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RESEARCH ARTICLE

ESTIMATION OF ANTI-INFLAMMATORY ACTIVITY AS WELL AS APOPTOTIC ACTIVITY OF ETHANOLIC EXTRACTS OF *CROCUS SATIVUS*

B. Arirudran[1], P. Priyadharshini[1], US Mahadeva Rao[2*]
[1]*PG Department of Biochemistry, SRM Arts and Science College, Kanchipuram district, Tamilnadu, India.*
[2]*School of Basic Medical Sciences, Faculty of Medicine, Universiti Sultan Zainal Abidin, Kuala Terengganu, 20400 Malaysia.*

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***Address for Correspondence:**
Dr. US Mahadeva Rao, School of Basic Medical Sciences, Faculty of Medicine, Universiti Sultan Zainal Abidin, Kuala Terengganu, 20400, Malaysia. Phone:+60-1116547654 E-mail: *raousm@gmail.com*

Abstract

Objective: Inflammation is a body reaction which embroils cellular and biochemical responses, which is not only symptom for shared diseases but also known to be an initial phase for certain serious Alzheimer's, cancer, heart vascular diseases. In order to overcome these drawbacks, there is an urgent need for nutraceuticals with excellent anti-inflammatory response with minimum side effects. An attempt has been made to evaluate the anti-inflammatory activity along with gene expression analysis on ethanolic extracts of *Crocus sativus* (CSEE).
Methods: Dried stigmas of *C. sativus* were analyzed for anti-inflammatory activity by macrophage scavenging assay. In this study, the phagocytic activity of the extract was tested on oxidative burst reduction of macrophages. RT-PCR was performed to analyze the anti-apoptotic gene expression during cell death, as a result of the compound treatment on cancer cells.
Results: The CSEE unveiled high phagocytic activity on the oxidative burst reduction, presenting intracellular killing and the enhancement of lysosomal enzyme activity, showing the active degranulation of macrophages.
Conclusion: These findings suggest that *C. sativus* possessed excellent anti-inflammatory as well as apoptotic activities. Hence it was proposed that *C. sativus* could be exploited against oxidative stress, anti-inflammatory, cancer and ageing therapy to justify their use in traditional medicine as a nutraceutical.
Keywords: Anti-inflammatory, *Crocus sativus*, macrophage, nutraceutical, oxidative stress.

INTRODUCTION

Inflammation is a physiological reaction which involves cellular and biochemical responses, which is not only symptom for common diseases but also known to be an early phase for some serious diseases such as alzheimer's disease, cancer, heart vascular diseases etc[1]. Non-steroidal anti-inflammatory drugs like ketoprofen, ibuprofen, aceclofenac, algesia and pyresis are under current clinical usage for the treatment of inflammation[2], but due to the decrease in production of prostaglandins in tissue[3] and due to the direct contact of free carboxylic group with the gastric mucosa they were associated with major drawbacks of gastrointestinal disorders like dyspepsia, and gastric ulcers[4,5]. In order to overcome these drawbacks, there is an urgent need for nutraceuticals with excellent anti-inflammatory response and minimum side effects. The term "nutraceuticals" combines two words "nutrient" (a nourishing food component) and "pharmaceutical" (a medical drug). The name was coined in 1989 by Stephen DeFelice, founder and chairman of the Foundation for Innovation in Medicine, an American organization located in Cranford, New Jersey. The philosophy behind nutraceuticals is to focus on prevention, according to the saying by a Greek physician Hippocrates (known as the father of medicine) who said "let food be your medicine". Their role in human nutrition is one of the most important areas of investigation, with wide-ranging implications for consumers, health-care providers, regulators, food producers and distributors. Nutraceuticals are increasingly being used as nutritional supplements in treatment of diseases. Due to the plant origin of these supplements they are considered safe for human consumption. However, the levels of the active substance consumed vary when taken as a whole food, as compared to a nutritional supplement[6,7]. Very few studies have reported on long-term effects of nutrition supplements in humans. Among the nutraceuticals, *C. sativus* is an important crop cultivated as the source of its spice for at least 3,500 years. Dried stigmas of

saffron flowers compose the most expensive spice which has been valuable since ancient times for its odoriferous, coloring and its medicinal properties[8]. Saffron has been also used as a drug to treat various human health conditions such as coughs, stomach disorders, colic, insomnia, chronic uterine haemo-rrhage, femine disorder, scarlet fever, smallpox, colds, asthma and cardiovascular disorders[9-11]. Earlier reports say that extractive of saffron shows antitumor effect against different malignant cells[12] and different tumors as well as cancers in ancient time[13].The present study is focused to evaluate the anti-inflammatory activity along with apoptotic activity of ethanolic extracts of *C. sativus* (CSEE).

