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Phytochemical analysis of bioactive fraction from the extract of *Crocus sativus* petal by LC-MS and its effect on secretion of nerve growth factors in rat c6 glioma cells

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Abstract: This article was aimed to analyze the bioactive ethyl acetate fraction from the extract of *Crocus sativus* petal by LC-MS and to study its antidepressant effect. Antidepressant test was performed employing rat C6 glioma cells model. The major compounds from ethyl acetate fraction were cinnamic acid derivatives, which primarily included methylcinnamaldehydes, carboxycinnamylic acid carboxyphenethyl ester, carboxyvinylcinnamyl alcohol propinate, 3-acetoxy-4-propoxycinnamylic acid, carboxycinnamylic acid glycol ester, vinnylcinnamylic acid hexylene glycol ester and cinnamylic acid. In C6 glioma cells test, the ethyl acetate extract of *C. sativus* petal significantly upregulated nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) expression. *C. sativus* petal has antidepressant effect, and cinnamic acid derivatives shoud be the main antidepressant constituents. The mechanism may be related to the inhibition of NGF and BDNF reuptake.

Keywords: *Crocus sativus,* petals, antidepressant, NGF, BDNF.

INTRODUCTION

Crocus sativus L., commonly known as saffron crocus, is a species of flowering plant of the *Crocus* genus in the Iridaceae family. It is best known for producing the spice saffron from the filaments that grow inside the flower. Human cultivation of saffron crocus and use of saffron have taken place for more than 3500 years and spans different cultures, continents and civilizations. *C. sativus* is currently known to grow in the Mediterranean, East Asia, and Irano-Turanian Region. *C. sativus* contains a number of bioactive components which are believed to be largely responsible for its health promoting properties including treating various disorders like asthma, atherosclerosis, painful menstrual periods and even depression. (Mousavi and Bathaie, 2011) The clinical findings suggest that *C. sativus* is a safe and effective plant. Depression and anxiety are symptoms of some physical diseases and side effects of some drugs and medical treatments. Depressed mood is also a symptom of some mood disorders such as major depressive disorder or dysthymia. In several studies, saffron stigma antidepressant effects were also found to be similar to the antidepressant medications of fluoxetine, imipramine and more effective than placebo for the treatment of major depressive disorder particularly in determining optimal treatment dosages and length of treatment. (Akhondzadeh *et al*, 2005; Akhondzadeh *et al*, 2008; Afshin *et al*, 2007; Hausenblas *et al*, 2013; Moshiri *et al*, 2006; Noorbala *et al*, 2005) There have been some reports on therapeutic effects of the active constituents of C. sativus stigma, crocins and safranal in treatment of animals' anxiety. The improving effects of crocin on anxiety might be also effective on other species and their behavioral paradigms. (Hosseinzadeh *et al*, 2004; Wang *et al*, 2010) Clinical trials also indicated that the supplementation of *C. sativus* petal improve symptoms of depression in adults. (Akhondzadeh *et al*, 2005; Akhondzadeh *et al*, 2008; Moshiri *et al*, 2006; Noorbala *et al*, 2005) The lower expression of nerve growth factor family is involved in the physiological and pathological process of depression diseases. The aim of the present study was to study active constituents of the petals of *C. sativus*, evaluate the effects of its extracts on synthesis and secretion of neurotrophins in rat C6 glioma cells.

MATERIALS AND METHODS

Drugs and chemicals

HPLC-grade acetonitrile (Fisher, Fair Lawn, NJ, USA) and ultra-pure water were used for all analyses. AR-grade ethanol, ethyl acetate and dimethyl sulfoxide (DMSO) for plant extraction was purchased from Shanghai Chemical Corporation. Nerve growth factor (NGF) kit (LF-EK50289) and brain derived neurotrophic factor (BDNF) kit (LF-EK50009) were from Abfrontier (Shanghai, China). C6 glioma cell strain was from Cyagen Biosciences Inc (Guangzhou, China).

