Avicenna Journal of Phytomedicine
Received: Feb 11, 2011; Accepted: April 18, 2011
Vol. 1, No. 1, Summer 2011, 43-50

Effects of aqueous saffron extract on nitric oxide production by two human carcinoma cell lines: Hepatocellular carcinoma (HepG2) and laryngeal carcinoma (Hep2)

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Abstract

Objective: A number of studies have demonstrated the potential antitumor effects of saffron and its constituents on different malignant cells in vitro. It has been reported that a novel glycoconjugate isolated from corms and callus of saffron possesses cytotoxic activity against different tumor cellswith nitric oxide (NO) production. These data suggest that the cytotoxic effect of saffron extract may be related to an effect on nitric oxide production. The aim of the study was to investigate the effect of whole saffron extract on NO production by the hepatocellular carcinoma cell line (HepG-2) and laryngeal carcinoma cell line (Hep-2).
Materials and Methods: The cell lines were treated with a saffron extract. The morphologic changes were observed and recorded after 24, 48 and 72 of incubation. The MTT test was used to assess cell viability and the quantitative changes in NO production was evaluated using Griess test in the aforementioned time intervals.
Results: The morphologic images showed qualitative changes in both cell lines. The MTT assay results indicated that there was an increase in cytotoxic effect by adding the extract at concentrations of 0, 200, 400 and 800 µg/ml. However, the NO concentration decreased significantly after 6, 12, 18, 24, 48 and 72 hours of incubation, respectively. IC_{50} of 400 µg/ml was obtained for HepG2 cells; however, Hep2 and L929 cells did not respond to any extract concentrations.
Conclusion: This study suggested that the saffron extract had a cytotoxic effect on HepG-2 and Hep-2 cell lines. The cytotoxic effect was probably related to a decrease in the NO concentration.

Keywords: Nitric oxide (NO), Saffron extract, Hepatocellular carcinoma cell line, Laryngeal carcinoma cell line.

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Introduction

Nitric oxide (NO) acts as an intercellular messenger molecule in many pathological and physiological processes (Moncada et al 1991). NO has a major role in cardiovascular system (Furchgott and Zawadzki 1980; Ignarro et al 1987; Katsuki et al 1977; Palmer et al 1987). In 1982, it became clear that the NO concentration increases during the immune defense (Wagner et al 1984; Hegesh and Shiloah 1982). In mammals' immunity which also includes defense against tumors, macrophages have a major role, and the cytotoxic effects of macrophages have been explained by Hibbs (Hibbs et al 1987). Recent studies have shown that cytokines and numerous factors can increase NO production in macrophages by increasing regulation in expressing inducible nitric oxide synthase gene (iNOS) (Macmicking et al 1997). During some processes, the biosynthesis of NO causes an antitumor effect (Xu et al 1998; Garban and Bonavida1999; Xie et al 1995; Juang et al 1998). Many direct and indirect mechanisms about the antitumor effect of NO have been proposed; these mechanisms include destruction of DNA, controlling DNA synthesis, controlling the ribonuclotid reductase enzymes, reducing the activity of Cis–econitase aconitase, and reducing the iron deposition. A better understanding of the role of arginine-derived NO in tumors may lead to novel antineoplastic and chemopreventative strategies.

Research into the effect of saffron on neoplastic cells has seen a renaissance in the last decade, and a growing body of evidence indicates that saffron and its major components possess anti-carcinogenic and antitumor activities in vivo and in vitro (Salomi et al 1991; Nair et al 1991; Daly 1998; Nair et al 1991; Nair et al 1994; Nair et al 1992; Garcia-Olmo et al 1999; Chang et al 1996).

The antitumor effect of saffron has been attributed to a proteoglycan that can be isolated from the saffron corm which showed cytotoxic activity in the human cancerous cells. High accumulation of this proteoglycan is reported to cause increased macrophages activation and subsequent production of NO (Ying and Hofseth 2007). It is therefore possible that the antitumor effect of saffron is associated with the nitric oxide production (Katia Aquilano et al 2011). In this research, the cytotoxity effect of the whole saffron (from the quantitative and morphologic point of view) and the amount of NO production in $HepG_2$ and Hep2 cells have been investigated.