MATERIALS AND METHODS

Chemical

Nitrobluetetrazolium dye, Dimethysulfoxide, Ethanol and all other chemicals and solvents were purchased from Sigma Chemical Co, St. Louis, MO, USA.

Sample collection:

Fresh stigma of *C. sativus* samples were purchased commercially from Nilgiris. Sample was authenticated based on organoleptic, macroscopic examination (PARC/2012/1254) and certified by Dr. P. Jayaraman, Director "National institute of Herbal Science and Plant Anatomy Research Centre" (PARC), West Tambaram, Chennai, Tamilnadu, India.

Preparation of *CSEE*

The *CSEE* was prepared as described by the standard method[14]. Ten grams of *C. sativus* dried stigma was coarsely powdered and weighed. The dried powder was soaked with ethanol for 48 hr with intermediate shaking separately. At the end of the extraction, it was passed through Whatman filter paper No.1 (Whatman Ltd., England). Then the filtrate was concentrated by distillation over boiling water bath and the last traces of solvent were removed under vacuum. The yield of the extract was calculated (1.0443 g), stored in dry sterile container and used for further study.

Macrophage scavenging assay

Nitrobluetetrazolium dye reduction assay was carried out for macrophage scavenging assay[15]. Briefly, 20µl of the macrophage suspension and 40 µl of Roswell park memorial institute medium (RPMI) were added in a flat bottom 96-well plate. Twenty micro liter of the solution containing the *CSEE* dissolved in 0.1% Dimethysulfoxide (DMSO) in phosphate buffer saline solution was added in each well at final extract concentrations of 10 µg/ml, 100 µg/ml, 500 µg/ml and 1000 µg/ml. The 0.1% DMSO in phosphate buffer alone used as a control. After incubation for 24 hr at 37ºC in 5% CO_2 humidified atmosphere, 20 µl of the heated inactivated yeast (*Saccharomyces cerevisiae*) suspension (5×10^7 particles/ml) and 20 µl of Nitrobluetetrazolium solution in phosphate buffer (1.5 mg/ml) were added and the mixture was further incubated under the same conditions. After incubation for 60min, the adherent macrophages were rinsed vigorously with RPMI medium and washed for four times with 200 ml methanol. After air-dried, 120 µl of 2M KOH and 140 µl of DMSO were added. The absorbance was measured at 570 nm by a well reader (Biorad Plate reader) and the percentage of NBT reduction was calculated by the following equation.

$$\text{NBT reduction (\%)} = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD negative control}} X100$$

The EC_{50} value represents the effective concentration required for 50% enhancement of oxidative burst reduction activity.

RT-PCR

RT-PCR was performed to analyze the gene expression during cell death, as a result of the compound treatment on cancer cells. Cells were harvested after treatment with active fraction. Total RNA was separated and cDNA was synthesized according to the manufacturer's protocol (Sigma Aldrich, USA). Using this cDNA as template, PCR was performed with Tnf and GAPDH gene specific primers.

Total RNA isolation

Total RNA from cell lines was separated using ONE STEP-RNA solution (phenol and guanidine isothiocyanate). It is a ready to be used reagent for the isolation of total RNA from cells and tissues. The reagent, mono-phasic solution of phenol and guanidine isothiocyanate, represents an improvement to the single step RNA isolation method developed by Chomczynski and Sacchi[16]. In order to decrease the possibility of RNA degradation during the procedure, all glassware and plastic ware were treated by incubating them overnight in 0.01% DEPC water (RNase-free) to decrease or reduce the risk of RNA begin depredated by RNase[17]. After incubation, all of the materials used for isolation were autoclaved and dried in the oven. Approximately $5\text{-}10x10^6$ cultured cells were taken to ensure for RNA isolation. Cells were pelleted by centrifugation at 1000 rpm, 5 min and 1 ml of ONE STEP-RNA reagent was added. Cell lysis was performed by repeated pipetting. Homogenized samples were incubated at 15 to 30℃ for 5 min to allow the complete dissociation of nucleoprotein complexes; 0.2 ml of chloroform per 1 ml of ONE STEP-RNA reagent to the sample. Tubes were shaken vigorously by hand for 15 sec and incubated at 15 to 30℃ for about 2 to 3 min and then the samples were centrifuged at 12,000 rpm for 15 min at 2 to 8℃. The mixture separated into two phase, lower phenol-chloroform inter-phase of cloudy white and upper colorless aqueous phase.