Extraction and chemical analysis of C. sativus petals

The petal of *C. sativus* was collected and authenticated by the authors in 2016 from Shanghai. The materials were pulverised and dried to constant weight before use. 250g petals were extracted with ethanol (2500 mL×2) for 3h under reflux and the combined extracts were concentrated

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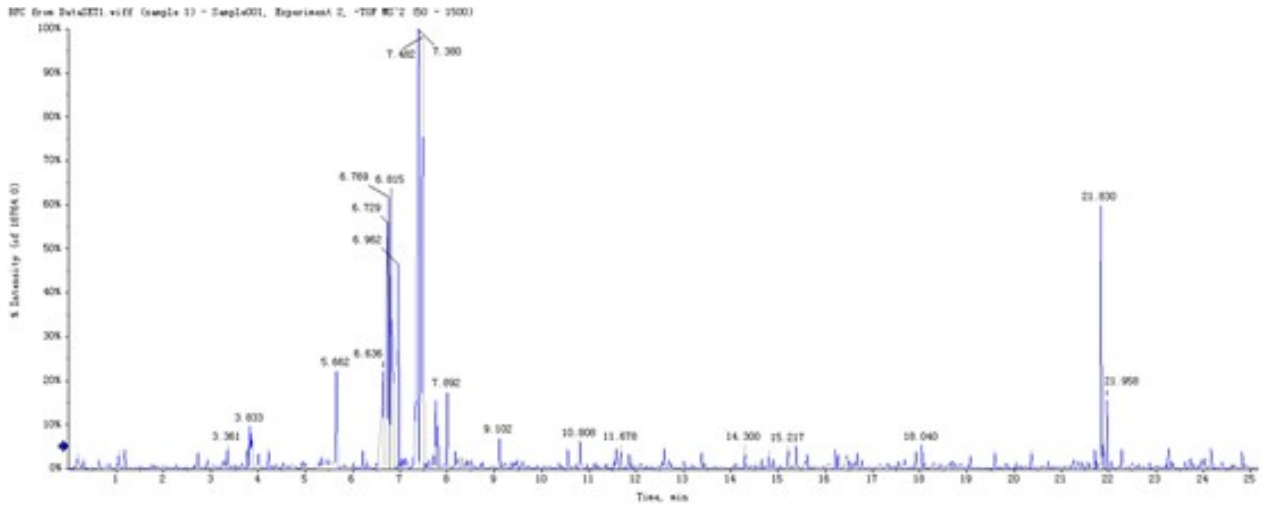

Fig. 1: HPLC-MS spectrum of ethyl acetate extract of *C. sativus* petals

in vacuo. The resulting extract (50 g) was then suspended in H_2O (50 mL) and extracted successively with acetidin. The combined acetidin layers were concentrated under vacuum to leave the residue (19g). For HPLC/MS analysis, an aliquot acetidin extract (20mg) was dissolved in ethanol (25mL). The solution was filtered through 0.2 μm micro porous membrane before use and a 10μL aliquot was injected into HPLC instrument for analysis. The Waters Alliance 2690 HPLC chromatographic system with a Waters 996 photodiode array detector was used for the analysis. The sample was separated on a Zorbax Extend C 18 column (250 × 4.6mm, 5.0μm, Agilent). The mobile phase was a gradient elution which was mixed with solvents A (water) and B (acetonitrile). The gradient program was as follows: initial 0-25 min, linear change from A-B (70: 30, v/v) to A-B (0: 100, v/v). The flow rate was 1.0 mL/min; the detector was monitored at 210 nm; column temperature was set at 30ºC. A Micromass Q/TOF Mass Spectrometer was connected to the Waters Alliance 2690 HPLC instrument via an electrospray ionisation interface. High-purity nitrogen was the nebulising gas. The Q/TOF MS condition were set as follows: ionising source was electrospray ionization (positive ion mode), drying gas (N_2) flow rate was 10.0 L/min; drying gas temperature was 320ºC; capillary voltage was set to 3000 V; fragmentation voltage was set to 120 V; the full-scan second-grade mass spectra of the investigated compounds from m/z 50-500 Da were measured using 500 ms for collection time and three micro scans were summed.

