ORIGINAL ARTICLE

PHARMACOGNOSTIC AND PHARMACOLOGIC SCREENING OF *CROCUS SATIVUS* OF GREEK ORIGIN

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Abstract

The main aim of the present study was to characterize and asses the antioxidant and biological activities of hydrophilic and lipophilic extracts from *Crocus sativus* from Greece. The bioactive compounds were analysed using high performance liquid chromatography (HPLC), the most important identified compounds being kaempferol derivatives, safranal and picrocrocin. Total phenolic content was also determined. The antioxidant activity of hydrophilic and lipophilic extracts of *Crocus sativus* of different origins was investigated, the hydrophilic extract of Greek origin showing a good antioxidant activity compared to the other types of extracts. The impact of Greek sample of *Crocus sativus* on memory in an Alzheimer's disease rat model was also studied, showing promising results in restoring short-term and long-term memory impairment induced by administration of beta-amyloid peptide (Aβ-(25-35)).

Rezumat

Principalul obiectiv al studiului de față a fost reprezentat de caracterizarea și evaluarea potențialului antioxidant și a activităților biologice a unor extracte obținute de la specia *Crocus sativus* de proveniență grecească. Compușii bioactivi au fost analizați folosind cromatografia de lichide de înaltă performanță (HPLC), cei mai importanți compuși identificați fiind safranalul și picrocrocina. În cadrul studiului a fost determinat și conținutul în polifenoli totali. În ceea ce privește activitatea antioxidantă, s-a observat că extractul hidrofil al probei de origine greacă a prezentat o acțiune mai bună în comparație cu extractul lipofil. Totodată, a fost studiat și efectul extractelor asupra memoriei în cadrul unui model experimental de Alzheimer indus cu beta-amiloid (Aβ-(25-35)) la șobolan, observându-se o îmbunătățire a memoriei de scurtă durată și a memoriei de lucru. Rezultatele obținute indică o posibilă utilizare a unor extracte de șofran în tulburări neurologice.

Keywords: *Crocus sativus*, antioxidant activity, Alzheimer's disease, beta-amyloid peptide

Introduction

Saffron is the vernacular name for the stigmata from the flowers of *Crocus sativus* L. (*Iridaceae*). The area where *Crocus sativus* is grown spreads from the Mediterranean Sea through Persia to India, Tibet and China. Relatively new cultivations have been created in Mexico and Australia. Quality saffron is characterized by its typical combination of bitter taste, aromatic smell and intense red colour. Its bitter taste originates from picrocrocin, a β-D-glucoside of hydroxysafranal [9, 10, 17]. According to ISO commercial, saffron is defined as the stigma of *Crocus sativus* dried, dark red in colour and trumpet shaped, serrated or indented at the distal end. The length measures 20 - 40 mm. The stigmas may be isolated or joined in pairs or in triplicate at the end of the portion of the style, which is whitish yellow in colour [8]. The characteristic aroma of saffron is given by safranal, a mono-terpene aldehyde that accounts for almost 70% of the components in the volatile oil of the species [2, 9, 13]. Other important components are crocins, water-soluble glycoside carotenoids which represent the most important pigments that determine saffron's characteristic red colour [2, 13, 17]. Among its numerous potential biological effects, saffron might also play a key role in the prevention and treatment of Alzheimer's disease [2, 16, 17]. The mechanism has not been clearly established, but it might be related to the inhibition of amyloid fibrils production [12, 13].

Materials and Methods

Plant material. For our study we used a sample of *Crocus* stigma purchased from Greece in the summer of 2015. Prior to extraction, the dried sample was grounded in a laboratory grinder. 2 g of stamens were added to the flask with ethyl acetate and 60% ethanol respectively. The ground saffron stamens were mixed and macerated with solvent. For efficient extraction, the samples were kept in an ultrasound

bath shaker for 2 h. Magnetic stirring and continuous rotary shaking were employed to enhance molecular interactions during extraction process. To ensure maximum extraction of the secondary metabolites, the extraction process was repeated 5 times and the extracts were pooled together [10]. Samples were filtered and subjected to centrifugation in order to remove any floating particulate matters. The obtained extracts were concentrated at reduced pressure in a rotary evaporator at 40ºC to prevent degradation of heat-sensitive compounds. The compounds were then identified by chromatographic methods.

