp cytotoxicity of the ethanolic extract from the aerial part of *Crocus alatavicus* L.

Abstract: The aim of this study is to investigate the phenolic profiles and evaluate the brine shrimp cytotoxic activity of the ethanolic extract from the aerial part of *C. alatavicus*, an endemic species of Kazakhstan flora. Nine phenolic compounds were identified and quantified in the extract by high-performance liquid chromatography method. Preliminary cytotoxicity of the extract was determined by the brine shrimp (*Artemiasalina*) assay. The results reveal that the ethanolic extract from the aerial part of *Crocus alatavicus* exhibit a high cytotoxicity with LC_{50} 15.71 μg/mL.
Key words: *Crocusalatavicus*, phenolic compound, cytotoxicity, brine shrimp, extract.

Introduction

Phenolic compounds are the substances containing benzoic nucleus with one or several hydroxylic groups and their derivatives. Higher plants synthesize several thousand known different phenolic compounds. These compounds are the products of secondary metabolism. Phenolics are produced in plant organism for protection against environmental stress, such as high light, low temperatures, UV radiation, various pathogens and nutrient deficiency. Phenolics play important roles in plant development, provide structural integrity and scaffolding support to plants [1].

Crocus alatavicus is an early spring ephemeral and geophytic-geocarpic species of the Iridaceae family. It is an endemic of Kazakhstan flora belonging to the group of bulbous and tuberous plant. Wild habitat of this species is the Kora river middle flow, Kopal district, Taldykurgan region and South-East Kazakhstan [2]. The species is interested as a source of naturally active substances that have many useful biological properties. Accumulation of the bioactive compounds is associated with the geophytic life-form of this plant and its ephemeral development cycle.It was usedas spasmolytic, anti-inflammatory, bactericidal and antiviral agents in traditional medicine [3]. The flowers were usedas diuretic, for treatment of abdominal illness and to improve hormonal regulation of women [4]. Dried stigmas are appliedfor coloring food products and impart flavor for them [5].

Phytochemical constituents and biological activities of this species insufficiently explored. It is revealedthe predominant content of secondary metabolites in the aerial part of this plant [6]. The leaves contain ascorbic acid and the stigmas of flowers contain yellow pigment available for food colorant [7]. Anthocyanins such as delphinidin 3-*O*-β-rutinoside and petunidin 3-*O*-β-rutinoside and flavonoids such as myricetin, quercetin, kaempferol have been investigated in flowers [8].Total antioxidant, radical scavenging and antibacterial activities of the various extracts from different part of *C. alatavicus*were reported [6].

Investigation of the phytochemical constituents and potential biological properties of plants is importance for their use in different industries. In recent years, the medicinal properties of plants are investigated due to their potent pharmacological activities. Among the metabolites of plants, phenolicshave been examined for their biological activities including antioxidant, radical scavenging, antibacterial and cytotoxic activities.

The purpose of this study is to investigate the phenolic profiles and evaluate the cytotoxic effect of the ethanolic extract from the aerial part of *C. alatavicus*.

Materials and methods

Plant material and preparation of the extract

The aerial part of *C. alatavicus* was collected in Almaty region (43^{0}22’14’) at the flowering phase in March, 2015. Collected materials were cleaned, air dried in the shade at room temperature.

Powdered samples (10 g) were three times extracted with 100 ml of 96% ethanol at 60 ^{o}C for 6

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hours in a water bath shaker. After the filtration with Watman No: 1 filter paper the solvent was concentrated under vacuum (48-49 °C) by evaporating to dryness. The extract were stored at -20 ºC until use.

HPLC analysis was performed according to the method of Caponio [9]. The extract sample was prepared by dissolving the 0.1 g of the dried extract in 1 ml methanol followed by filtration over 0.45 µM Nylon Syringe Filter. 9 standard phenolic compounds (gallic acid, 3.4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, cinnamic acid) have been analyzed.

