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Cytotoxicity, Antioxidant and Antibacterial Activities of Crocus Sativus Petal Extract

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Abstract

Crocus Sativus Petals are a dusted waste that contains bioactive compounds. The aim of this study was to evaluate the cytotoxicity, antioxidant, antibacterial activities of Crocus Sativus petals extract (CSPE). The cytotoxic effects of CSPE on human liver cancer cells (HepG2) were performed using MTT assay. Then, cell morphology was determined by inverted microscope. The antioxidant activity of CSPE was evaluated using DPPH and ABTS assays. Antibacterial activity of CSPE was tested against gram-negative bacteria *Escherichia coli (E. coli)* and gram positive bacteria *staphylococcus aureus (S. aureus)* using macrodilution method. CSPE inhibited the proliferation of the HepG2 cell line in a time and dose-dependent manner, which was consistent with morphological changes. Extract showed the enhanced antioxidant properties and also were found effective against the bacterial strains. Due to the anti-proliferative, antioxidant, antibacterial effects of CSPE, it can be used in pharmaceutical industry, including the production of anticancer, antioxidants and antibacterial drugs.

***Keywords*:** Crocus Sativus petal extract, HepG2, antioxidant activity, antibacterial activity, pharmaceutical industry

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Introduction

Since the importance of natural products in modern medicine is well-known(1), Researchers interested in studying plants with the aim of separating new drugs to replace antibiotics (2, 3) and chemotherapy drugs. The use of natural products from plant sources can be of great help in the treatment of various diseases (4) and a template for the correction of artificial products (1). Therefore, it is essential that the safety, quality, toxicity, and proper amount of plants be considered as a useful medicinal agent (5). According to epidemiological studies, the long-term use of some plant compounds is associated with a reduction in the incidence of cancer (6), antimicrobial resistance and antioxidant effects (7).

Antioxidants play a vital role in neutralizing free radicals (8). High levels of free radicals cause oxidative stress in the cell, causing damage to essential macromolecules, such as DNA, lipids, and proteins (5). The damage of macromolecular may contribute to the development of some chronic diseases such as cancer, cardiovascular disease and inflammation (9). Secondary metabolites, including phenolic compounds and carotenoids, are responsible for biological activities of plants (5).

Crocus Sativus L. (C. Sativus), commonly known as saffron, is a perennial stemless herb of Iridaceae family (10). This plant is widely cultivated in Iran (11). It has a biting taste and a sweet smell which has been in use in the preparation of various foods and considered as an important herb in cosmetics, medical and hygienic industries (12, 13). Its flower consists of various chemical compounds which have been used in folk medicine for more than 3000 years (14). It has 150 volatile and nonvolatile substances in Crocus Sativus (12). The volatile compounds include Safranal, which acts as the Crocus Sativus aroma factor (13). Crocin (color agent), crocetin and picrocrocin (bitter taste) classified as non-volatiles agents (12). The different parts of Crocus Sativus contain three stamens, three red stigma and six petals. Petals are usually distilled as a waste and contain sugar, protein, amino acids, minerals and crude fiber, small amounts of pigments such as anthocyanins, alpha carotene, beta-carotene, and xanthan (15). Studies have shown that phenolic compounds(13,14), crocin, crocetin, and kaempferol may play an important role in protecting against oxidative stress (16). The aqueous and ethanolic extracts of Crocus Sativus can be used in the treatment of various disorders as anti-depressant (17), anti-inflammatory, anti-coagulant (18), analgesic, antihypertensive (16), anti-cancer (1) and anti-tumor (13, 14, 19). Most studies have examined the effects of Crocus Sativus, but so far no comprehensive study has been conducted on the CSPE. The purpose of this study was to evaluate the cytotoxic effects of CSPE on human liver cancer cells (HepG2), antioxidant activity and its antibacterial activity.

Materials and Methods

Preparation of Crocus Sativus Petal Extract:

Crocus Sativus plants have been cultivated in the region of Torbat Heydariyeh (East of Iran) without any chemical treatments. Various parts of the plant were collected manually from October to November. Crocus Sativus petals dried in the shade and powdered with the help of the mill. Dried milled powder of Crocus Sativus petals (1g), were mixed with 100 ml of distilled water and heated to 100 ° C. The extract was filtered using Whatman filter paper.

