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Effect of *Crocus sativus* (Saffron) on Muscarinic Receptors of Guinea Pig Tracheal Chains

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ABSTRACT

The effects of aqueous-ethanolic extracts of *Crocus sativus* (Iridaceae) on muscarinic receptors were examined on tracheal chains of guinea pigs. The effects of three concentrations of aqueous-ethanolic extract, safranal, atropine and saline on muscarinic receptors were tested (n=8). The EC_{50} (effective concentration of methacoline causing 50% of maximum response) obtained in the presence of atropine, all concentrations of the extract and safranal were significantly greater than those of saline ($p<0.05$ to $p<0.001$). Maximum responses to methacholine obtained in the presence of different concentrations of saffron extract were significantly lower than that in saline ($p<0.01$ to $p<0.005$). There were parallel rightward shift in concentration response curves obtained in the presence of only low concentration of saffron and safranal. These results may indicate an inhibitory effect of *Crocus sativus* and especially for safranal on muscarinic receptors.

Keywords: *Crocus sativus*, guinea pig, inhibitory effect, Iridaceae, muscarinic receptor, trachea

INTRODUCTION

Crocus sativus L. is a small perennial plant from the iris family (Iridaceae) which is cultivated in many regions particularly in Iran. The main constituents of the stigma of this plant are crocins, safranal, picrocrocin, ketoisophorone, isophorone, glycosidic terpenoids (Tarantilis *et al.* 1995).

Saffron is used in traditional medicine as an antispasmodic, eupeptic, gingival sedative, anticatarrhal, nerve sedative, carminative, diaphoretic, expectorant, stomachic, aphrodisiac, and emmenagogue (Rios *et al.* 1996; Abdullaev *et al.* 2004). The part of the plant used medicinally is its stigma (central part of the flower, female sexual organ).

Previous studies indicated different pharmacological effects for this plant including: anticonvulsant (Hosseinzadeh *et al.* 2002a, 2005a), antidepressant (Akhondzadeh *et al.* 2005; Hosseinzadeh *et al.* 2005b; Akhondzadeh Basti *et al.* 2007), anti-inflammatory (Hosseinzadeh *et al.* 2002b), radical scavenger and anti-oxidant properties (Abe *et al.* 1999; Assimopoulou *et al*. 2005; Papandreou *et al.* 2006; Kanakis *et al.* 2007) and antitumor effects (Escribano *et al.* 1996; Rios *et al.* 1996; Abdullaev *et al.* 2004; Das *et al.* 2004; Chryssanthi *et al.* 2007). The plant has also learning and memory improving properties (Pitsikas *et al.* 2006). Saffron extract also has chemo preventive and geno protective effects and protects from geno toxins-induced oxidative stress in mice (Premkumar *et al.* 2001, 2003, 2006). A lowering blood pressure effect (Rios *et al.* 1996) and relaxant effect on vascular (Fatehi *et al.* 2003) and tracheal smooth muscle (Boskabady *et al.* 2006) has also been described for this plant.

To elucidate the mechanism(s) responsible for the relaxant effect of the plant in tracheal smooth muscle, the effect of aqueous-ethanolic extracts of *Crocus sativus* on muscarinic receptors was examined on tracheal chains of guinea pigs.

MATERIALS AND METHODS

Plant and extracts

Crocus sativus was collected from Torbat Heydarieh (Eastern Iran) and a voucher specimen was preserved in the Herbarium of the School of Agriculture, University of Ferdowsi, Mashhad (Herbarium No: 143-0319-1). The stigma (part of the plant used in traditional medicine) of the identified plant was isolated and used in the study. The aqueous-ethanolic extract of the isolated stigma was prepared as follows: 10 g of chopped, dried (in the absence of sun light in room temperature) isolated stigma of the plant was extracted in 50 mL of ethanol 50% (25 mL distilled water and 25 mL ethanol) using a Soxhlet apparatus. The solvent was then removed under reduced pressure and 2.5 g of extract was obtained from 10 g of stigma. The plant ingredient concentration in the final extract was adjusted to 0.1 g/mL by adding distilled water to the dried extract (under reduced pressure at room temperature).