The RNA remains exclusively in 60% volume of upper aqueous phase of ONE STEP-RNA reagent used for homogenization. RNA was precipitated from the aqueous phase by mixing it with isopropyl alcohol. Samples were incubated at 15 to 30℃ for 10 min and centrifuged at 12,000 rpm for 10 min at 2 to 8℃. The RNA precipitate, often invisible before centrifugation, supernatant was removed and the gel-like RNA pellet at the bottom was washed once with 75% ethanol by centrifuging at 7,500 rpm for 5 min at 2 to 8℃. RNA pellet was dried by vacuum-dry for 5 to 10 min and finally dissolved in DEPC treated water and stored in -20℃.

cDNA preparation

After RNA isolation, RNA was immediately reverse transcribed with Easy Script Plus™ Reverse

Transcriptase. For RT-PCR, 1-2 µg of RNA was used corresponding to 1-10 µl of total RNA isolate. RNA isolated from fresh tissue samples was reverse transcribed, where oligo-dT was used as a primer, into a 1.5 ml eppendorf PCR tube, 1-2 µg of RNA, 2 µl of oligo-dT (stock was 10 µM) was added and the total volume was made up to 12.5 µl with DEPC treated water. The tube was incubated at 65ºC for 5 min and chilled on ice. Then, 4 µl of 5X reverse transcriptase buffer (final concentration 1X), 2 µl of 2 mMdNTP mix (final concentration 0.2 mM) and 0.5 µl of RNase inhibitor (40 U/µl) were added in the indicated order. After incubating at 42ºC for 5 min, 1 µl of Easy Script Reverse Transcriptase (200 U/µl) was added. The reaction was carried out at 42ºC for 50 min. Finally, the tube was heated up to 70ºC for 10 min and chilled on ice. The samples were stored at -20ºC until further use.

PCR

The cDNA obtained was amplified by PCR. Gene specific PCR was used to amplify Tnf. A constitutively expressed gene, namely GAPDH was selected in order to assess the quality of PCR. The primers for the study were purchased from Eurofins Genomics India Pvt Ltd., Bangalore, India. Anti-apoptotic gene expressions were studied using primers intF and intR primers (Table 1, Table 2 and Table 3). Amplification was carried out in a 20 µl volume containing 0.3 µM of each primer (Eurofins, India), 0.2 mM deoxy nucleotide triphosphates (dATP, dCTP, dGTP and dTTP) (Biotools, Spain), 100 ng of template DNA sample and 1U of Prime Taq DNA polymerase (Genetbio, Korea). The reaction tubes were subjected for thermal cycling reactions consisted of an initial denaturation (3 min at 94°C) followed by 32 cycles of denaturation (30 sec at 94°C), annealing (1 min at 49°C) and extension (1 min 20 sec at 72°C), with a final extension (7 min at 72°C). The procedure was repeated for GAPDH gene. PCR products were visualized using 1.5% agarose gel stained with EtBr (20 mg/ml). The molecular weight of the bands was estimated using 1Kb DNA Ladder as reference.

Agarose Gel Electrophoresis of PCR Products

In a total volume of 25 ml, 1.5% agarose and 1X TAE buffer were prepared and poured onto a gel tray. The PCR product was mixed with the loading dye. The mixture was loaded to each well along with 1 kb ladder as a reference. The gel was run at 50V for 90 min and visualized.

Expression folds calculation

Expression ratio was derived by analyzing the gel photos in software-Image J (Java based image processing). Expression ratio was obtained using the formula:

$$\text{Target gene} = \frac{\text{gene expression}}{\text{internal control}} X100$$