The operating mode of HPLC:

Chromatograph: Shimadzu Prominence

Column: Agilent Eclipse XDB-C18 (250 mm x 4.6 mm, 5 µm particle size)

Mobile Phase: A: 3% formic acid; B: methanol

Injection Volume and Flow Rate: 20 µl, 1 ml/min

Detector: DAD (SPD-M20A) (λ=280 nm)

Column Oven and Temperature: CTO-10 ASvp, 30°C

Pump: LC-20 AT

Auto Sampler: SIL-20ACHT

Computer Program: LC Solution

Brine Shrimp Test

The test was performed as described in Meyer [10] and McLaughlin [11]. The extract was dissolved in artificial seawater and was tested at the concentrations of 10, 50, 100, 500 and 1000 µg/ml. Brine shrimp eggs (*Artemia salina* Leach, USA) were hatched in artificial sea water at room temperature.

After 48 h, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Ten nauplii were placed in each vial containing 4.5 ml of brine solution and 0.5 ml of the plant extractand maintained at room temperature for 24 h under the light. After 24 h, the number of surviving shrimps at each concentration of the extract were counted and LC50 values were calculated using EPA probit analyzer version 1.5. The test was performed in triplicate.

Results and their discussion

According to the previous study [6] among the various extracts from *C. alatavicus* the highest total phenolic content (72.29 mgGAE/g) was observed in the ethanolic extract from the aerial part. It is the reason to investigate some phenolic compounds in this extract.

In this study, 9 standard phenolic compounds (gallic acid, 3.4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, cinnamic acid) have been analyzed.

The phenolic compounds were identified based on their retention times (RT) and quantified according to the respective standard calibration curves. Each of the phenolic compound was expressed as µg/g of extract.

The chromatograms of the standards and the ethanolic extract from the aerial part of *C. alatavicus*are presented in Figure 1.

Quantitative results (Table 1) show that 4-hydroxybenzoic acid (1491.1 ± 0.13 µg/g) has the highest concentration value over other benzoic acid derivatives: gallic acid = 289.8 ± 1.03µg/g, vanillic acid = 156.7 ± 0.03 µg/g and 3.4-dihydroxybenzoic acid = 59.6 ± 0.82 µg/g. Hydroxicinnamic acid derivatives as caffeic acid (468.7 ± 0.44 µg/g), ferulic acid (397.3 ± 0.02 µg/g), chlorogenic acid (77.5 ± 0.09 µg/g) and p-coumaric acid (20.2 ± 0.28 µg/g) also were identified and quantified in the extract. Unsaturated carboxilic acid namely cinnamic acid was determined in quantities of 238.3 ± 0.61 µg/g extract.

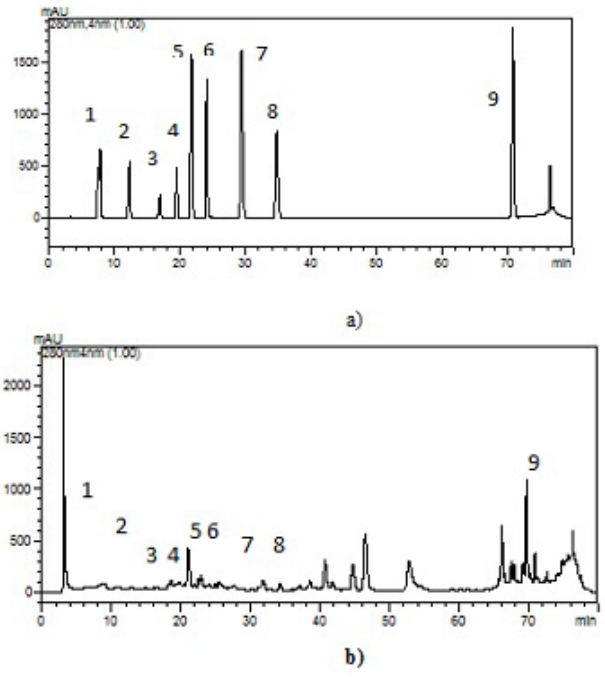

Figure 1 – HPLC profiles: a) chromatogram of the standards; b) chromatogram of the phenolic compounds in ethanol extract from the aerial part of *C. alatavicus:*
1 – gallic acid; 2 – 3.4-dihydroxybenzoic acid; 3 – 4-hydroxybenzoic acid; 4 – chlorogenic acid; 5 – vanillic acid; 6 – coffeic acid; 7 – p-coumaric acid; 8 – ferulic acid; 9 – cinnamic acid.