Figure 1. Plant Crocus Sativus

Cell lines and culture method:

HepG2 human liver cancer cells were purchased from the Pasteur Institute of Iran Cell Bank. Cells were cultured in DMEM (Bio-Idea, Iran) with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin / streptomycin (Gibco, USA) and maintained in a

humidified cell incubator (5% CO2, 95% humidity and 37 ° C).

Cytotoxicity assay:

Cytotoxicity was evaluated using by 3- (4, 5-dimethyl-2-thiazole) -2, 5-diphenyl-2- hydroxide oxide bromide (MTT) (Sigma Aldrich, USA) reagent. MTT is one of the yellow soluble tetrazolium salts that is reduced by active cellular dehydrogenases and then converted to insoluble purple formazan crystals (20). In this method, 2×10^5 HepG2 cancer cells were placed in 96-well microplates and incubated for 24, 48 and 72 hours. Different concentrations of CSPE were prepared and added to the cultured cells. Then, 50 µl of MTT solution was added to each plate and incubated for 4 h at 37 ° C and then formazan crystals in dimethyl sulfoxide (DMSO) (100 µl) were dissolved. Finally the optical density (OD) of plates was read at 570 nm by a microplate reader (Bio-Tek Instruments Inc., Vermont, and USA). The cytotoxicity of CSPE on HepG2 cells was expressed as IC_{50} (a concentration of the extract, which reduces 50% of cell growth compared with control). The percentage of viable cells was calculated as follows:

Percentage of viable cells = (Mean absorbance of the control sample / Mean absorbance of the treated sample) × 100

Study of cell morphology:

In order to observe the morphological changes of HepG2 cells treated with CSPE, an inverted microscope with a magnification of 40X was used. The cells were then treated with IC_{50} concentrations of CSPE (18.75-75 µg / ml) for 48 hours. Untreated cells were used as negative controls.

Antioxidant assays:

The materials used in this research included DPPH (2, 2-dipheny-1-picrylhydrazyl), ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6 – sulphonic acid), BHA solution (Butylated hydroxyanisole) from Sigma-Aldrich USA, EDTA and ethanol were purchased from the German Merck company. The two antioxidant methods for scavenging of free radicals are described as below.

DPPH assay:

Different concentrations of CSPE were added to the solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read using a UV spectrophotometer at 517 nm. DPPH solution with ethanol solution as negative control and BHA as standard antioxidant (positive control) was used to compare with CSPE activity (21). The percentage of DPPH scavenging was calculated using the following equation:

% scavenging (DPPH) = (absorption of control-absorbance of sample / absorption of control) × 100

ABTS assay:

ABTS cation solution was prepared by mixing the ABTS solution with sodium persulfate at room temperature in the dark for 16 h. The solution was diluted with ethanol to reach a concentration of 0.756. The absorbance of reaction mixture was measured at 734 nm. The percentage of inhibition of free radical by ABTS was calculated in the following formula:

% scavenging (ABTS) = (absorbance of control-absorbance of sample / absorbance of) × 100

Antibacterial assay:

Two standard bacterial strains (*Staphylococcus aureus* (ATCC25923) and *Escherichia coli* (ATCC25922)) were used for evaluation of the antibacterial activity of CSPE were obtained from the Iranian Pasteur Institute. The macrodilution method was used to evaluate antibacterial activity. This method involves the preparation of 2 dilution series of an antimicrobial agent in a liquid medium, which is distributed to tubes containing a minimum volume of 2 milliliters, and then 10 µl of bacterial suspension added in test tubes for 24 hours at 37 ° C. The lowest concentration of CSPE that inhibits the growth of the bacterial strain is considered as the minimum inhibitory concentration (MIC). The minimum bactericidal concentration (MBC) can be determined after the MIC test. For this purpose, MIC tubes, that did not observed any growth, at the surface of the Muller Hinton Agar culture medium was cultured. The plates were then incubated for 24 hours. After incubation, the lowest CSPE concentration, which did not show any growth on

the solid medium, was considered as MBC for the CSPE (4).

