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Can Bioactive Compounds of *Crocus sativus* L. Influence the Metabolic Activity of Selected CYP Enzymes in the Rat?

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Summary

Safranal and crocin are biologically active compounds isolated from *Crocus sativus* L., commonly known as saffron. Clinical trials confirm that saffron has antidepressant effect, thus being a potential valuable alternative in the treatment of depression. The aim of the present study was to determine, whether systemic administration of safranal and crocin can influence the metabolic activity of CYP3A, CYP2C11, CYP2B, and CYP2A in rat liver microsomes (RLM). The experiments were carried out on male Wistar albino rats intragastrically administered with safranal (4, 20, and 100 mg/kg/day) or with intraperitoneal injections of crocin (4, 20, and 100 mg/kg/day). Our results demonstrate the ability of safranal and crocin to increase the total protein content and to change the metabolic activity of several CYP enzymes assessed as CYP specific hydroxylations of testosterone in RLM. Crocin significantly decreased the metabolic activity of all selected CYP enzymes, while safranal significantly increased the metabolic activity of CYP2B, CYP2C11 and CYP3A enzymes. Therefore, both substances could increase the risk of interactions with co-administered substances metabolized by cytochrome P450 enzymes.

Key words

Crocin • Safranal • CYP • Rat liver microsomes

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Introduction

Crocus sativus L. is a perennial flowering plant and a member of the Iridaceae family. Saffron, the dried stigmas of *Crocus sativus* flowers, is a well-known spice. Chemical analysis of saffron extracts has revealed about 150 different compounds from which the most studied are crocin, safranal, and picrocrocin (Winterhalter and Straubinger 2000, Bathaie and Mousavi 2010). Crocin as a water-soluble carotenoid gives saffron its typical reddish or yellowish color. Safranal is a monoterpenic aldehyde responsible for saffron's characteristic odour, and the bitter taste of saffron is attributed to picrocrocin, which is also a precursor of safranal (Tarantilis *et al.* 1995, Melnyk *et al.* 2010). Saffron has not always been primarily a spice (for flavoring and coloring of food), but rather a medicinal plant, which was used in traditional medicine as e.g. sedative, anxiolytic, expectorant, aphrodisiac, antispasmodic, and for the treatment of premenstrual syndrome, asthma, or pain (Schmidt *et al.* 2007, Ríos *et al.* 1996).

Several food supplements containing saffron extracts are already available worldwide. The antidepressant effect of saffron extract was described in several double blind, randomized, placebo-controlled clinical trials (Moshiri *et al.* 2006, Akhondzadeh *et al.* 2005) and its activity was comparable to the effect of clinically used antidepressants, namely fluoxetine (Basti *et al.* 2007, Noorbala *et al.* 2005) and imipramine (Akhondzadeh *et al.* 2004). Thus, saffron may become a valuable alternative to classical antidepressants. Safranal and crocin have been intensively studied in the

last few years also for other biological effects, such as antiproliferative, antioxidative, hypnosedative, etc. (Rezaee and Hosseinzadeh 2013, Alavizadeh and Hosseinzadeh 2014, Hosseinzadeh and Noraei 2009, Moshiri *et al.* 2014).

It was described that the metabolic activity of cytochrome P450, which is one of the most important enzymatic systems for xenobiotic biotransformation, is influenced by a large number of natural substances, including carotenoids. The modulation of CYP metabolic activity could lead to clinically relevant changes in plasma concentrations of concurrently administered drugs, and thus also to changes in their pharmacological properties. The knowledge of the influence of saffron or its compounds on CYP enzymes is lacking. Therefore, it is important to reveal whether the activity of CYP enzymes could be affected. The aim of the present study is to determine the effect of systemic administration of safranal and crocin on the metabolic activity of CYP2C11, CYP3A, CYP2B, and CYP2A, and the total protein and total cytochrome P450 (CYP) content in rat liver microsomes (RLM).

Methods

Chemicals

Crocin and safranal were purchased form Sigma-Aldrich (St. Louis, MO, USA). Other compounds used in the study were following: NADP, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, $MgCl_2$, EDTA, prednisone, testosterone, KH_2PO_4, NA_2HPO_4, sucrose, and KCl, all of them provided by Sigma-Aldrich (St. Louis, MO, USA). The metabolites of testosterone, namely 2β-, 2α-, 7α-, 6β-, 16α-, and 16β-hydroxy-testosterone were purchased from Steraloids Inc. (Newport, RI, USA). Chemicals and organic solvents for HPLC analysis (acetonitrile, methanol, and dichloromethane) were provided by Lach-ner (Neratovice, CZ).