Materials and Methods

Materials

MTT (Dimethyl thiazole – diphenyl – tetrazolium–bromide), N – 1 [naphtyl] – Ethylene diamine, sodium nitrate, sulphanylamide, lipopolysaccharide – *Eshershia coli*, SNAP as a positive control, NMMA as a negative control, human γ- interferon obtained from Sigma Company and dimethyl sulfoxide (DMSO) and glycine from Biogen Company.

Freshly dried and purified stigma of saffron (*C. sativus L.*) was bought from local market, and was kept at 4°until use.

Methods

Extract preparation

An extract of saffron was prepared using Soxhlet procedure in pharmacology section of Ghaem Hospital, Mashhad, Iran and stored at -80^{0}C. To do this, 15g of ground saffron was put in flask containing 150 ml distilled water and was gradually warmed up. The extraction procedure was continued overnight till the water becamecolorless.

Cell toxicity assay

The HepG2 (Human hepatocellular carcinoma cells) and Hep2 cell lines which were isolated from the initial

phases of liver and larynx cancer growth and were grown and kept in the laboratory culturing environment (DMEM media, serum 5% and CO_2 5%), were obtained from Bu-Ali research Institute. Also the L929 cells (which are rat's fibroblast cells and were used as normal cells in this research) were obtained from Bu-Ali research Institute.

To investigate the effect of whole saffron extract on the growth of provided cell lines, experiments were designed in two stages.

Qualitative investigation of cells: in this stage, cells from the morphological point of view, level of stickiness on the matrix, level of granulation of cytoplasm and nucleus were evaluated. For each cell line, small flasks each contained 1×10^6 cell in 5 ml culturing environment (cultured in DMEM) were provided. After 24 hours that the attachment of cells was assured, the extract was added with concentrations of 0, 200, 400, and 800 µg/ml. To prepare the extract, different concentrations of the lyophilized extract without the serum was dissolved in culture environment (using DMEM media) and then was added to flasks containing cultured cells. For the morphological comparison of cells, their degree of stickiness and degree of granulation, pictures were taken from the flasks containing cells after 24, 48 and 72 hours, respectively using an HP–American–camera with inverted microscope 200 magnification. The pictures were then saved on computer for the final comparison and inference.

Quantitative investigation of cells with the MTT assay procedure: MTT is tetrazolium salt dissolved in water. When this compound is prepared in culturing environment, with salt solution that lacks red phenol, it results in a yellowish solution. This compound in living cells mitochondria is converted into an insoluble compound named 'formazan' by dehydrogenase enzyme. When this product is dissolved by solvents such as acidic isopropanol or DMSO, it gives purple (lilac) appearance. Absorption of this color at 570 nanometer wavelength was measured with microplate instrument ADYNA–Tech, MR Elisa plate reader (600).

The effects of the extract were assessed after 24, 48, 72 hours, respectively by measuring the levels of cellular proliferation through the light absorption (OD). By using the following formula, he percentage of living cells, after the effect of the extract compared to those cells that did not receive any extract was calculated:

$$\frac{\text{Light absorption for cells treated with the extract in each well}\times100}{\text{Light absorption mean value for the control cells}}$$

Nitric oxide (NO) measurement with Griess procedure

It is difficult to measure NO directly because of its radical nature and very short half-life. Nitrite was measured by the Griess colour reaction. Nitrite reacts with sulfonyl amide and N (1–naphthyl) ethylenediamine to produce the AZO – color.

Preparation procedure for Griess reagent

By adding distilled water, the volume of 0.05 gram N-1-naphthylene-dihydrochloride was increased to 50 milliliters, and 0.5 gram sulfonyl amide was dissolved in 5% phosphoric acid, then two equal volumes of these compounds were mixed. The mixture reagent is called Griess reagent which is useable for up to 12 hours and should be kept cool during this period. Both components of the reagent can be kept separately in the fridge for up to two months.