Experimental grouping, cell culture and biochemical assay

There were six groups: normal control group, blank solvent (DMSO) group, acetidin extract low dosage group (15μg·mL^{-1}), acetidin extract high dosage group (60 μg·mL^{-1}), water extract low dosage group and water extract high dosage group. C6 glioma cels were maintained in DMEM medium supplemented with 10% (v/v) fetal bovine serum, 100U/mL penicillin and 100 μg/mL streptomycin at 37^{o}C in a 5% CO_2 incubator for 72 hr. The culture medium was changed every other day. The cell number was adjusted to 5×10^5/ml and 160μL cell suspensions were transferred to 96-well cell culture plate. Each well of cell cultures were successively incubated for 24 hr. The cell supernatants NGF and BDNF levels were measured according to the ELISA kit instructions.

STATISTICAL ANALYSIS

Results are expressed as mean values ± SD. Statistical evaluations were made using t-test, and values were considered significantly different when $P < 0.01$. T-test was performed in Microsoft Excel 2013.

RESULTS

Using high performance liquid chromatography, the main detected peaks in ethyl acetate extract of *C. sativus* petals were assigned to cinnamic acid derivatives, which primarily included methylcinnamaldehydes, carboxycinnamylic acid carboxyphenethyl ester, carboxyvinylcinnamyl alcohol propinate, 3-acetoxy-4-propoxycinnamylic acid, carboxycinnamylic acid glycol ester, vinnylcinnamylic acid hexylene glycol ester and cinnamylic acid (fig. 1). A detail chemical composition is presented in table 1.

To reveal the mechanism of *C. sativus* petal against depression and anxiety, we studied its ability to regulate the levels of neurotrophins in rat C6 glioma cells. As shown in table 2, the ethyl acetate extracts of *C. sativus* petal upregulated NGF and BDNF levels in a dose-dependent manner. These results showed that the ethyl acetate extract of *C. sativus* petal treatments could promoted the synthesis and secretion of neurotrophins, which might be one of the mechanisms of promoting the repair of nerve injury.

Table 1: Chemical constituents of ethyl acetate extract of *C. sativus* petals identified by LC/M S

Retention time (min)	m/z	Molecule Weight	Compound	Relative content (w/%)
5.662	408, 348, 306, 245, 202, 161	408	4-Acetoxycinnamylic acid-6′-acetylglucoside	3.40
6.729	145	146	2-Methylcinnamaldehyde	8.65
6.815	145	146	3-Methylcinnamaldehyde	9.15
6.962	145	146	4-Methylcinnamaldehyde	6.72
7.380	263, 201, 161	264	3-Acetoxy-4-propoxy-cinnamylic acid	7.38
7.482	319, 275, 191, 147, 145	320	Carboxycinnamylic acidcarboxyphenethyl ester	14.27
7.514	360, 276, 191, 175	360	Carboxylvinylbenzoic acidcarboxylvinylphenylpropanol ester	0.77
7.774	147, 146, 103	148	Cinnamylic acid	2.88
7.892	235, 191, 147, 106	236	Carboxycinnamylic acidgylcol ester	3.17
9.102	205, 161, 117	206	Carboxymethylcinnamylic acid	1.25
10.808	203, 157, 131	204	3-Ethyl-4-ethyoxylcinnamaldehyde	1.16
11.678	179, 135, 91	180	Methylbenzenedicarboxylic acid	1.25
12.621	242, 198, 196	242		1.12
14.300	179, 135, 91	180	Methylbenzenedicarboxylic acid	1.22
14.322	355, 337, 309, 265, 245	356	Crocetinethyl ester	0.19
14.910	207, 163, 119, 75	208	4-Carboxymethyl-phenylpropanoic acid	0.70
15.193	212, 168, 141, 121	212	3,4,5-Trimethoxybenzoic acid	1.15
15.217	179, 135, 91	180	Methylbenzenedicarboxylic	0.96
15.382	179, 135, 91	180	Methylbenzenedicarboxylic	0.86
15.617	212, 168, 141, 121	212	3,5-Drimethoxy-4-hydroxy-benzoic acidmethyl ester	0.97
16.208	179, 135, 91	180	Methylbenzenedicarboxylic acid	0.77
16.465	207, 163, 119, 75	208	3-Carboxymethyl-phenylpropanoic acid	0.71
16.677	207, 163, 119, 75	208	2-Carboxymethyl-phenylpropanoic acid	0.79
17.927	175, 131, 87	176	3-Hydroxy-3-ethyl-pentanedioic acid	0.95
21.830	259, 215, 171, 159, 115	260	Carboxylvinylcinnamyl alcoholpropionate	13.52
21.958	273, 255, 173, 127	274	Vinylcinnamylic acid hexylene glycol ester	3.08

