Running title: Cytotoxic Effect of Saffron Stigma Aqueous Extract- Ahmadnia et al.

Cytotoxic Effect of Saffron Stigma Aqueous Extract on Human Prostate Cancer and Mouse Fibroblast Cell Lines

Hassan Ahmadnia[1]*, Jalil Tavakkol Afshari[2], Jamshid Tabeshpour[3], Mehdi Younesi Rostami[4], , Ehsan Mansourian[5], Alireza Akhavan Rezayat[5] and Azam Brook[2]

[1]*Urology, Endoscopic & Minimally Invasive Surgery Research Center, Ghaem Hospital, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

[2]Immunology Research Center, Bu-Ali Research Institute, Mashhad University of Medical Sciences, Mashhad, Iran

[3] Faculty of Pharmacy, Damghan Bransh, Islamic Azad University, Damghan, Iran.

[4]Department of Urology, Imam Hospital, Mazandaran University of Medical Sciences, Sari, Iran

[5]Department of Urology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Abstract

Purpose: Several lines of experimental evidence have shown that saffron has anticarcinogenic effects. This study aimed at evaluating the possible anticancer effect of saffron stigma aqueous extract on human prostate cancer (PC3) and mouse fibroblast cells (L929) as non-cancerous control cells.

Materials and Methods: Saffron stigma aqueous extract at concentrations of 100, 200, 400, 600, 800, 1600 and 3200 µg/mL were prepared. PC3 and L929 cells were incubated with different concentrations of saffron extracts in different time intervals (24, 48, 72, 96 and 144 hours). MTT assay was used for each cell line to investigate the cytotoxic effect of saffron. Morphological alterations were also observed under light inverted microscope.

Results: In fibroblast cell line after 24 hours, Saffron extract did not affect significantly the normal cells and they were intact in morphologic view. After 96 hours in the cells with highest concentration (1600 µg/mL), cell death and cellular form changes as well as severe granulation was observed. In prostate cell line after 24 hours, the only changes were observed in cells with the concentration of 1600 µg/mL. The cells were granulated and the form of the cells were spherule. After 72 hours, in group with the concentration of 1600 µg/mL, severe granulation was observed and the cell count decreased and some cells were dead.

Conclusion: Saffron aqueous extract has an *in vitro* inhibitory effect on the proliferation of

human prostate cell and mouse L929 cells which is dose-dependent.
Key words: Saffron aqueous extract**,** L929 cells, PC3 cells**,** anticancer effect**,** MTT assay**,** cytotoxic effect.

INTRODUCTION

The most prevalent visceral malignant neoplasm in men, prostate cancer is responsible for one third of all such cancers.[1] According to American Cancer Society, 220,000 Americans have been diagnosed with prostate cancer, annually.[2] Different kinds of treatments have been introduced for prostate cancer such as cabazitaxel, docetaxel and mitoxantrone.[3] But, unfortunately, a variety of adverse effects following synthetic medicines are observed in the clinical settings.[3] To minimize such adverse effects, many researchers have been persuaded to search for herbal therapies with the least adverse effects such as *Adiantum venusutum*[4], *Abelmoschus moschatus*[5] and *Aspidosperma tomentosum*.[6]

Herbal medicine is a new alternative therapy in cancer patients.[7] Consumption of certain botanicals could be associated with reduced cancer incidence. One of these botanical agents is *Crocus sativus* L., commonly known as saffron. It is a stemless herb of the Iridaceae family that is widely cultivated in Iran and other countries. In the world, 205 tons of saffron are produced every year, while Iran has an important role in this production as the grand producer (80 percent of total). Khorasan province, sited at north east of Iran, accounts for 137 tons of the above-mentioned totals.[8-10]