HPLC determination. HPLC analyses were carried out on an UltiMate3000 Thermo instrument equipped with a P580 binary high-pressure pump, a PDA-100 detector, a TCC-100 Column Compartment and an ASI-100 Automated Sample Injector. Collected data were processed through a Chromeleon Chromatography Information Management System v7.20. Chromatographic runs were performed using reverse-phase column (Accucore XL C18 column 50 x 4.6 x 4). Analyses were run by using 250 µL of the 0.1% working solutions further diluted to 1 mL with water. Saffron metabolites were eluted with a gradient of acetonitrile in water (from 5% to 80% in 25 min). The solvent flow rate was 1 mL/min, the temperature was kept at 25ºC and the injector volume selected was 20 µL. Identification was carried out at 257 nm for picrocrocin and at 330 nm for all crocetin esters [3].

Total phenolic content (TPC). The improved Folin-Ciocalteu method outlined by Singleton and Rossi specifies several conditions which were adapted to our lab as follows: 0.04 mL of sample was mixed with 0.6 mL of 20% Na_2CO_3 and 0.2 mL of Folin-Ciocalteu reagent [5]. After proper mixing in a Vortex, the samples were incubated for 2 h at 20°C and its absorbance was measured at 765 nm. Results were expressed as mg gallic acid equivalents (GAE)/g extract.

In vitro antioxidant activity. We used two well-known methods to assess the antioxidant potential: DPPH and ABTS techniques. The activity against DPPH radical was measured according to the previously described method and the absorbance was measured at 517 nm [5, 11]. ABTS radical cation was generated by incubation of equal volumes of 7 mM ABTS and 2.45 mM potassium persulfate solutions in the dark at room temperature, for 12 - 16 h. The ABTS stock solution was diluted to get an absorbance of 0.70 ± 0.02 at 734 nm [15]. Free radical scavenging activity was determined by mixing 100 µL of each extract (0.62 - 20 mg/mL in DMSO) with ABTS radical solution in a total volume of 2 mL; the decrease in absorbance was measured after 6 min.

Rutoside and butylated hydroxyanisole (BHA) were used as references. The scavenging activity (A%) against both DPPH and ABTS was estimated by the following formula:

$$A\% = 100 \times (A_{control} - A_{sample})/(A_{control}),$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance in the presence of extracts/positive controls.

Biological activity in vivo. 60 male Wistar rats (200 ± 30 g, housed in a temperature and light-controlled room, fed and allowed to drink water *ad libitum*) were divided into 6 groups (10 animals per group): (1) control group with saline treatment (0.9% NaCl); (2) beta-amyloid peptide 25-35 (Aβ(25-35)) alone-treated group (by intracerebroventricular injection of 400 µmol of beta-amyloid peptide 25-35, Rat, Sigma-Aldrich, Germany), 20 days prior to testing, (3) Aβ(25-35) - treated group received by i.p. administration 5 mg of *Crocus sativus* hydrophilic extract Aβ(25-35) + (a); (4) Aβ(25-35) - treated group received by i.p. administration 10 mg of *Crocus sativus* hydrophilic extract Aβ(25-35) + (b); (5) Aβ(25-35) - treated group received by i.p. administration 5 mg of *Crocus sativus* extract lipophilic Aβ(25-35) + (c); (6) Aβ(25-35) - treated group received by i.p. administration 10 mg of *Crocus sativus* lipophilic extract Aβ(25-35) + (d). The first two groups of animals were caged in similar conditions and did not receive saffron extracts. Tested animals were treated in accordance with the guidelines of animal bioethics in compliance with the European Council Directive of 24th November 1986 (86/609/EEC) with the approval of the local Ethics Committee.

Y-maze task

Short-term memory was assessed by spontaneous alternation behaviour in the Y-maze task. The Y-maze used in the present study consisted of three arms (35 cm long, 25 cm high and 10 cm wide) and an equilateral triangular central area. The maze was cleaned with a 10% ethanol solution and dried with a cloth before each animal was tested. 60 min after the administration of *Crocus sativus* extracts, rats were placed at the end of one arm and allowed to move freely through the maze for 8 min.