Tissue preparations

Male Dunkin-Hartley guinea pigs (400-700 g) (purchased from Razi Institute, Mashhad, Iran) were sacrificed by a blow on the neck and the trachea were removed. Each trachea was cut into 10 rings (each containing 2-3 cartilaginous rings). All the rings were then cut open opposite the trachealis muscle, and sutured together to form tracheal chain (Holroyde 1986). Tissue was then suspended in a 10 mL organ bath (organ bath 61300, Bio Science Palmer-Washington, Sheerness, Kent U.K.) containing Krebs-Henseleit solution with the following composition (mM): NaCl 120, $NaHCO_3$ 25, $MgSO_4$ 0.5, KH_2PO_4 1.2, KCl 4.72, $CaCl_2$ 2.5 and dextrose 11.

The Krebs solution was maintained at 37°C and gassed with 95% O_2 and 5% CO_2. Tissue was suspended under isotonic tension (1 g) and allowed to equilibrate for at least 1 hr while it was washed with Krebs solution every 15 min.

The study was approved by the University's Ethics Committee. The allowance number of the relevant ethical committee for the animal experiments is 85301.

Received: 13 April, 2009. Accepted: 25 January, 2010.

Original Research Paper

Protocols

The inhibitory effect of *C. sativus* on muscarinic receptors was examined by producing the cumulative log concentration-response curve of methacholine hydrochloride (Sigma Chemical Ltd, U.K.) induced contraction of tracheal chains 10 min after the exposure of tissue to one solution. Different tested solutions were included: 0.1 µg/mL concentration of atropine sulphate (Sigma), three concentrations of aqueous-ethanolic extract (25, 50 and 100 µg/mL), safranal (0.63, 1.25 and 2.5 µg/mL, Fluka, Italy, Catalogue No. C4915, purity 75%), or 0.2 ml saline (n=8 for each solution). The consecutive concentrations of methacholine were added every 2 min (range 0.1 - 1000 µM); and the percentage of contraction due to each concentration in proportion to the maximum contraction, obtained in the presence of saline, was plotted against the log concentration of methacholine. The effective concentration of methacholine causing 50% of maximum response (EC_{50}) in each experiment was measured using the log concentration-response curve of the corresponding experiment. The slopes of concentration-response curves (in steepest part of the curve) and EC_{50} were measured using Graph Pad Prism 5 software. The shift of cumulative log concentration-response curves obtained in the presence of extract and atropine were examined by comparing the EC_{50} obtained in the presence of each solution with that of saline. In addition the maximum responses to methacholine obtained in the presence of extract and atropine in all sets of experiments was compared with that of saline. To examine the parallel rightward shift, the slope of the methacholine-response curve of each experiment was measured and was compared with that of saline. In experiments with parallel shift in methacholine-response curve, the concentration-ratio minus one (CR-1) as an index of the competitive antagonism effect was calculated by the following equation:

[EC_{50} obtained in the presence of effective solutions/EC_{50} obtained in the presence of saline] - 1

All of the experiments were performed randomly with 1 hr resting period of tracheal chains between each two experiments (examining the effect of atropine, saffron and safranal) while washing the tissues every 15 min with Krebs solution. In all experiments responses were recorded on a kymograph (ET8 G-Boulitt, Paris) and measured after fixation.

Statistical analysis

All data were expressed as mean±SEM. The EC_{50}, the slope, and the maximum response obtained in the presence of extract, safranal and atropine were compared with those obtained in the presence of saline using the paired *t*-test. The values of (CR-1) obtained in the presence of extract with those obtained in the presence of atropine were also compared using the paired *t*-test. Significance was accepted at $p<0.05$.