After 6, 12, 18, 24, 30, 48, and 72 hours, by collecting the surface liquid of culture environment and using the Griess method, the level of NO production was

assessed. To manage this, 100 microliters of sample was poured into the flat 96-pitted plates and 100 µL of Griess reagent was added; meanwhile, the culture which lacked any cell was used as the blank. After thirty minutes of incubation at room temperature, a spectrum of purple color was formed which could be read at the wavelength of 540 nm.

Results

Morphological changes

With or without extract, normal cells (L929) did not show any clear morphologic modifications and during the first to third days at extract concentration of 400 µg/ ml, the morphological results did not show much difference in the quality of cells growth (Figure 1).

In HepG2 cells, the control of cells together or individually was accompanied with star-shaped morphology, wasting, small size, increase in vacuole size, cytoplasm reduction, and nucleus pigmentation (nucleus wasting and pigmentation are final characteristic of cell's programmed death). During the first to third days at extract concentrations of zero and 200 µg/ml, the morphological results did not show much difference in the quality of cells growth. However, in day 3 with the use of extract at concentration of 400 µg/ ml, gradually pigmentation and granulation of cells which are the signs of programmed cell death were seen (Figure 2). During the second and third days at concentration of 800 µg/ ml, reduction in the number of cells was clearly observable. In addition, increase in the number of dead cells which were floating on the surface of culture environment was visible. Data was analyzed using the Statistical Package for the Social Sciences (SPSS) 15.5.0.

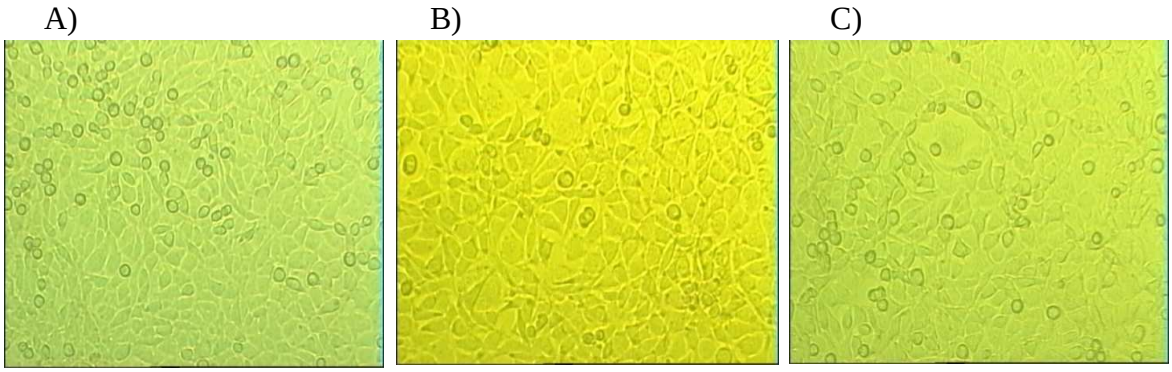


Figure1. Use of extract at concentration of 400 µg/ml in L929 cell line in A) day 1, B) day2 C) day 3.

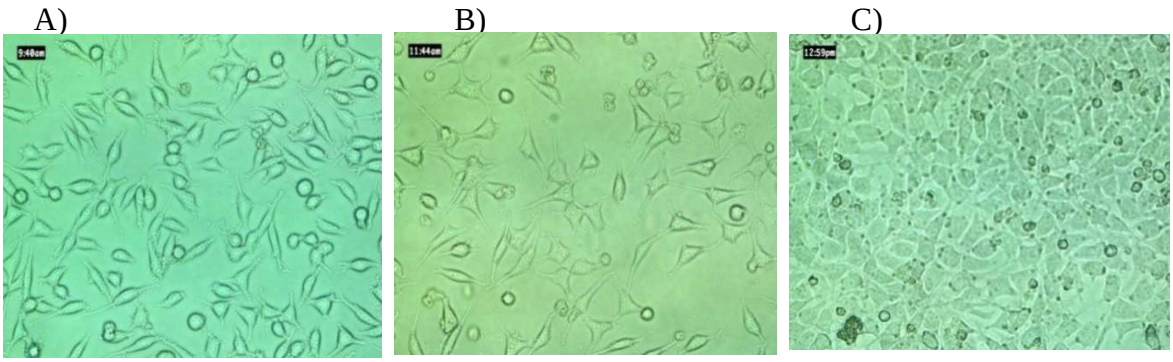


Figure 2. Use of extract at concentration of 400 µg/ml in HepG2 cell line in A) day 1, B) day 2 C) day 3.