Spontaneous alternation behaviour was defined as entry (the hind paws were completely in the arm) into all three arms on consecutive choices. The number of maximum spontaneous alternation behaviour was the total number of arms entered minus 2, whereas spontaneous alternation percentage was calculated as the ratio between the actual alternations and maximum alternations and multiplied with 100. Spontaneous alternation behaviour is considered to reflect spatial working memory, which is a form of short-term memory.

Radial arm-maze task

The radial arm-maze (32 cm in diameter) used in the present study consisted of 8 extending radially

arms. Each arm had at its end a food cup with a single 50 mg food pellet (bait). Prior to the test, the animals were kept on restricted diet (body weight maintained at 85%) over a week period, with water being available *ad libitum*. After accommodation to the maze, when the rats could explore for 5 min and take the food freely, the training for 4 days (5 consecutive trials per day) consisted in getting animals to the end of the arms and eating the bait. The end of the trial was considered either when all 5 baits were consumed or when 5 min have elapsed (set as the performance criteria). For the actual test, each animal was placed individually in the centre of the maze (cleaned with a 10% ethanol solution and dried between animals) and subjected to reference and working memory tasks, in which same 5 arms were baited as in training trial. Since animals prefer to solve the maze using an adjacent arm selection, we altered adjacent arm patterning behaviour by only baiting 5 arms (nos. 1, 2, 4, 5 and 7) forcing the tested animals to change their strategy to avoid the empty arms. Working memory errors (entering twice a baited arm), reference memory errors (entering an arm that was empty) and the necessary time to consume all food were registered.

Results and Discussion

Regarding HPLC determination, the identified compounds were: kaempferol glycosides, safranal, picrocrocin, catechin, luteolin, caffeic acid and gallic acid. The richest extract in safranal and picrocrocin was the lipophilic extract. Crocin esters were also present in the investigated samples. Such results were previously published in scientific literature for samples harvested from Italy [3].

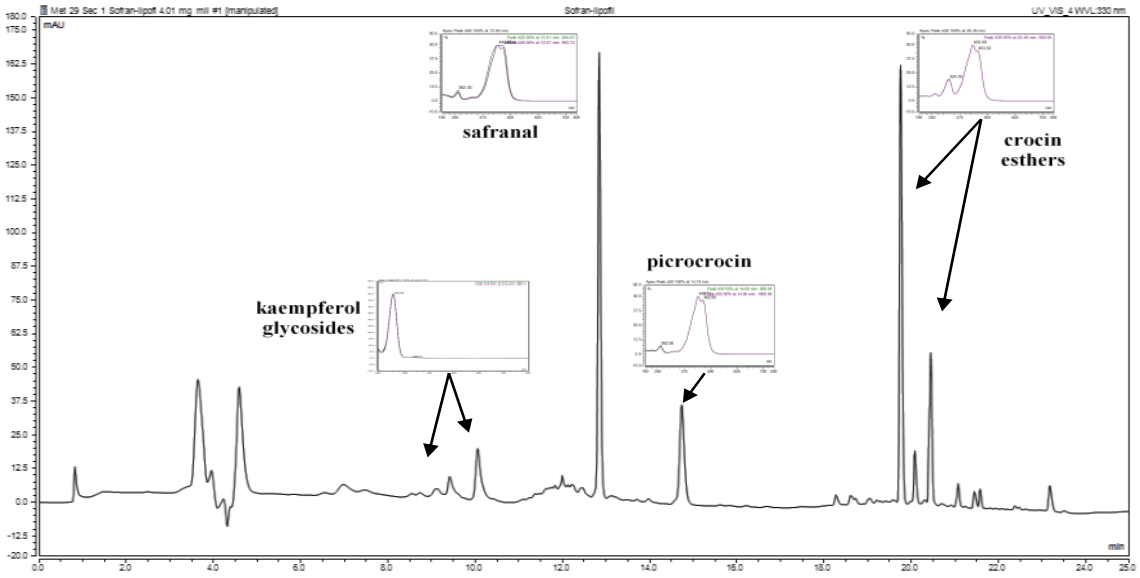

Figure 1.
Chromatogram for the lipophilic extract of *Crocus sativus* of Greek origin

Therefore, our results indicated that the investigated sample is genuine *Crocus sativus* L. and it was not falsified with other species. The most important marker compounds are glycosylated kaempferols, safranal and crocins which were present in our sample. Although gas chromatography is preferred for safranal detection, the HPLC method is often employed by other researchers [9, 10]. Moreover, in recent years, the concern regarding the quality of this spice has grown and therefore certifying the plant material identity is very important.