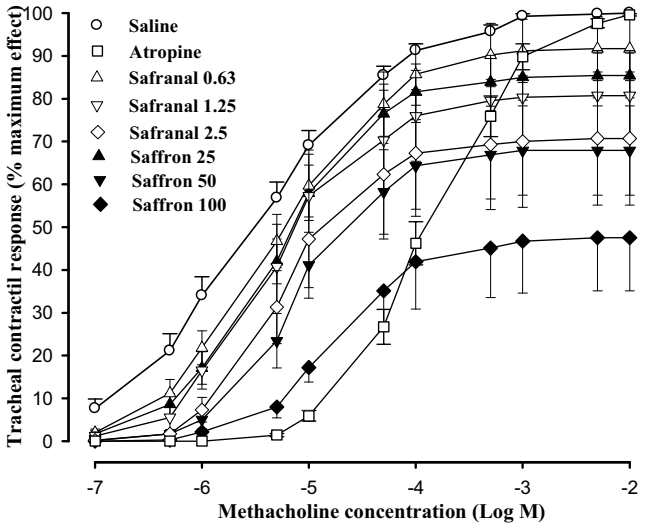

Fig. 1 Cumulative log concentration-response curves of methacholine-induced contraction of guinea pig tracheal chains, in the presence of saline, three concentrations of aqueous ethanolic extract (saffron), three concentrations of safranal (the unite of all concentrations is µg/mL) and 0.1 µg/ml concentration atropine (n=8).

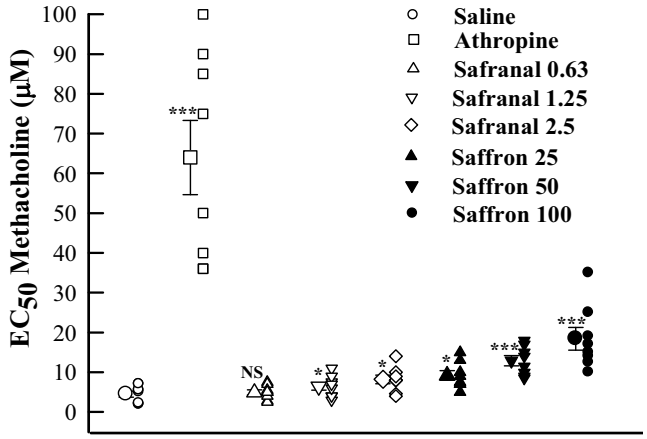

Fig. 2 EC_{50} methacholine obtained in the presence of three concentrations of aqueous-ethanolic extract from saffron (25, 50 and 100 µg/mL), safranal (0.63, 1.25 and 2.5 µg/mL), 0.1 µg/ml atropine, and saline (n=8). Statistical comparison in EC_{50} between saline and other solutions NS: non-significant difference, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

RESULTS

Shift in cumulative log concentration-response curves

Cumulative log concentration-response curves of methacholine obtained in the presence of all concentration of the extract, safranal and atropine showed clear rightward shift compared to methacholine curves produced in the presence of saline (**Fig. 1**).

Tracheal response to methacoline (EC_{50})

The EC_{50} methacholine obtained in the presence of atropine and all concentrations of safranal and extract except low concentrations of safranal (0.63 µg/ml) were significantly higher than that of saline ($p<0.05$ to $p<0.001$) (**Fig. 2**).

Maximum response to methachline

The maximum responses to methacholine obtained in the presence of different concentration of saffron were significantly lower than that of saline ($p<0.01$ to $p<0.005$) (**Table 1**). However, there were no significant differences in maximum responses obtained in the presence of different concentrations of safranal with that of saline (**Table 1**).

Slope of methacholine -response curves

There were parallel right ward shift in concentration response curves obtained in the presence of only low concentration of saffron and safranal (**Table 1**). However, the slopes of methacholine -response curves obtained in the presence of medium and high concentrations of the extract (50 and 100 µg /mL) and safranal (1.25 and 2.5 µg/mL) were significantly higher than that of saline ($p<0.05$ to $p<0.001$) (**Table 1**).

Shift in methacholine concentration-response curves (CR-1)

The values of CR-1 obtained in the presence of all concentrations of the extract and safranal were significantly lower than that of atropine ($p<0.001$ for all cases) (**Fig. 3**).

