Assessment of in vitro cytotoxicity of saffron (Crocus sativus L.) on cervical cancer cells (HEp-2) and their in vivo pre-clinical toxicity in normal Swiss albino mice

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Abstract
Cervical cancer is the second most common cause of death in women followed by breast cancer. Chemotherapy is the most feasible format to handle but their clinical consequences such as tumor relapse, drug resistance accompanied with severe toxicity is uncontrollable. Developing non-toxic chemotherapeutic drug is major goal of cancer researchers. Medicinal herbs and plants remain as important source of drugs. In this study we explored in vitro anti-cancer potential of Crocus sativus (common name - Saffron) extract (CSE) on cervical cancer cells (HEp-2) and to study their in vivo toxicity profile in normal Swiss albino mice. HEp-2 and vero cell viability was measured by MTT assay and morphology of cells was observed after 24 h of treatment of CSE (25, 50, 75 and 100 µg/ml). CSE elicited considerable dose-dependent cell death on HEp-2 cells whereas vero cells survived after 24 h of treatment. Further HEp-2 cells lost their adherence and morphology significantly after 24 h of treatment. In vivo toxicity profile by histological studies revealed no observable histopathologic differences in the liver, kidney, spleen, lungs and heart of CSE treated and untreated groups. Further we found that crocin is the principal component of CSE extract by high performance liquid chromatography (HPLC). Thus our present study compels us to continue to examine the effects of CSE on other human and murine neoplasms with the final goal of transforming this formulation to clinic. However, more detailed mechanistic studies are needed to fully delineate the role of C. sativus in cancer treatment.

Keywords: Cervical cancer, Crocus sativus, saffron, vero cells, toxicity, crocin

1. Introduction
Cervical cancer is the second most common cause of death in women, and is considered a serious public health problem worldwide [1]. If not found in the early stages, cervical cancer can spread from the surface of the cervix, deep into the tissue of the cervix as well as the surrounding tissues within the vagina and uterus [2]. The compounds used in chemotherapy are only effective when applied to patients with small tumors detected in an early stage, so chemotherapy is not effective in patients with terminal cancer or metastasis. Furthermore, the compounds used generate severe side effects associated with their necrotic activity [3, 4], a fact that indicates the need to seek new compounds, ones with high anti-proliferative activity on cancer cells but low necrotic effects on normal cells.

Natural products from plant sources have extensive past and present use in treatment of diverse diseases and serve as compounds of interest both in their natural form and as templates for synthetic modification. The importance of natural products in modern medicine has been well recognized. Scrutiny of medical indications by source of compounds has demonstrated that natural products and related drugs are used to treat 87% of all categorized human diseases such as infectious and non-infectious [5]. Recently, CSE was found to have anti-cancer activities against leukaemia, osteosarcoma, fibrosarcoma and ovarian carcinoma cells [6]. Crocin, a main constituent of the CSE, exhibits a variety of pharmacological effects in mice including inhibition of skin tumor growth [7], improvement of learning behavior previously impaired by ethanol [8], anti-hyperlipidemic effects [9]. In recent past, some of our work on Crocin have shown potential free radical scavenging and lipid peroxidation inhibition [10]. Cytotoxic and apoptogenic effect of CSE on different human cancer cell lines [11] and in vitro xenograft inhibition of daltons lymphoma [12], DNA Fragmentation and cell cycle arrest of CSE in a human pancreatic cancer cell line [13], and cytotoxicity activity against breast and lung cancer cell lines [14]. To extend our observation on anti-cancer efficiency of CSE, in the present study, we evaluated in vitro cytotoxicity effect of CSE on HEp-2 cell line and in vivo toxicity profile on normal Swiss albino mice.
2. Materials and methods
2.1 Chemicals and reagents
Trypsin-EDTA solution, 3-(4,5 dimethylthiazole- 2-yl)-2,5- Dimethyl tetrazolium bromide (MTT), penicillin streptomycin 100 units, Dimethyl sulfoxide (DMSO), minimum essential medium (MEM), RPMI 1640 medium, fetal bovine serum were purchased from Sigma Aldrich chemical Co (St. Louis, USA). All the other chemicals used in this study were of pure analytical grade.

2.2 Plant material and extraction
Saffron stigma was powdered using mortar and pestle. Powdered materials were extracted with ethanol and it is store in -20°C until use for experiment. The active component was elucidated by different spectral analysis such as IR, NMR, MS (data not shown).

2.3 Drug Preparation
Different concentration (25, 50, 75 and 100 µg/ml) of CSE was freshly prepared by reconstituting them in cell culture medium.

2.4 Cell lines and Culture Method
HEp-2 and Vero cells were purchased from ATCC, USA. Cells were cultured in minimum essential medium and RPMI 1640 medium respectively with 10% fetal bovine serum and 1% antibiotics (Penicillin/streptomycin) and maintained in humidified cell incubator at 37 °C and 5% CO2.

2.5 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) cell proliferation assay
HEp-2 and Vero cells (1X10^5/well) were seeded in 96 well plate (100 µl/well) and allowed to adhere firmly overnight in respective cell culture medium with 10% FBS. Then cells were treated with different concentration of CSE for 24 h. Then medium was removed and cells were incubated with MTT reagent (5mg/ml) for 4 h and violet crystals dissolved in DMSO and absorbance was read at 540/690 nm using 96 well plate reader (Biotech, UK). Absorbance of control (without treatment) considered as 100 % cell survival. Doxorubicin was used as positive control. Morphology of cells was photographed after treatment period by Olympus microscope at 100X magnification.