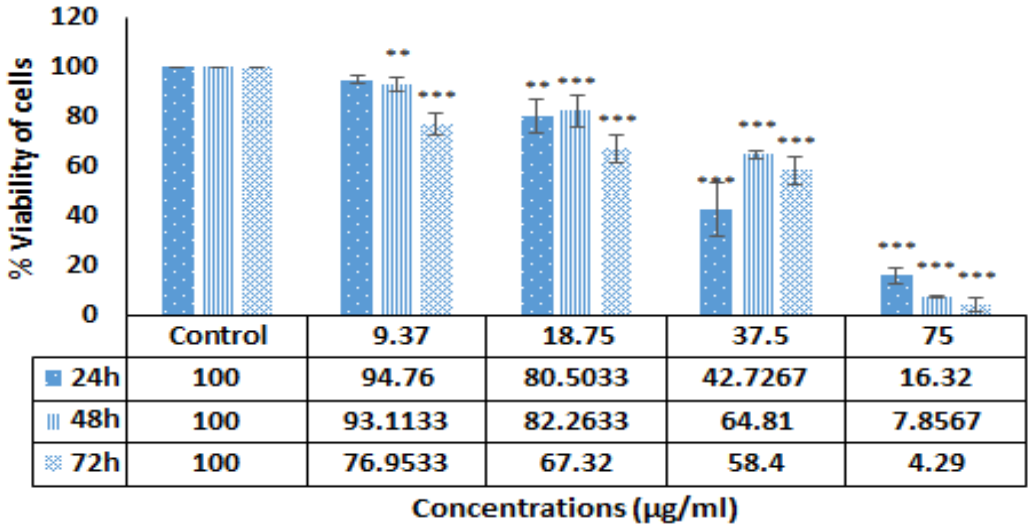
Statistical analysis:

All experiments were performed in three replicates. Statistical analysis was performed using SPSS software v18.0 (SPSS Inc., Chicago, USA). One-way analysis of variance (ANOVA) was used for analysis of variance and comparison of the meanings was done by least significant difference or LSD. Data is expressed as mean ± standard deviation.

Results

Cytotoxicity assay:

In our study, the cytotoxicity of various concentrations of CSPE was evaluated in HepG2 cells in vitro using MTT assay. Experimental results showed that CSPE reduced cell viability by dose and time dependent, and inhibited the proliferation of the HepG2 cell line (Figure2). The dose inducing 50% cell growth inhibition (IC_{50}) against HepG2 cells was 37. 5 µg/ml treatment with CSPE after 72h (P<0. 001) and showed that CSPE had a long lasting effect.



	Control	9.37	18.75	37.5	75
24h	100	94.76	80.5033	42.7267	16.32
48h	100	93.1133	82.2633	64.81	7.8567
72h	100	76.9533	67.32	58.4	4.29

Figure 2. The cytotoxic effect of CSPE on HepG2 cells. * P <0. 05, ** P <0.01 and *** P <0. 001

Cellular morphology analysis:

Figure3 shows the morphological changes of HepG2 cells treated with different concentrations of CSPE (18.75, 37.5 and 75 µg / ml) for 48 hours. In the control, the cells have a cylindrical shape. At a concentration of 18.75 µg / ml, the CSPE inhibits the growth of HepG2 cells after 48 hours compared with control. By increasing the concentration of CSPE, the cellular morphology of the spherical to cylindrical shape has been altered, and more cell growth has been inhibited and, as compared to the control group, the granulocyte content of the cells increases and cell size is reduced and the cells seem to shrink.

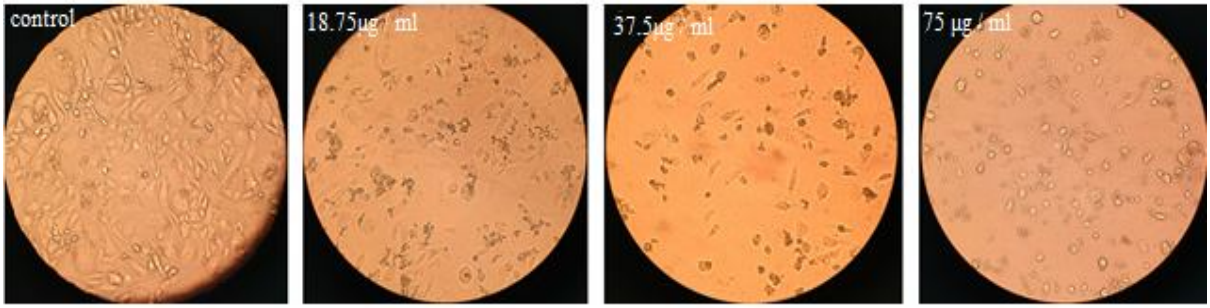


Figure 3. Morphological changes of HepG2 cells treated with different concentrations of CSPE

Antioxidant activity:

Antioxidant activity of CSPE was evaluated by both DPPH and ABTS methods. The results of the antioxidant activity show that by increasing the concentration of CSPE, the DPPH (Figure4a) and ABTS radicals (Figure4b) also increase. The radical release activity of DPPH and ABTS at the highest concentration of CSPE (500µg / ml) was 80.98% and 93%, respectively.