Total phenolic content. Knowing that polyphenols are important secondary metabolites for the growth of the plants, further studies were conducted to establish possible correlations between the quantity of the chemical compounds and its activity.

The results confirmed that the quantity of total phenols in the investigated extracts is quite low in the lipophilic extract (0.139 mg GAE/g dry extract), as compared to the hydrophilic extract (0.846 mg GAE/g dry extract). This quantification is often related to the total antioxidant capacity of a plant extract. Nevertheless, only this parameter is not enough to justify a good antioxidative potential.

The antioxidant activity in vitro. For a better understanding of the importance of the investigated sample, we included also in the tests an Iranian sample (saffron II, according to ISO standards). The Iranian saffron (Pers) sample was used just as comparison standard for the antioxidant test, for which we obtained a lipophilic and a hydrophilic extract by the same methods employed for the Greek sample.

The hydrophilic and lipophilic extracts of *Crocus sativus* of Greek origin exhibited a better anti-oxidant capacity compared to the extracts of Iranian origin, the hydrophilic extract showing the most promising results (71.36 ± 1.03 µg/mL).

Table I

Inhibitory concentration for the investigated samples of saffron

Sample/positive control	IC_{50} (µg/mL)	
	DPPH scavenging assay	$ABTS^{\bullet+}$ scavenging assay
Gr L	242.2 ± 1.02	152.92 ± 2.38
Pers L	167.1 ± 0.45	619.31 ± 1.65
Gr H	45.3 ± 0.30	71.36 ± 1.03
Pers H	56.3 ± 0.89	140.50 ± 0.89
Rutoside/BHA	1.24 ± 0.05	1.51 ± 0.05

Gr L – Greek lipophilic extract, Gr H – Greek hydrophilic extract, Pers L – Iranian lipophilic extract, Pers H – Iranian hydrophilic extract, BHA – butylated hydroxyanisole

This data points out that the hydrophilic extracts are better radical scavengers than the lipophilic fractions and do not necessarily indicate a valuable *in vivo* activity.

Biological activity in vivo. Generally, memory is a complex feature that comprises different mechanisms affected variably by aging. The most affected type of memory is the one that lets you remember things automatically, almost without effort. Deficits of incidental memory are easier to notice in elderly. It is known that memory deficits start to show and deepen with age, moreover for, patients with mild cognitive impairment or age-related dementia [5, 6]. However, the approved treatment strategies for Alzheimer's disease (AD) and other similar dementia are not satisfactory, due to their side effects [1, 4]. In such a context, our research is aiming to prove that spices such as saffron might be a future alternative in the prevention and treatment of memory deficits.

Therefore, in our research we tried to find out if the Greek sample of *Crocus sativus* might have an impact of the memory in an AD rat model induced by intracerebroventricular injection of amyloid Aβ-(25-35). This peptide fragment has shown neuro-toxic activities in cells by formation of β-sheets within 7 hours from administration. Previous studies on Aβ-(25-35) peptide suggested that the fragment 25-30 might participate in interpeptide β-sheet formation, whereas the remaining part (31-35) forms a β-turn conformation at the C-terminal end [12, 14]. The novelty of the present study is represented especially by this peptide fragment used for AD model, saffron extracts not being evaluated before on this type of Aβ-(25-35) peptide.

Our results indicated that saffron extracts sustain memory formation in a rat model of AD Aβ-(25-35) - induced. The statistic calculation showed that short working memory was improved by both type of extracts in a dose correlated pattern, although for group b (treated with 10 mg of hydrophilic extract) there is an inverse in the activity intensity. Nevertheless, all the doses had a significant positive impact on short memory (Figure 2).