2.6 Animals
Swiss albino (Mus musculus) male mice (25 – 30 g) were maintained under standardized, environmental conditions (22-28°C, 60-70 % relative humidity, 12 h dark/light cycle and water ad libitum). Then animals were divided in to two groups (n=16). Group I treated with vehicle (saline) and group II treated with CSE (300 mg/kg of body weight) at weekly intervals of 60 days. All the experiments were conducted under the guidelines of Institutional Animal Ethical Committee.

2.7 Histopathological analysis
At the end of experimental period, animals were euthanized by cervical dislocation and liver, kidney, spleen, lung and heart were dissected and tissue sections were stained with hematoxylin and eosin staining as described earlier [15]. Microscopic evaluation was done blindly by pathologist.

2.8 Statistical evaluation
Data presented as mean ± SD of four duplicates of three independent experiments. Experimental data were evaluated by students‘t’ test and one or two way analysis of variance (ANOVA). Significant difference between each set of data were considered at the confidence level of p<0.05 & p<0.001.

3. Results
3.1 Identification of active component from CSE
CSE was fractionated by HPLC and crocin is the major active principle found in CSE (Figure 1). Further structure of crocin (Figure 2) was elucidated by different spectral analysis such as IR, NMR, MS (data not shown).

3.2 In vitro cytotoxicity of CSE on HEp-2 cells and Vero cells (normal)
We tested different concentration of CSE for cytotoxic activity against HEp-2 cells and vero cells. We found that CSE can elicit considerable dose dependent cell death on HEp-2 cells whereas vero cells survived after 24 h of treatment (Figure 3). This results indicates that extract sensitize the HEp-2 cells compare to Vero cells. Further HEp-2 cells lost their adherence and morphology significantly after 24 h of treatment (Figure 4A and 4B).

3.3 In vivo toxicity of CSE in normal Swiss albino mice
To address the preclinical toxicity issue of CSE, we tested the maximum dose (300 mg/kg) of CSE in normal Swiss albino mice. After 60 days of treatment period major organs were dissected and examined for tissue damage and compared with untreated mice. H&E staining of tissue sections evidenced that CSE administration does not elicit significant damage in different organs such as liver, kidney, spleen, lung and heart (Figure 5A, B, C).

4. Discussion
Scientists worldwide are more attracted to indicate that consumption of saffron positively correlates with a lower risk of many types of cancers, and they also investigated the attribution of the large number of phytochemicals in saffron. Among these phytochemicals, crocins, crocetin, picrocrocin, and safranal are considered medicinally bioactive and the most frequently examined in many in vitro and in vivo studies [10]. In this study, we found that CSE can elicit significant toxicity on HEp-2 cells by sparing normal Vero cells (Figure 3). This might be due to presence of crocin in CSE which target specifically tumor cells. The current study is in agreement with our previous reports [10, 11, 13, 14]. Toxicity in many tissues following chemotherapy is a major clinical concern. Therefore, the search for safe, well tolerated regimen has been major goal of clinical cancer research. There are many natural agents such as vinca alkaloids and taxanes, although effective treatment agents are known to be cytotoxic to dividing cells [17]. To determine whether 300 mg of CSE (per kg of body weight) treatment for 60 days results in toxicities to normal tissues, we examined the liver, kidney, spleen, lung and heart of treated and untreated groups. Evaluation of numerous histological sections of these tissues from animals treated with CSE did not indicate any detectable pathologic abnormalities as examined by H&E staining. There were no observable histopathologic differences in these tissues. The liver showed normal hepatic lobular architecture, intact central vein with trapped red blood cells in a liver section from CSE treated animals. The kidney revealed normal glomeruli, proximal and distal tubules, interstitium, and blood vessels (solid arrow heads) (Figure 5A). The splenic follicles and vascular sinosoids were indistinguishable between the CSE treated and untreated control groups. The lung tissue showed normal alveoli (solid arrow heads) (Figure 5B).
The heart muscle showed normal morphology among the two groups (Figure 5C). To date, the exact mechanism of anticancer effect of saffron is not clear. However, most medicinally bioactive of saffron belongs to carotenoids. In our earlier studies, we reported that carotenoids such as crocin exhibit various pharmacological activities as antioxidant, anticancer, and immunomodulatory responses. Thus, our present study compels us to continue to examine the effects of CSE on other human and murine neoplasms with the final goal of taking it to the clinics. However, before coming to a conclusive statement, more research is needed to fully delineate the role of saffron in cancer treatment.

Fig 1: HPLC profile of Crocin

Fig 2: The chemical structure of Crocin

Fig 3: Anti proliferative effect of CSE on HEp-2 cells and vero cells

Values are presented as mean ± SD of four duplicates. Asterisks indicate the significant difference compared to control (*: P<0.05, **: P<0.001).

Fig 4A: Morphology of HEp-2 cells after 24 hrs treatment of CSE

Fig 4B: Morphology of Vero cells after 24 hrs treatment of CSE

Fig 5A: Organ toxicity (Liver and kidney) profile of CSE treated and untreated mice
Fig 5B: Organ toxicity (spleen and lungs) profile of CSE treated and untreated mice

Fig 5C: Organ toxicity (Heart) profile of CSE treated and untreated mice

5. Conflict of interest
Authors have no competing conflict of interest.

6. References