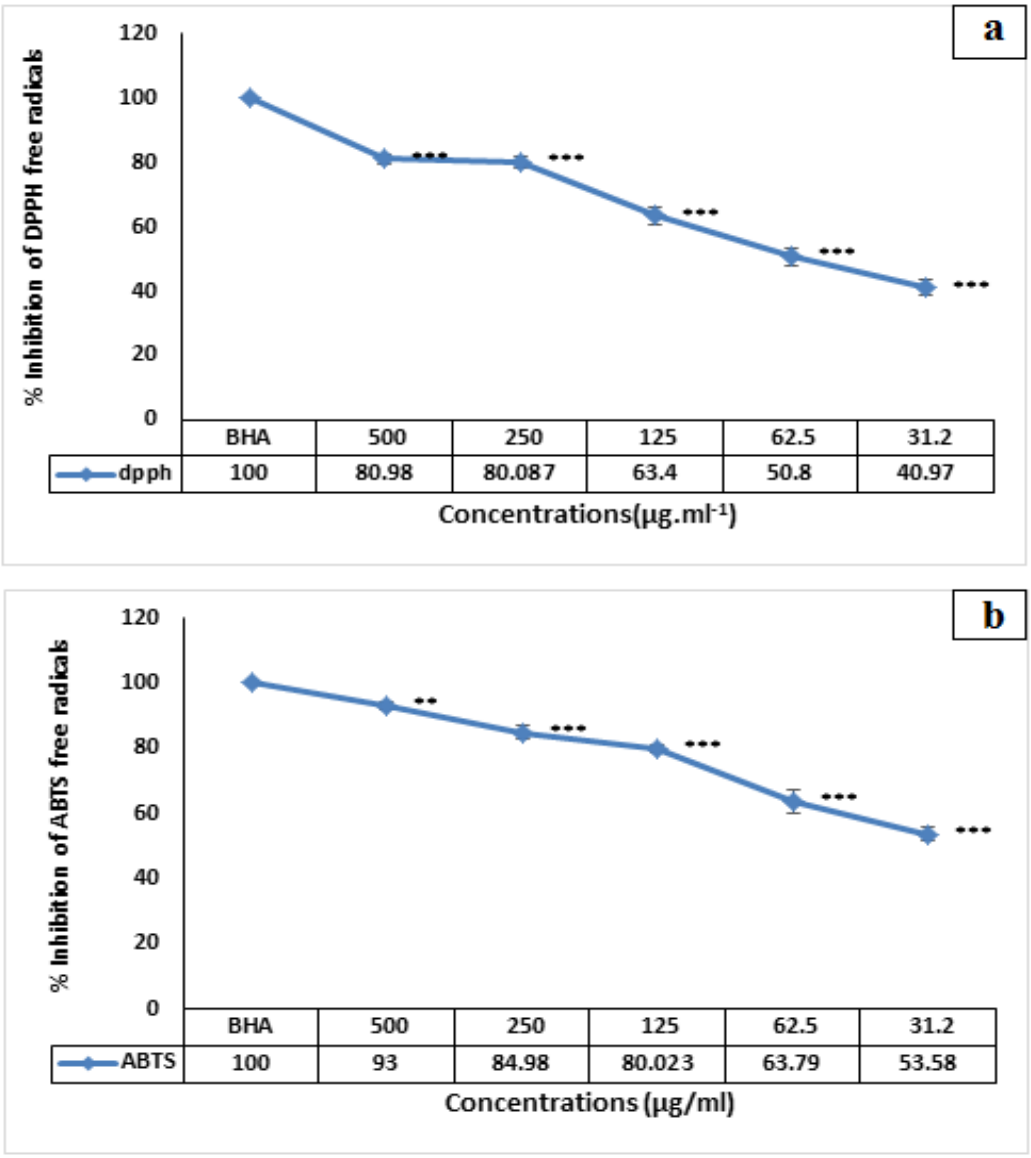


Figure 4. Antioxidant activity of CSPE using: a) DPPH method and b) ABTS method. BHA was used as the standard. *** P <0.001 and ** P<0.01

Antibacterial activity:

CSPE showed inhibitory effects on pathogenic bacterial strains. The results of MIC for CSPE (Table 1) against Escherichia coli and Staphylococcus aureus were respectively 62.5 µg / ml and 250 µg / ml and had a significant antibacterial effect on gram-negative bacteria.