Animal procedures

The experiments were carried out on male Wistar albino rats (280±20 g), which were housed under standard laboratory conditions (22±2 °C room temperature; 55±5 % room humidity; 12:12 h light/dark cycle). Animals had free access to water and food and they were sacrificed 24 h after the last drug administration. All experiments were performed in accordance with the Czech act No. 246/1992 and with the approval of both the local and national Czech Central Commission for Animal Welfare.

In the Experiment I, after 5 days of acclimatization, rats were randomly divided into four groups per 9-10 animals and were treated intragastrically with safranal dissolved in a mixture of propylene glycol and 5 % glucose (1:1) at the doses of 4, 20, and 100 mg/kg/day. The administrations were repeated for 10 consecutive days. The control group was administered with appropriate volume of vehicle (2 ml/kg). Safranal was administered intragastrically to simulate the same route of administration of food supplements and due to its poor solubility in aqueous solvents and thus inability to prepare appropriate parenteral solution.

In the Experiment II, after 5 days of acclimatization, rats were randomly divided into four groups per 9-10 animals and were treated intraperitoneally with crocin dissolved in saline at the doses of 4, 20, and 100 mg/kg/day for 9 consecutive days. The control group was administered with appropriate volume of vehicle (1 ml/kg). Crocin was administered intraperitoneally due to its poor absorption through the gastrointestinal tract (Singla and Bhat 2011).

Preparation of rat liver microsomes

The RLM were isolated from 3 grams of liver tissue of individual animals by differential ultracentrifugation (19 000 × g and 105 000 × g) in 20 mM Tris/KCl buffer (pH=7.4) including washing with 0.15 M KCl and finally diluted in 0.25 M Tris/sucrose buffer (pH=7.4). The total protein content in the microsomal preparations was assessed according to Lowry *et al.* (1951) using bovine serum albumin as a standard. Determination of total CYP content was assessed using CO-difference spectroscopy method according to Omura *et al.* (1964).

Determination of cytochrome P450 activity in rat liver microsomes

Assessment of the activity of CYP2A, CYP2B, CYP2C11, and CYP3A enzymes was based on the rate of testosterone biotransformation in RLM with a NADPH generating system according to the previously described method of Wójcikowski *et al.* (2008) with a slight modification. The incubation mixture of final volume of 0.5 ml contained phosphate buffer (50 mM; pH=7.4), EDTA (1.1 mM), NADP (1.2 mM), glucose-6-phosphate (4.4 mM), $MgCl_2$ (3.2 mM), glucose-6-phosphate dehydrogenase (0.5 U in 0.5 ml), RLM (25 µl), and

testosterone, which was added to be in the final concentration of 400 μM. The reaction was stopped after 15 min of incubation at 37 °C by adding 50 μl of methanol and by cooling down in ice.

Table 1. CYP specific metabolites of testosterone (modified from Wójcikowski *et al.* 2008, Kot and Daniel 2008, Chovan *et al.* 2007, Kobayashi *et al.* 2002).

CYP	Metabolites of testosterone
2A	7α-hydroxytestosterone
2B	16β-hydroxytestosterone
2C11	2α-hydroxytestosterone
2C11	16α-hydroxytestosterone
3A	2β-hydroxytestosterone
3A	6β-hydroxytestosterone

The concentrations of testosterone and its specific metabolites were measured by a HPLC system (Shimadzu LC-10) with the UV detector (Shimadzu SPD-M10AVP) by the modified method of Haduch *et al.* (2006). After addition of the internal standard (prednisone), the analytes were extracted from the microsomal suspension with dichloromethane (4 ml), and the residue obtained after evaporation of extracts was dissolved in 200 μl of 50 % methanol. An aliquot (45 μl) was injected into the HPLC system, and the mobile phase was used in the following gradient mode: time 0 to 12 min 1:59:40 (v/v/v acetonitrile/water/methanol), from 12:00 to 17:50 min 1:48:51 and from 17:50 to the end of the analysis in the 25:00 min 1:59:40. The flow rate was 0.8 ml/min. Analytical column (Kinetex 2.6u PFP 100A, 150 × 4.60 mm) was purchased from Phenomenex (Torrance, CA, USA), and the absorbance was measured with DAD detector at the wavelength 245 nm. Metabolic activities of all CYP enzymes were studied by measuring the rates of CYP specific reaction (Table 1) and expressed as the metabolite molar concentration/min/mg of total protein in RLM.