Table 1 – RTs (min) and values of regression (r^2) for standard phenolic compounds and their content (μg/g) in the ethanolic extract from the aerial part of *C. alatavicus*

Peak no	Phenolic compound	RT ± SD	r^2	Content in the extract: mean ± SD (μg/g)
1	Gallic acid	7.8 ± 0.05	>0.9999	289.8 ± 1.03
2	3.4-Dihydroxybenzoic acid	12.2 ± 0.03	>0.9998	59.6 ± 0.82
3	4-Hydroxybenzoic acid	16.9 ± 0.05	>0.9999	1491.1 ± 0.13
4	Chlorogenic acid	19.4 ± 0.04	>0.9994	77.5 ± 0.09
5	Vanillic acid	21.7 ± 0.06	>0.9991	156.7 ± 0.03
6	Coffeic acid	24.6 ± 0.08	>0.9997	468.7 ± 0.44
7	p-Coumaric acid	29.3 ± 0.05	>0.9998	20.2 ± 0.28
8	Ferulic acid	34.7 ± 0.08	>0.9998	397.3 ± 0.02
9	Cinnamic acid	70.7 ± 0.07	>0.9998	238.3 ± 0.61

The same content of p-coumaric acid (25.36 μg/g) was detected by HPLC in the methanolic extract of *C. baytopiorum*[12]. Cumaric, chlorogenic and gallic acid was identified in the methanolic extracts of *C. sativus* stigmas [13]. Karimi et al. [14] have the concentration of gallic acid found in *C. sativus* stigmas to be 1.82 mg/g dry sample. It is markedly higher compared to the gallic acid content (289.8 μg/g) in ethanolic extract from aerial part of *C. alatavicus*.

Brine shrimp bioassay is one of the most useful and rapid tool for the screening of biochemical activity and it is used to determine the toxicity of a wide variety of products. This method is considered as a broad measure of antitumor activity [15]. It is the first study on cytotoxicity of *C. alatavicus*.

The results (Table 2) reveal that the extract from the aerial part of *C. alatavicus* exhibit LC_{50} with 15.71 μg/mL. According to Meyer [10], extracts obtained from natural products which have $LC_{50} \leq 1.0$ mg/mL are known to possess toxic effects.

The ethanolic extract from the aerial part of *C. alatavicus* possesses high brine shrimp cytotoxic activity and it probably due to the presence of phenolic hydroxyl group in the extract.

Table 2 – Brine shrimp cytotoxicity of *C. alatavicus*

Part of plant	Concentration, μg/ml	Number of shrimps surviving after 24 h			Total number of surviving shrimps	Lethality,%	LC_{50}, μg/ml
		T_1*	T_2	T_3			
Aerial part	10	3	2	2	7	76.6	15.71±0.5
	50	2	2	1	5	83.3	
	100	0	2	1	3	90.0	
	500	0	1	0	1	96.6	
	1000	0	1	0	1	96.6	
T*- parallels of the experience							

In many studies it is reported the high cytotoxicity of *C. sativus*. Safronal andcrocin isolated from *C. sativus* shows cytotoxicity against *Artemiasalina* with LC_{50} 14.3 μg/mL and 147.036 μg/mL, respectively.

High inhibitory effects of the main activity substances of saffron were found in suspension of *Agrobacterium tumefaciens* (LC_{50} 0.31 and 2.34 μg/mL) [16].

Anticancer, anti-tumor and cytotoxic effects of saffron and its constituents have been studied on another test-systems [17].

Conclusions

This is the first report that shows the phenolic compound analysis and brine shrimp cytotoxicity of *C. alatavicus* extract from aerial part. Some phenolic compounds were identified and quantified by HPLC. Analized extract showed a high brine shrimp cytotoxicity and can be considered as a promising candidate for a plant derived antitumor agent. Future studies should be carried out to determine the cytotoxicity of *C. alatavicus* on cancer cell lines *in vitro*.

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