Table 1. MIC and MBC CSPE against Escherichia coli and Staphylococcus aureus

	Staphylococcus aureus		Escherichia coli	
Tested sample	MBC(µg.ml-1)	MIC(µg.ml-1)	MBC(µg.ml-1)	MIC(µg.ml-1)
CSPE	500	250	250	62.5

Discussion

Toxicity in many cells after chemotherapy is a major concern. Therefore, trying to achieve a healthy and reliable diet was the main target of cancer research (1). Before using new drugs such as extract of plants, research on their cytotoxic evaluation is required (18). CSPE showed significant cytotoxic against HepG2 cells. The high sensitivity of HepG2 to CSPE can be part of the difference in growth rates between cancer cells and the cytotoxic behavior of CSPE is selective for HepG2 cells. To date, the precise CSPE anticancer mechanism is not clear. We guess that cancer cells are targeted by the presence of active compounds of crocin and crocetin in CSPE. The present study is consistent with previous studies (1). The use of crocetin in lung cancer reduces the peroxidation of lipids and glutathione metabolizing enzymes, which is an antitumor agent (13). Some studies have shown that Crocus Sativus carotenoids directly bind to DNA minor grooves and thus cause changes in the structure of DNA and induce apoptosis in cancerous cells (22). Stimulation of apoptosis by Crocus Sativus plays an important role in inhibiting the proliferation of human cancer cells, including HepG2, Hela (23), A549 (18), and MCF-7 (24). Morphological results confirmed our MTT results. Morphological changes are concentration-dependent. However, the cells were completely eliminated at the highest concentration of CSPE. Previous studies have shown that Crocus Sativus extract reduced the number of survival cells, rounded the nucleus in A549 cells (18), and also had normal morphology on cardiac cells (1). In this study, CSPE showed significant antioxidant activity. The release of radicals by any material is due to its antioxidant capacity (4). Crocus Sativus petals are a source of natural compounds, kaempferol, crocin and crocetin, and may play an important role in protecting against diseases caused by oxidative stress (16). The aqueous and ethanolic extracts of Crocus Sativus petals had antinociceptive and anti-inflammatory effects in mice (25). We anticipate that this is the antioxidant effect of the CSPE, which is responsible for the observed activities. Active substance of Crocus Sativus (crocin) reduced lipid peroxidation products and increased antioxidant power in injured rat kidneys (26). Due to its antioxidant activity, crocin inhibits liver toxicity and safranal can be a candidate for preventing liver damage by protecting against oxidative stress and enhancing the activation of antioxidant enzymes (27). Long-term use of antibiotics leads to resistant to infectious diseases, so researchers are looking for new antimicrobial agents from a plant source for treatment (3). CSPE inhibitor activity may be due to antibacterial compounds in CSPE. Reports indicate that methanol stigma and callus extracts of Crocus Sativus showed significant antibacterial activity against bacterial strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, but not effective in inhibiting *Escherichia coli* and *Shigella flexneri* growth (4). Another study has shown that Crocus Sativus has a stronger antibacterial effect on *Staphylococcus aureus* (3). In the present study, we predict that the sensitivity of the Gram-negative bacteria in comparison with the Gram-positive bacteria to CSPE is related to the cell wall structure, physiology, and degree of contact of the bacterial cell.

Conclusion

The importance of Crocus Sativus in traditional medicine is known for various types of diseases. Crocus Sativus petals are discarded as waste or used only as fertilizer. Our research is the first complete report that shows the effects of CSPE in vitro and the results are satisfactory and CSPE has significant cytotoxic, antioxidant, and antibacterial effects. Crocus Sativus petals have a rich source of natural compounds with antioxidant activity. Therefore, we assume that release of free radicals is responsible for some activities,

including cytotoxicity, anti-aging and anticancer. It is recommended that the CSPE be used in pharmaceutical industries, including the production of anticancer drugs, antioxidant and antibacterial. Farmers and researchers may not see the Crocus Sativus petals as waste, but it can be used as an important plant source in the pharmaceutical industry. Further studies are needed to fully recognize the biologically active ingredients, quantification and identification of molecular profile. and mechanisms involved in the death by Crocus Sativus petals.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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