Statistical analysis

The results were statistically evaluated using the non-parametric Kruskal-Wallis test, and performed using the Statistica 12 software (StatSoft, Inc. 2013). Results were regarded as statistically significant when $p \leq 0.05$.

Results

Experiment I (safranal)

The analysis revealed that the systemic administration of the highest dose (100 mg/kg) of safranal significantly increased the total protein content (149 % of the control group value) and also the total CYP content (151 % of the control group value) (Fig. 1A). Other doses of safranal did not show any significant changes at the levels of the total protein and CYP content in RLM. However, both parameters were slightly elevated in comparison to the controls.

Changes in the metabolic activity were detected in all selected CYP enzymes except CYP2A (measured as a rate of 7α-hydroxylation of testosterone) (Fig. 2). The metabolic activity of CYP2B (measured as a rate of the 16β-hydroxylation of testosterone) was significantly increased only at the highest dose of safranal. The effect of safranal on CYP3A is not convincing because the rate of 6β-hydroxylation was significantly increased, while 2β-hydroxylation of testosterone was not changed (both believed to reflect CYP3A metabolic activity). The lowest dose of safranal significantly increased the rate of 2α- and 16α-hydroxylation of testosterone, which is considered as CYP2C11 specific reaction.

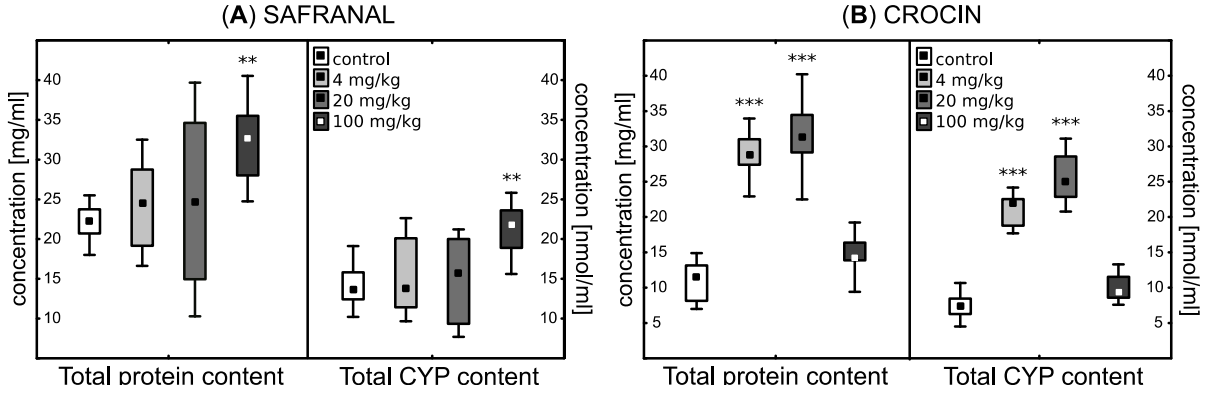

Fig. 1. The effect of systemic administration of safranal (**A**) and crocin (**B**) on the total protein and the total CYP content in RLM. All values are expressed as box plots with median (box 25 % – 75 %; whiskers Min-Max without outliers). Statistical significance with respect to the control group is indicated with **p≤0.01, ***p≤0.001.