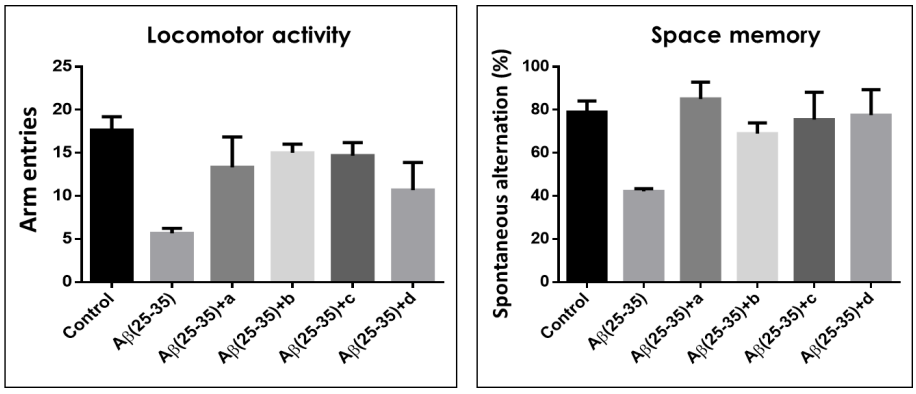

Figure 2.

Locomotor activity and space memory in Y-maze test for animals treated with saffron extracts

On the other hand, the working memory errors were shown in radial arms maze as compared to regular memory after the animals learned the maze. The results showed significant improvement for all groups treated with saffron extracts, meaning fewer errors when executing the test (Figure 3).

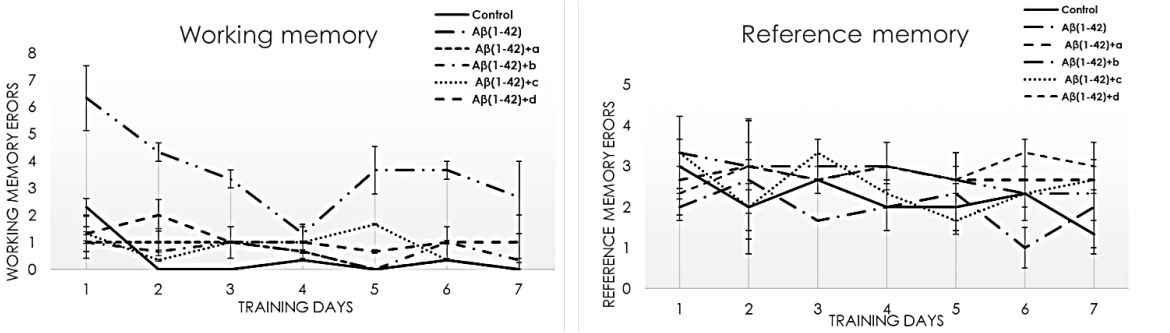

Figure 3.
Working memory errors compared to standard memory errors for the investigated extracts

The time taken to consume all five baits was taken into account, and the time difference from regular training and testing showed the potential of the administered extracts. The lowest values for errors were registered for 10 mg of the lipophilic extract that was administered. Corroborating the results from both *in vivo* and *in vitro* tests one can conclude that the lipophilic extract might be a better choice for further testing and formulation, relatively low doses (5 mg) of extract were potent enough to improve the memory deficits induced by the neurotoxicity of Aβ-(25-35). Also, shorter time to consume the food treats and a more active movement were observed for the same group of rats.

Conclusions

The present study indicated that doses of 5 mg to 10 mg of *Crocus sativus* L. extracts could effectively restore memory impairment and alleviate the toxicity induced by administration of beta-amyloid peptide. Moreover, the used quantities were very low, even if they were calculated for human use the doses would be ranging from 375 mg to 750 mg for a bodyweight of 75 kg. This also indicates the potency of such extracts on memory impairment. Therefore, *Crocus sativus* L. standardized extracts could be potential candidates for further preclinical and clinical studies aimed at long-time prevention and treatment of cognitive deficits in neurological disorders.

Conflict of interest

The authors declare that they have no potential conflicts of interest to disclose.

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