RESULTS AND DISCUSSION

In this study, the phagocytic activity of the C*SEE* was tested on oxidative burst reduction of macrophages. The Figure 1 shows that CSEE enhanced the NBT reduction at 10, 100, 500 and 1000 µg/ml by 5% ($p< 0.01$), 35% ($p< 0.01$), 55% ($p< 0.01$) and 65% ($p< 0.01$) respectively. The higher reduction in NBT assay represented higher activity of the oxidase enzyme reflecting the stimulation of phagocytes in proportion to the foreign particles ingested[15]. *CSEE* exhibited high phagocytic activity on the oxidative burst reduction, presenting intracellular killing and the enhancement of lysosomal enzyme activity, showing the active degranulation of macrophages. The maximum phagocytic activity of the extract on the NBT dye reduction was found and the % of NBT dye reduction was found to be 1000µg of *CSEE*, with an EC_{50} value of 150 mg/ml. Crocins, *Crocus* glycosides, exhibited an anti-inflammatory effect in some models of inflammation[17]. Flavonoids such as rutin, quercetin, luteolin, hesperidin and bioflavonoid produced significant antinociceptive and anti-inflammatory activities[19-21]. Flavonoids, tannins, anthocyanins, alkaloids and saponins exhibited antinociceptive effects in chemical pain test as well as acute and chronic anti-inflammatory activity[22-23].

Table 1: Sequence of the primer used in the RT-PCR.

Tnf F	5' ATGATGGATCTTGAGAGTCAG 3'
Tnf R	5' TCATAAAGCAAACACCCCAAAGAA 3'
GAPDH Forward	5` TCCCATCACCATCTTCCA 3`
GAPDH Reverse	5` CATCACGCCACAGTTTCC 3`

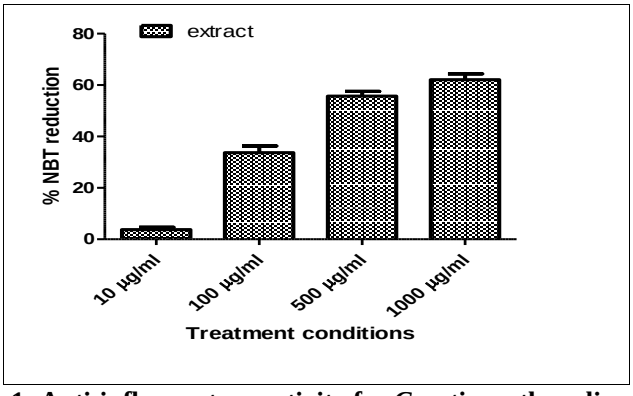

Figure 1: Anti-inflammatory activity for *C. sativus* ethanolic extract.

Table 2: PCR reaction setup for GAPDH and Tnf genes.

	Stock Concentration	Final Concentration	Final Volume (for 20 µl)
Mili Q Water	-	-	11.4 µl
Taq Buffer (with $MgCl_2$)	10X	1X	2 µl
dNTPs	2 mM	0.2 mM	2 µl
$MgCl_2$	25 mM	2.5 mM	2 µl
Primer-Forward	3 nM	0.3 µM	0.2 µl
Primer-Reverse	3 M	0.3 µM	0.2 µl
Template cDNA	-	10% of the reaction	2 µl
Taq Polymerase	5 U/µl	1 U	0.2 µl

Table 3: PCR reaction conditions for GAPDH and Tnf genes.

	Tnf	GAPDH
Initial denaturation	94°C for 2 min	94°C for 2 min
Denaturation	94°C for 30 sec	94°C for 30 sec
Annealing	56°C for 1 min	53°C for 1 min
Extension	72°C for 1 min 20sec	72°C for 1 min 20sec
Final extension	72°C for 7 min	72°C for 7 min
Hold	4°C	4°C
Total number of cycles	32	32

It was reported that tannins has an important role in antinociceptive and anti-inflammatory activities[24]. Phenolic compounds have been shown to possess antioxidant activity based on their (hydroxyl group) donation to free radicals. Moreover, phenolic compounds also possess a wide spectrum of biological activities such as antimutagenic, anticarcinogenic, anti-inflammation, anti-allergic, as well as the ability to modify gene expression[25-32]. The authors have had phytochemical study in CSEE and reported that *C. sativus* extract has a rich amount of secondary metabolites like carbohydrates, tannins, saponins, flavonoids, alkaloids, quinones, cardiac glycosides, phenols, coumarins, phyto steroids, anthroquinones[33]. The anti-apoptotic activity related gene expression study results were well aligned with other researcher's findings with different extracts (*Musa paradisiaca*, *Vernonia amygdalina*, *Melastoma malabathricum*, *Persea americana*, *Monopterus albus* and *Channa straitus* extracts) as well as secondary metabolites (syringin and scopoletin)[34-40].