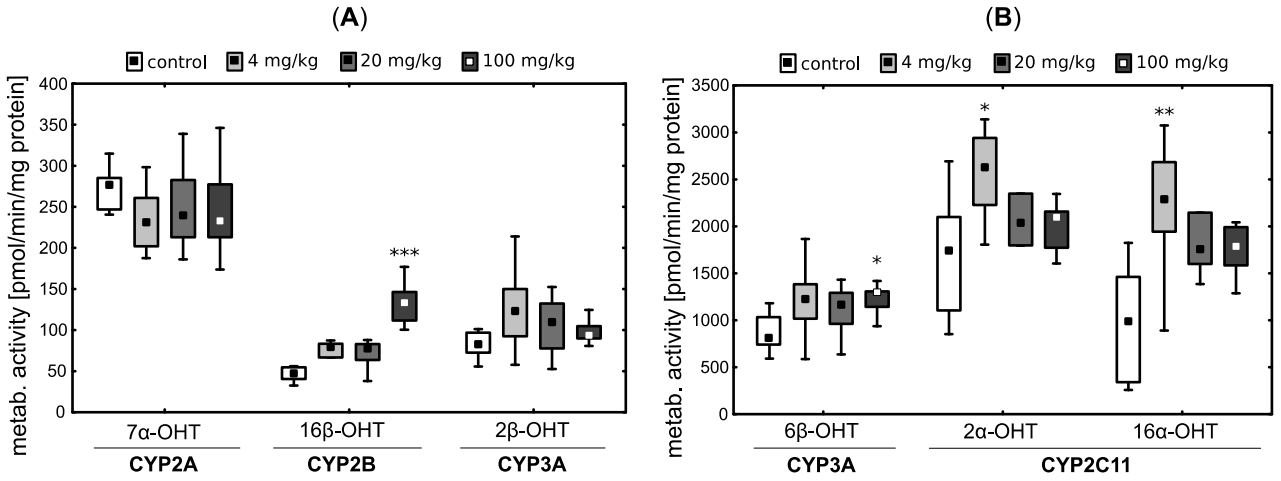

Fig. 2. The metabolic activity of selected CYP enzymes in RLM after safranal systemic administration, measured as the rate of testosterone hydroxylation. (**A**) The metabolic activity of CYP2A, CYP2B and CYP3A (2β-hydroxylation). (**B**) The metabolic activity of CYP3A (6β-hydroxylation) and CYP2C11. All values are expressed as box plots with median (box 25 % – 75 %; whiskers Min-Max without outliers). Statistical significance with respect to the control group is indicated with *p≤0.05, **p≤0.01, ***p≤0.001.

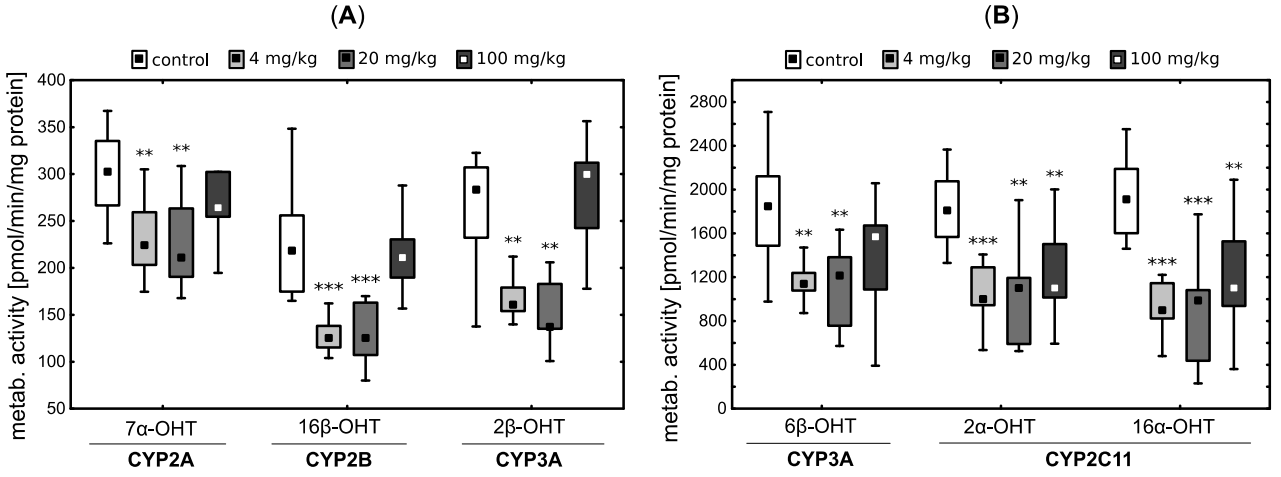

Fig. 3. The metabolic activity of selected CYP enzymes in RLM after crocin systemic administration, measured as a rate of testosterone hydroxylation. (**A**) The metabolic activity of CYP2A, CYP2B and CYP3A (2β-hydroxylation). (**B**) The metabolic activity of CYP3A (6β-hydroxylation) and CYP2C11. All values are expressed as box plots with median (box 25 % – 75 %; whiskers Min-Max without outliers). Statistical significance with respect to the control group is indicated with *p≤0.05, **p≤0.01, ***p≤0.001.