Mostly used as a spice, flavoring agent, food coloring and herbal medicine, saffron is produced from dried red stigma with a small portion of the yellowish stamina attached.[9] The stigmas of saffron has been demonstrated to have several components including carotenoids, crocin, crocetin, picrocrocin, anthocyanin, lycopene, monoterpene aldehydes and safranal as well as proteins, sugars, vitamins, flavonoids, amino acids and minerals (Fig. 1).[11-15] Anticancer[9], anti-inflammatory[16], antidepressive[17], antioxidant[18] and antibacterial[19] activities of saffron have been established by many researchers as its pharmacological effects. Since 1990, research

has been focused on anti-carcinogenic activity of saffron.[7, 8] Saffron and its main components have shown anti-tumor and anti-carcinogenic activities both *in vitro* and *in vivo*.[7-9, 20]

As far as we know, the possible effects of saffron on prostate cancer has not been evaluated and reported. Therefore, this study aimed to evaluate the *in vitro* cytotoxic effects of saffron stigma aqueous extract on prostate cell line and non-neoplastic fibroblast cells of mouse as a normal cell line.

MATERIALS AND METHODS Preparation of Saffron Extract

Saffron harvested from saffron farms of a city of Khorasan province (Ghaen), was used in this experiment. 15 g of ground petal stigma was mixed with 400 mL of distilled water in a Soxhlet extractor for 18 h, to prepare saffron aqueous extract. Then, to be sterilized, it was concentrated to 100 mL with a rotatory evaporator in low pressure and filtered through a 0.2-mm filter. The obtained solution was kept at 4°C to 8°C. Different concentrations of saffron extract (100, 200, 400, 600, 800, 1600 and 3200 μg/mL) were prepared immediately and refrigerated before the experiments.

Morphologic Observation of Cell Lines

Human prostate carcinoma cells (PC3) and mouse fibroblast cell lines (L929) were bought from the National Cell Bank of Iran *(*Pasture Institute, Tehran, Iran*)*. Both of the cell lines were cultured and passaged. Trypan blue test was used to determine cell viability. Equal parts of 0.4% trypan blue dye to the cell suspension were added to obtain a 1 to 2 dilution and weremixed by pipetting up and down. The incubation time was less than three minutes at room temperature. The percentage of viable cells were calculated by dividing the number of viable cells by the number of total cells and multiplying by 100 or % viable cells. Six-well plates were used for both cell lines. In each well, 5×10^5 PC3 cells or 2×10^5 L929 cells were cultivated in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St Louis, Missouri, USA) with 10% fetal calf serum (Gibco, Paisley, UK). The media were supplemented with 100 IU/mL

streptomycin and 100 IU/mL penicillin *(*Jaberebn-e-Hayan, Tehran, Iran*)*. The cells were incubated at 37°C in a humidified 5% CO2 atmosphere for 24 h. Then, the cells were exposed to saffron extract as follows: The media (2 mL capacity) were replaced with fresh media and plates were incubated with different concentrations of saffron extract (100, 200, 400, 600, 800, 1600 and 3200 µg/mL) in the situation aforementioned for 24, 48, 72, 96 and 144 h, and the cells were observed by light inverted microscope for morphological alterations. Each extract concentration was observed 3 times to check their reliability. The viability of the cells was determined to be higher than 95% during the experiment.

Quantitative Assessment

Surviving cells (percent) compared to the controls = $\frac{\text{optical density of treated cells in each well} \times 100}{\text{mean optical density of control}}$

The absorbance of trypan blue dye was read using fluorescence microplate reader (Ex: 488 nm, Em: 585 nm).

Statistical Analysis

Data are presented as mean ± SD. Statistical analyses were performed in Graph pad Prism software version 8.0.

RESULTS

Effect of saffron on L929 cell viability and morphological alterations

Incubation of L929 cells with different concentrations of saffron extract for 24 h, did not significantly affect the cells and they were intact in morphological view. In addition, there were no changes in number, cytoplasm, and nucleus of the cells. Incubation of the cells with 1600 µg/mL of saffron extract for 48 and 72 hours, showed a slight granulation of the cells while no changes in number, cytoplasm, and nucleus of the cells were observed. After 96 and 144 hours, at the concentration of 800 µg/mL, the cells were larger and granulated while at the concentration of 1600 µg/mL, cell death, cellular form changes and severe granulation were noticed (Fig. 2).