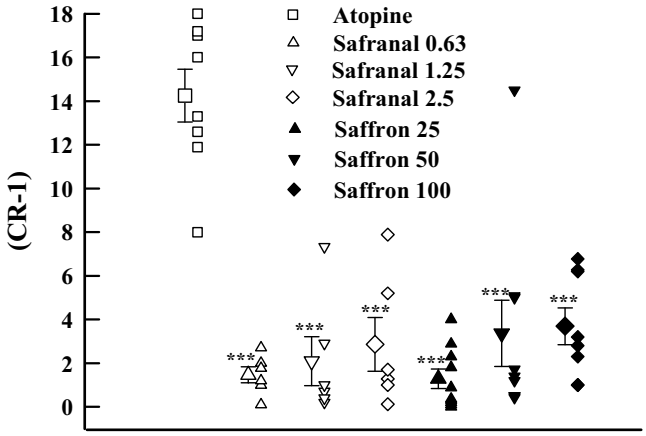

Fig. 3 The values of CR-1 obtained in the presence of three concentrations of aqueous-ethanolic extract from saffron (0.025, 0.05, and 0.1 µg/mL), safranal (0.63, 1.25 and 2.5 µg/mL), and 10 nM atropine, (n=8). Statistical comparison in (CR-1) between atropine and other solutions NS: non-significant difference, *: p<0.05, **: p<0.01, ***: p<0.001. The unit of (CR-1) is the proportion of EC_{50} obtained in the presence of tested solutions/EC_{50} obtained in the presence of saline – 1.

Table 1 Differences in maximum response and slope obtained in the presence of atropine, different concentration of saffron and safranal with those of saline.

Solutions	Concentration	Maximum Response	Slope
Atropine		NS	NS
Saffron	25 µg/mL	p<0.05	NS
	50 µg/mL	P<0.05	p<0.01
	100 µg/mL	P<0.005	p<0.05
Safranal	0.63 µg/mL	NS	NS
	1.25 µg/mL	NS	p<0.05
	2.5 µg/mL	NS	p<0.05

SN: non significant

Table 2 EC_{50} (µM) of methacholine obtained in the presence of aqueous-ethanolic extract from stigma of *Crocus sativus* (saffron), safranal, atropine and saline.

Solutions			Saffron	Safranal	Saffron *vs* Safranal
Saline		4.51 ± 0.77			
Concentration	1		9.19 ± 1.19	4.91 ± 0.63	p< 0.01
	2		12.87 ± 1.29	6.44 ± 0.97	P< 0.005
	3		18.44 ± 2.86	8.20 ± 1.10	P< 0.005
Atropine		64.00 ± 9.33			

Values are presented as mean ± Standard Error of Mean. NS: non-significant difference. Tested concentrations for extract were 25, 50 and 100 µg/mL and for safranal were 0.63, 1.25 and 2.5 µg/mL

Differences between the extract of *Crocus sativus* and safranal

The values of EC_{50} obtained in the presence of all concentrations of the extract were significantly greater than those of safranal (p<0.05 t0 p<0.005) (**Table 2**). The maximum response to methacholine and slopes of methacholine concentration response curves obtained in the presence of different concentrations of safranal were greater than those of the extract the values of CR-1 were lower but were not statistically different (**Tables 3-5**).

Table 3 Maximum response of methacholine obtained in the presence of aqueous-ethanolic extract from stigma of *Crocus sativus* (saffron), safranal, atropine and saline.

Solutions			Saffron	Safranal	Saffron *vs* Safranal
Saline		100.0 ± 0.00			
Concentration	1		85.4 ± 5.22	89.3 ± 6.35	NS
	2		67.9 ± 10.43	80.7 ± 10.52	NS
	3		47.5 ± 12.41	70.7 ± 15.55	NS
Atropine		99.6 ± 0.27			

For abbreviations see **Table 2**.

Table 5 Values of (CR-1) of methacholine obtained in the presence of aqueous-ethanolic extract from stigma of *Crocus sativus* (saffron), safranal and atropine.

Solutions			Saffron	Safranal	Saffron *vs* Safranal
Concentration	1		1.28 ± 0. 44	1.46 ± 0.37	NS
	2		3.37 ± 1.52	2.09 ± 1.127	NS
	3		3.69 ± 0.84	2.86 ± 1.23	NS
Atropine		14.25 ± 1.21			

For abbreviations see **Table 2**.

DISCUSSION

In this study, the inhibitory effect of the aqueous-ethanolic extract of the plant and one of its constituent, safranal, on muscarinic receptors was examined on isolated guinea pig tracheal preparations in order to verify one possible mechanism responsible for the observed relaxant effect seen for stigma of *Crocus sativus* (saffron) on tracheal chains. One possible mechanism responsible for the relaxant effect seen for saffron on tracheal chains (bronchodilatory) in our previous study (Boskabady *et al*. 2006) might be its inhibitory effect on muscarinc receptors because the relaxant effect of muscarinic receptor inhibition has been shown previously (Loenders *et al.* 1992). However the other major mechanisms responsible for the relaxant effect of the extract and safranal on tracheal muscle are including stimulation of β-adrenergic receptors and inhibition of histamine H_1 receptors of the plant, because indication of the relaxant effect of these mechanisms also have been shown previously (Popa *et al.* 1984; Linden *et al.* 1993).