Table 2: NGF and BDNF contents in culture supernatant of C6 glioma cells in various groups

Group	NGF (pg/ml)	BDNF (pg/ml)
Normal	20.91±3.01	50.01±6.31
Solvent	19.61±3.02	49.11±6.12
Acetidin extract 15 μg·mL^{-1}	25.01±4.10**	60.10±6.76**
Acetidin extract 60μg·mL^{-1}	77.23±8.04**	194.23±19.64**
Water extract 15 μg·mL^{-1}	21.99±3.41	50.36±5.81
Water extract 60 μg·mL^{-1}	22.25±3.27	51.21±5.57

**$P < 0.01$ compared to normal control.

DISCUSSION

The pathogenesis of depression and anxiety is still not clear, even though there are various hypotheses. The popular antidepressants used on clinical practice are monoamine transmitters reuptake inhibits, such as such as fluoxetine and venlafaxine. However, these drugs are not always effective for some patients, which suggested that monoamine transmitters are not the only target of antidepressant medication. Recent studies found that neurotrophic factors are also the important pathogenesis of depression. Neurotrophins are a family of proteins that induce the survival, development, and function of neurons. They belong to a class of growth factors, secreted proteins that are capable of signaling particular cells to survive, differentiate, or grow. Neurotrophins mainly include NGF, BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), which signals predominantly through the TrkA, TrkB and TrkCreceptors to affect the survival and repairing of neurons. Clinical studies have shown that serum neurotrophin levels in depression patients are low, especially NGF and BDNF.(Aydemir *et al.*, 2005; Diniz *et al.*, 2012; Moosavi *et al.*, 2016; Okamoto *et al.*, 2008; Schmidt *et al.*, 2007; Schmidt *et al.*, 2008) So neurotrophic factors were important for diagnosis of depression and the potential target for drug development.

Stigma of *C. sativus* is the most expensive spice and apart from its traditional value as food additive. Recent studies indicate its potential as antidepressant. Because petal is

not expensive compared to stigma, there will be economical interests for further investigations by pharmaceutical industries. Findings from clinical trials conducted to date indicate that the supplementation of *C. sativus* petal and stigma improve symptoms of depression in adults. (Akhondzadeh *et al.*, 2005) In order to investigate the antidepressant components of *C. sativus* petal, we studied the effects of its extracts on synthesis and secretion of neurotrophins in rat C6 glioma cells, and identified chemical constituents by HPLC-ESI-MS/MS. The experimental results showed the ethyl acetate extract of *C. sativus* petals significantly upregulated NGF and BDNF expression (table 2), and that various cinnamic acid derivatives should be the main antidepressant constituents. Crocins and safranal were reported to treat animal anxiety, and Crocin-1 might inhibit dopamine and norepinephrine reuptake, while safranal might be 5-hydroxytryptamine reuptake inhibitor (Hosseinzadeh *et al.*, 2004; Wang *et al.*, 2010). Hydroxycinnamic acid derivatives including caffeic acid phentyl ester were reported to enhance neuronal survival and promote neurite outgrowth *in vitro* (Moosavi *et al.*, 2016). Cinnamic acid derivatives were useful for designing more effective agents for management of neurodegenerative diseases, which needs further research in the future.

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