Effect of saffron on PC3 Cell viability and morphological alterations

PC3 cells were granulated and the form of the cells were spherule when they were exposed to 1600 µg/mL of saffron extract for 24 hours. After 48 and 72 hours of treatment (400, 600 and 800 µg/mL), the cells were granulated and spheroid, while at the concentration of 1600 µg/mL, severe granulation was observed and the cells count decreased and some cells were dead (Fig. 2, 3, 4).

The half maximal inhibitory concentration (IC50) is a measure of the potency of a substance in inhibiting a specific biological or biochemical function which was calculated for PC3 cells to be 400 to 800 µg/mL.

Granulated and dead cells were noticed at the concentration of 1600 µg/mL, after 144 h of treatment (Fig. 5).

DISCUSSION

Prostate cancer is a commonly diagnosed cancer in men, and dietary chemoprevention was considered due to its slow growth rate and its long incubation period. Different herbal extracts has shown noticeable benefits such as pomegranate, green tea in chemoprevention of prostate cancer.[21, 22]

Several effects were recognized for saffron such as anti-carcinogenic, decreasing blood pressure, and controlling tonic-clonic and absence seizures.[10] Saffron can cure coronary heart disease and hepatitis, and promote immunity.[23] There are several reports demonstrating the anti-carcinogenic effects of saffron extract, either inhibition of new tumors formation or shrinkage of existing tumors. The anticancer activity of saffron against a wide spectrum of tumors, such as leukemia, transitional cell carcinoma, ovarian carcinoma, colon adenocarcinoma, rhabdomyosarcoma, papilloma, squamous cell carcinoma, and soft tissue sarcoma have been demonstrated.[7, 9, 10, 15, 20, 23-26] The inhibitory effects of Saffron aqueous extract on the growth of both TCC 5637 and normal L929 cell lines have been reported[10] as we observed in our

research. Moreover, increasing saffron concentrations results in a reduction in the cell survival percent of healthy L929 cells. Higher saffron concentrations seem to reduce the cell survival rate of healthy cells up to 50% in longer incubation time.

Salomi *et al*. reported anti-promoting and non-mutagenic activity of saffron extract.[7] Protective effect of crocin against adverse consequence of hepatocarcinogenic materials has been demonstrated.[15] In addition, different *in vitro* studies have reported the protective effects of crocin such as inhibition of intracellular nucleic acid synthesis[11] and inhibition of proliferation of promyelocytic leukemia cells in a dose-dependent manner.[12, 20] Saffron extract in combination of vitamin E has been shown to protect rats against cisplatin toxicity.[15] A significant inhibitory effect of ethanolic extract of saffron on the colony formation and intracellular DNA and RNA synthesis of Hela cells (cervix epitheloid carcinoma cells) was reported by Abdullaev and Frenkel.[26] Growth delay of papilloma, decreased incidence of squamous cell carcinoma and soft tissue sarcoma in mice treated by saffron, have been reported.[20]

Although saffron extract has well-documented antitumor effects, the cellular mechanisms responsible for these effects remain ill-defined. Different hypotheses have been suggested for the antitumor mechanism of saffron such as the inhibitory effect on DNA and RNA synthesis without any effect on protein synthesis and the inhibitory effect on free radicals which is thought to be due to carotenoid component of saffron.[10, 20] It is suggested that saffron (dimethyl-crocetin) disrupts DNA-protein interactions e.g. topoisomerases II, which is essential for cellular DNA synthesis.[20] The anti-tumor components of saffron were reviewed by some researchers. Crocin isolated from saffron, inhibits PC12 (rat's pheochromocytoma cell line) cell growth with increased synthesis of glutathione. The mechanism behind its antigrowth effect could be the possible decrease in tumor necrosis factor-alpha levels.[27]

CONCLUSION

This research demonstrates *in vitro* cytotoxic effects of saffron on human prostate cell lines. In fact, saffron extract possesses *in vitro* inhibitory effect on the proliferation of PC3 cells in a dose-dependent manner. More studies need to be accomplished to use saffron as a chemopreventive agent in prostate cancer treatment.