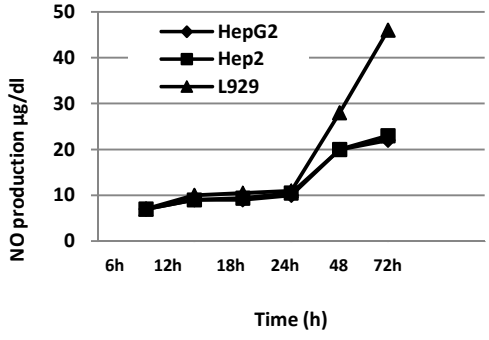
MTT Assay

Statistical results were performed using the ANOVA and Tukey multiple comparisons formula with computer software program. Graph 1 shows the effect of extract on normal and cancer cells after 24, 48 and 72 hours. From the statistical point of view, ANOVA test showed a meaningful difference among living cells after 24 hours of incubationand concentrations of whole extract of saffron (p=0.007) and cell line (p=0.029, Graph 1A). ANOVA test also showed a meaningful difference among living cells after 48 hours and concentrations of whole extract of saffron. (p=0.017, Graph 1B). There was also a meaningful difference among living cells after 72 hours and concentrations of whole extract of saffron (p=0.023) and cell line (p=0.05, Graph 1C).

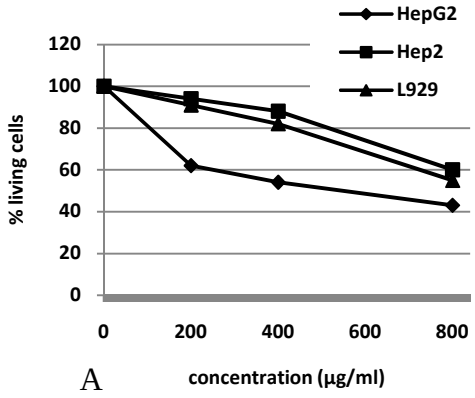
In all three Graphs, control concentration of 400 µl/ml for 50% cells growth (IC_{50}) was obtained for HepG2 cells; however, Hep2 and L929 cells did not respond to the extract concentrations.

Griess assay

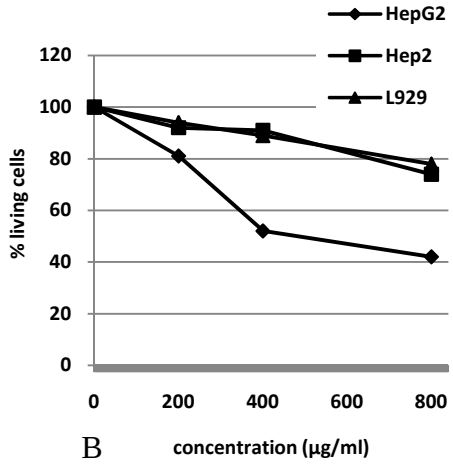
Statistically significant association was observed among the levels of produced NO in the presence of whole saffron extract after 6, 12, 18, 24, 48, and 72 hours (p=.09 ,.008 ,.010 ,.003 ,.003 ,.025) (Graph 2).



Graph 2. Comparison of No production at 800 µg/ml Concentration of saffron in three cell lines after 6 ,12, 18 ,24, 48,and 72 h.



A



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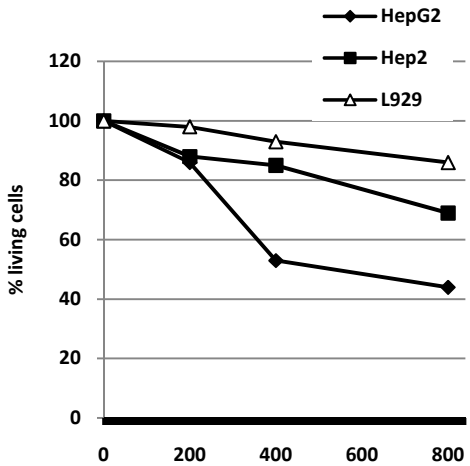

C

Graph 1. Percentage of living cells after A) 24 h, B) 48 h, C) 72 h.