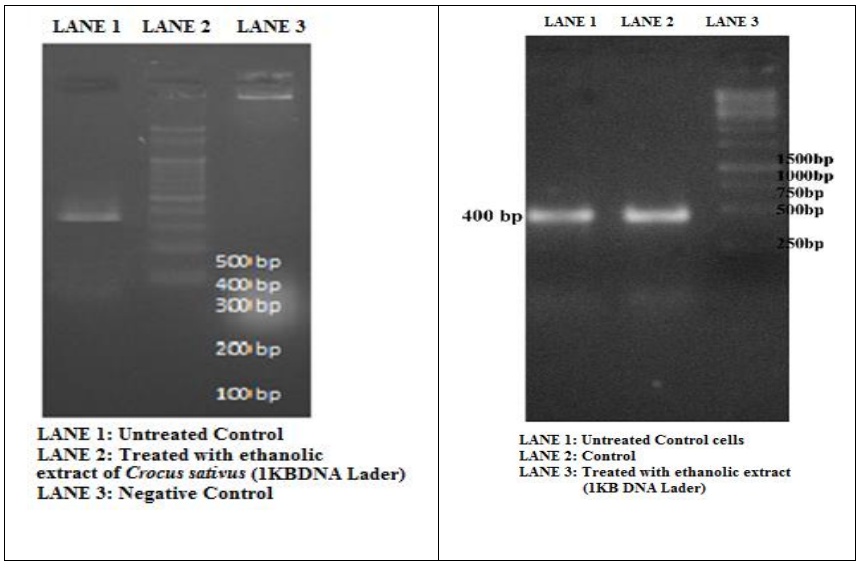

Plate 1 **Plate 2**

Figure 2: Plate 1: Expression of Tnf gene, Plate 2: Expression of GAPDH gene.

Henceforth, these findings advocated that, the phagocytic mediated to macrophage-lymphocyte defense system may be due to the presence of some secondary metabolic active principle compounds present in the CSEE and it is responsible for intracellular killing more than degranulation. This property of *C. sativus* may be a safe and effective choice in the treatment of anti-inflammatory disorders. In future, studies should be carried out to pinpoint the mechanism of respective phytochemical both in an animal model and cell lines to exploit the medicinal potential of *C. sativus*.

In the present study the plate 1 and 2 displayed that the RT-PCR was made with Tnf and GAPDH gene specific primers to amplify. In the plate 1, Tnf gene was expressed in 1 KB ladder of about 500 bp was observed, in the photographic plate 2 and GAPDH gene was expressed in 1 KB ladder of about 400bp.

In this model, CSEE caused the suppression and subsequent expression of mRNA for tumor necrosis factor, interleukin. It has been demonstrated that CSEE possesses anti-apoptotic effects on non-cancerous cells which incorporate out it into a model showing a possible mechanism for the anti-cancer effect of saffron by promoting apoptosis, inhibiting cell proliferation and blocking inflammation in carcinomas by Tnf expressions. Tumour necrosis factor Tnf is a cytokine that has a wide variety of functions. It can cause cytosis of certain tumor cell lines; it is involved in the induction of cachexia; it is a potent pyrogen, causing fever by direct action or by stimulation of interleukin-1 secretion; it can stimulate cell proliferation induced cell differentiation under certain conditions. These findings indicate that saffron provides an anticancer protective effect by promoting cell death apoptosis and inhibiting proliferation of cancerous cells and blocking inflammation.

CONCLUSIONS

In supposition, these preliminary findings indicated that *C. sativus* can be a potential source of natural immune stimulator as well as an antioxidant agent. In addition, *CSEE* (Saffron stigma and petal) exhibit antinociceptive, anti-inflammatory activity, along with potential free radical scavenger and act as an important tool in cancer prevention. Further studies are warranted to isolate the active constituent from *C. sativus* for herbal preparations against oxidative stress, inflammation, cancer, ageing etc, and justifying their use in traditional medicine.

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AUTHOR'S CONTRIBUTION

Arirudran B: writing original draft, conceptualization, methodology, investigation. **Priyadharshini P:** writing, review, and editing, supervision, resources. **Rao USM:** writing, review, and editing. **Ijaz AS:** writing, review, and editing, project administration. The final manuscript was read and approved by all authors.

DATA AVAILABILITY

The data and material are available from the corresponding author on reasonable request.

CONFLICT OF INTEREST

No conflict of interest associated with this work.

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