ACKNOWLEDGEMENT

Authors are thankful to the Vice Chancellor of Research, Mashhad University of Medical Sciences for financial support. The results described in this paper are part of a Medical thesis.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

REFERENCES

1. Schroder FH, Hugosson J, Roobol MJ, et al. Screening and prostate-cancer mortality in a randomized European study. N Engl J Med. 2009;360:1320-8.
2. Cokkinides V, Albano J, Samuels A, Ward M, Thum J. American cancer society: Cancer facts and figures. Atlanta: American Cancer Society. 2005;
3. de Bono JS, Oudard S, Ozguroglu M, et al. Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. The Lancet. 2010;376:1147-1154.
4. Viral D, Shivanand P, Jivani NP. Anticancer Evaluation of Adiantum venustum Don. J Young Pharm. 2011;3:48-54.
5. Gul MZ, Bhakshu LM, Ahmad F, Kondapi AK, Qureshi IA, Ghazi IA. Evaluation of Abelmoschus moschatus extracts for antioxidant, free radical scavenging, antimicrobial and antiproliferative activities using in vitro assays. BMC COMPLEM ALTERN M. 2011;11:64.
6. Kohn L, Pizao P, Foglio M, et al. Antiproliferative activity of crude extract and fractions obtained from Aspidosperma tomentosum Mart. Rev Bras Pl Med. 2006;8:110-115.
7. Salomi MJ, Nair SC, Panikkar KR. Inhibitory effects of *Nigella sativa* and saffron (*Crocus sativus*) on chemical carcinogenesis in mice. Nutr Cancer. 1991;16:67-72.
8. Bharali R, Tabassum J, Azad MR. Chemomodulatory effect of *Moringa oleifera*, Lam, on hepatic carcinogen metabolising enzymes, antioxidant parameters and skin papillomagenesis in mice. Asian Pac J Cancer Prev. 2003;4:131-9.
9. Abdullaev FI. Cancer chemopreventive and tumoricidal properties of saffron (*Crocus sativus L.*). Exp Biol Med (Maywood). 2002;227:20-5.
10. Feizzadeh B, Afshari JT, Rakhshandeh H, Rahimi A, Brook A, Doosti H. Cytotoxic effect of saffron stigma aqueous extract on human transitional cell carcinoma and mouse fibroblast. Urol J. 2008;5:161-7.
11. Abdullaev FI. Inhibitory effect of crocetin on intracellular nucleic acid and protein synthesis in malignant cells. Toxicol Lett. 1994;70:243-51.
12. Tarantilis PA, Morjani H, Polissiou M, Manfait M. Inhibition of growth and induction of differentiation of promyelocytic leukemia (HL-60) by carotenoids from *Crocus sativus L*. Anticancer Res. 1994;14:1913-8.
13. Escribano J, Alonso GL, Coca-Prados M, Fernandez JA. Crocin, safranal and picrocrocin from saffron (*Crocus sativus L.*) inhibit the growth of human cancer cells in vitro. Cancer Lett. 1996;100:23-30.
14. Giaccio M. Crocetin from saffron: an active component of an ancient spice. Crit Rev Food Sci