Discussion

Most of the effect of saffron extract appears against viability of HepG2. At the same time, with regard to the graphs for Griess test (Graph 2), the highest reduction in the level of NO was also seen in HepG2 cells (as the experimental evidence certify the necessity of NO presence in hepatocellular carcinoma (Ensor et al 2002)). It also needs to be mentioned that crocetin from saffron extract inhibits mRNA expression of inducible nitric oxide synthase in the liver of rats (Yang et al., 2006). These results suggest that the antitumoral effect of saffron, probably is coincidental to the reduction in NO production. At zero, 200 and 400 µg/ml concentrations, from the quantitative point of view of living cells, the Hep2 and L929 cells did not show any significant difference, but at 800 µg/ml concentration, the number of living cells was gradually reduced. At 400 µg/ml concentration the number of living cells of HepG2 cells was almost reduced to half, while at 800 µl/ml the number of living cells reached 40%.

In total, with regard to the MTT test in days 1, 2 and 3, it was observed that the extract in non-cytotoxic concentrations (less than 800 µg/ml) could easily cease the control of growth in hepatic carcinoma cells (HepG2), although it did not have much effect on normal cells.

Many studies have shown the antitumor effects of saffron extract against different cell lines, and there is a clear difference between the type of cancer cells and the level of their responses to the saffron extract.

To sum up, regarding the MTT test, the number of cancer cells are reduced gradually with rise in saffron concentration whereas in Griess test with increasing saffron concentration, the amount of NO produced in cancer cells is reduced gradually compared to normal cells. According to the results obtained, it seems that the whole extract of saffron had controlling effect on the growth of tumor, whereas the effect of different concentrations of saffron on HepG2 and Hep2 tumor cells probably caused a reduction in the production of NO. Therefore, this antitumor effect of the extract is probably due to a reduction in NO production.

Different studies have shown that increased and continuous NO production plays a pivotal role in the regulation of carcinogenic process (Ying and Hofseth 2007)

NO, through cGMP-dependent pathways, regulates different modifications in proteins. However, thiol groups in the proteins that are involved in the regulation of gene expression are also potential targets for posttranslational modifications by NO (Muntané and De la Mata 2010).

Taking into account the presence of carotenoids in saffron and their effect in scavenging free radicals such as NO, the cytotoxic activity and its effect on the reduction of NO production could be due to the presence of carotenoids in the whole extract of saffron.

To show a relationship between the effects on NO production and viability of the cell line, one would need to do blocking studies with NO inhibitors.

Limitation of study

Following limitations in the present study should be addressed in a separate study.

The question ethat why after three days of incubation, experiments were not continued while in practice, up to day 7, performing the experiments were continued. The fact is, after the day 4, because of excessive increase in the number of cells which caused a multilayer growth in the culturing plates, as a result number of cells died due to lack of space for growth (not the effect of extract), hence, in this study, the

results of working with cells were reported up to day 3.

The fact that why higher concentrationsthan 800 µg/ml were not used is because higher concentrations of 800 µg/ml caused the growth control of L929 cell (normal), and were considered as cytotoxic.

Although there was numerous cell lines available in Avicenna Research DInstitute cell bank, L929 cell was employed as normal cell because the growth rate of these three cell lines (L929, HepG2, Hep2) are similar; moreover, their growth characteristics is also similar in culture flasks, and they grow in form of single layer, attached to the bottom of flask.

With regard to the fact that there is a glycoprotein in saffron stigma which causes increase in NO production, this experiment was based on this preassumption that saffron antitumor effect could be because of increase in NO production whereas on the contrary, reduction of NO production was observed. To overcome the uncertainty, experiments were performed in three consecutive times, and each time similar results were obtained.

Finally, with all mentioned limitations in conditions of this experiment, it was observed that the saffron extract at non-cytotoxic concentrationcould causes growth control of carcinoma cells, probably by reduction of NO production.

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