Experiment II (crocin)

The obtained results showed that the systemic administration of crocin significantly increased the level of both the total protein and the total CYP content, at the doses 4 mg/kg (up to 259 % and 280 % of the control, respectively) and at 20 mg/kg (up to 278 % and 343 % of the control, respectively) (Fig. 1B). The amount of total protein and the total CYP content remained unchanged at the highest dose of crocin.

The administration of crocin, unlike safranal, caused reduction of the metabolic activity of selected CYP enzymes (Fig. 3). The determination of CYP2B metabolic activity revealed significant decrease at the doses of 4 and 20 mg/kg and the same result was observed in the metabolic activity of CYP2A. All doses of crocin significantly decreased the metabolic activity of CYP2C11. In the case of CYP3A, significant decrease was observed in the rate of both 6β-hydroxylation and 2β-hydroxylation of testosterone at the doses of 4 and 20 mg/kg.

Discussion

Our results demonstrate the ability of systemic administration of safranal and crocin to increase the total protein and the total CYP content in RLM and to change the metabolic activity of different CYP enzymes. Thereby, tested substances could raise the risk of

interactions with co-administered drugs metabolized by the same pathway. Several clinical trials confirmed that saffron may be of therapeutic benefit especially in the treatment of mild to moderate depression (Basti *et al.* 2007, Akhondzadeh *et al.* 2004, Moshiri *et al.* 2006). This is of interest because currently psychiatric patients with depression are treated by a wide range of synthetic antidepressants, but up to 30 % of them are pharmacoresistant without a significant clinical improvement (Micale *et al.* 2013). Food supplements containing saffron extract are relatively safe, and so they are a viable alternative to the conventional drugs. The daily dose of saffron extract is 30 mg according to clinical trials recommendations (Basti *et al.* 2007, Akhondzadeh *et al.* 2004, Moshiri *et al.* 2006).

To the best of our knowledge, no studies exist up to date dealing with the effects of safranal or crocin on CYP3A, CYP2C11, CYP2B, and CYP2A enzymes. However, it was described that metabolic activity of CYP enzymes is influenced by a large number of natural carotenoids, and the authors reported either decreased or increased CYP metabolic activity (Louisa *et al.* 2009, Jewell and O'Brien 1999, Satomi and Nishino 2013, Wang and Leung 2010). Our experiment showed that the metabolic activity of CYP2A, CYP2B, CYP2C11, and CYP3A was decreased after administration of carotenoid crocin. Safranal significantly increased the metabolic activity of CYP2B and CYP2C11, while the metabolic activity of CYP2A enzyme was without any significant changes. The effect of safranal on the testosterone hydroxylation *via* CYP3A was uncertain because 6β-hydroxylation was significantly increased, while 2β-hydroxylation of testosterone was not changed. Therefore, in our future experiments, we aim to use other probe substrates (nifedipine or midazolam) to determine the influence of safranal on CYP3A.

Interestingly, the total protein content was increased by safranal as well as by crocin. However, the metabolic activity of CYP2B, CYP2C11 and CYP3A enzymes was increased in the case of safranal and decreased in the case of crocin and CYP2A, CYP2B, CYP2C11, and CYP3A. The specific mechanism by which safranal or crocin influence CYP enzymes is unknown and we can therefore only hypothesize that this discrepancy could be explained by increase in CYP content including those CYP enzymes whose activity was not assessed in this study. Thus, we have observed apparent decrease in the CYP activity when the activity was calculated per mg of total protein or per nmol of total CYP. Other explanations could also exist, such as the negative feedback on reduced activity of main metabolic enzyme system. It is essential to carry out further experiments to confirm or reject these hypotheses.

To conclude, the obtained results showed that safranal and crocin influence the apparent metabolic activity of CYP enzymes in RLM. It would be necessary to take into account the risk of possible interactions with substances metabolized by CYP enzymes in the case of use of these substances in the clinical practice. This risk becomes more serious as food supplements containing saffron extracts are already available and their influence on the human CYP enzymes is not estimated yet. We report the ability of safranal and crocin to influence the activity at least of some rat liver CYP enzymes. Nevertheless, the daily intake of crocin and safranal from the food supplements is much lower in comparison to the doses administered to rats in our study and the substrate specificity of the rat CYP enzymes is not fully identical to human enzymes.

Conflict of Interest

There is no conflict of interest.

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