The results showed a non-parallel rightward shift in methacholine log concentration-response curves, obtained in the presence of the aqueous-ethanolic extract and safranal compared to those of saline. Mmaximum contraction effect to methacholine obtained in the presence of the extract was also lower than that of saline. However, the EC_{50} methacholine obtained in the presence of all concentrations of the extract and two higher concentrations of safranal was greater than that of saline. These results indicated a functional antagonistic effect of saffron and a possible competitive antagonistic effect of safranal at muscarinic receptors of guinea pig trachea (Arunlakshana *et al.* 1959; Ariens 1987; Linden *et al*. 1993). However, further studies are required to inhibit the other receptors involving in the relaxant effect of the extract and safranal in tracheal smooth muscle such as β-adrenergic receptors (Linden *et al.* 1993) and re-examining their inhibitory effect on muscarinic receptors to ensure of this effect of the plant and its consti-

Table 4 Slope of methacholine obtained in the presence of aqueous-ethanolic extract from stigma of *Crocus sativus* (saffron), safranal, atropine and saline.

Solutions			Saffron	Safranal	Saffron *vs* Safranal
Saline		0.990 ± 0.003			
Concentration	1		0.984 ± 0.004	0.988 ± 0.008	NS
	2		0.978 ± 0.005	0.961 ± 0.014	NS
	3		0.890 ± 0.014	0.973 ± 0.007	NS
Atropine		0.987 ± 0.00			

For abbreviations see table 2.

tuent and also to explore the receptor types contributing in the functional antagonism seen in the present study. Our previous study also suggested the inhibitory effect of the extract and safranal on muscarinc and histamine H_1 receptors and a β-adrenergic receptors stimulatory effect (Boskabady *et al*. 2006) which confirms the results of the present study. Our other study showed stimulatory effect of the plant on β-adrenergic receptors of tracheal chains using standard method of performing concentration-response curves to isoprenaline (Nemati *et al.* 2008) may also support the contribution of this type of receptors on functional antagonism of the plant on muscarinic receptors seen in the present study. However, values of both EC50 and CR-1 obtained in the presence of concentrations of the extract and safranal were significantly lower than atropine indicating lower inhibitory effect of the extract and safranal on muscarinc receptors compared to atropine at used concentrations. According to pharmacological principle if a substance caused parallel right ward shift in the concentration response curve to an agonist, it is a pharmacological indicator of the antagonistic effect of the substance on that receptor (Arunlakshana *et al.* 1959; Ariens 1987; Linden *et al*. 1993). However, the radioligand binding assay, a more precise approach for evaluating the inhibitory effect of a plant on muscarinic receptors, needs to be examined in further studies. In addition, the effect of both the extract and safranal should be examined on muscrinic receptors in the presence of β and H1 antagonists in further studies.

The results of the present study indicated the greater values of EC_{50} and (CR-1) obtained in the presence of hydroethanolic extract of saffron and lower values of maximum response to methacholine and slope of methacholine response curves compared to its constituent safranal. These results indicated that the pharmacological properties of the extract are not solely due to its constituent, safranal. In fact, in phytotherapy it is the extract which defines the efficacy, not some analytical substance, even if it contributes to the overall effect. The greater relaxant effect of the extract compared to safranal (Boskabady *et al.* 2006) also indicate that the relaxant effect of the plant is partially due to its constituent, safranal. Safranal of the saffron used in the present study was 0.26% (Hadizadeh *et al.* 2007) while the concentration of safranal was 1/40 of that of the extract. Therefore the lower effect of safranal seen in the present study is not due to its lower relative concentration.

In conclusion, the results of this study suggested a functional antagonistic effect of stigma of *Crocus sativus* (saffron) and a possible competitive antagonistic effect of safranal at muscarinc receptors. The results also suggested that the pharmacological properties of the extract are not solely due to its constituent, safranal.

ACKNOWLEDGEMENTS

This study was financially supported by Research Deputy Chancellor of Islamic Azad University, Mashhad Branch

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