Nutr. 2004;44:155-72.
15. Das I, Chakrabarty RN, Das S. Saffron can prevent chemically induced skin carcinogenesis in Swiss albino mice. Asian Pac J Cancer Prev. 2004;5:70-6.
16. Hosseinzadeh H, Younesi HM. Antinociceptive and anti-inflammatory effects of *Crocus sativus L*. stigma and petal extracts in mice. BMC Pharmacol. 2002;2:7.
17. Hosseinzadeh H, Karimi G, Niapoor M. Antidepressant effects of *Crocus sativus* stigma extracts and its constituents, crocin and safranal, in mice. J Med Plant Res. 2004;3:48-58.
18. Makhlouf H, Saksouk M, Habib J, Chahine R. Determination of antioxidant activity of saffron taken from the flower of *Crocus sativus* grown in Lebanon. Afr J Biotechnol. 2011;10:8093-8100.
19. Nakhaei M, Khaje-Karamoddin M, Ramezani M. Inhibition of Helicobacter pylori Growth *in vitro* by Saffron (*Crocus sativus L.*). Iran J Basic Med Sci. 2008;11:91-96.
20. Nair SC, Kurumboor SK, Hasegawa JH. Saffron chemoprevention in biology and medicine: a review. Cancer Biother. 1995;10:257-64.
21. Gasmi J, Sanderson JT. Growth Inhibitory, Antiandrogenic, and Pro-apoptotic Effects of Punicic Acid in LNCaP Human Prostate Cancer Cells. J Agric Food Chem. 2010;58:12149-12156.
22. Khan N, Adhami VM, Mukhtar H. Review: green tea polyphenols in chemoprevention of prostate cancer: preclinical and clinical studies. Nutr Cancer. 2009;61:836-41.
23. Deng Y, Guo ZG, Zeng ZL, Wang Z. [Studies on the pharmacological effects of saffron(*Crocus sativus L.*)--a review]. Zhongguo Zhong Yao Za Zhi. 2002;27:565-8.
24. Dufresne C, Cormier F, Dorion S. *In vitro* formation of crocetin glucosyl esters by *Crocus sativus* callus extract. Planta Med. 1997;63:150-3.
25. Nair SC, Pannikar B, Panikkar KR. Antitumour activity of saffron (*Crocus sativus*). Cancer Lett. 1991;57:109-14.
26. Abdullaev FI, Frenkel GD. Effect of saffron on cell colony formation and cellular nucleic acid and protein synthesis. Biofactors. 1992;3:201-4.
27. Ochiai T, Soeda S, Ohno S, Tanaka H, Shoyama Y, Shimeno H. Crocin prevents the death of PC-12 cells through sphingomyelinase-ceramide signaling by increasing glutathione synthesis. Neurochem Int. 2004;44:321-30.

*Corresponding author:

Hassan Ahmadnia, MD;

Urology, Endoscopic & Minimally Invasive Surgery Research Center, Ghaem Hospital, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

E-mail: ahmadniah@mums.ac.ir

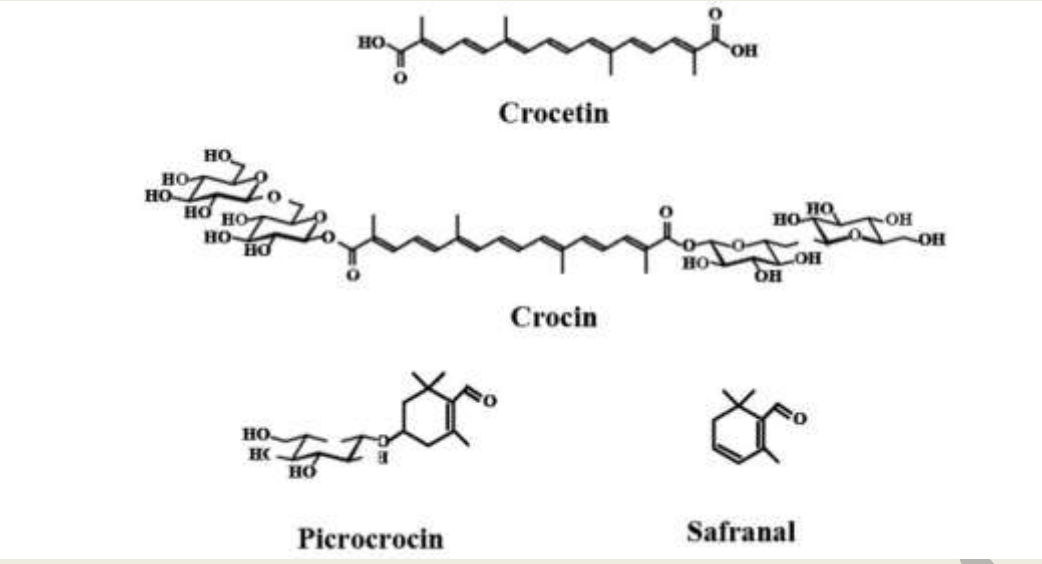

Figure 1. Molecular structures of the most important carotenoid secondary metabolites of saffron.

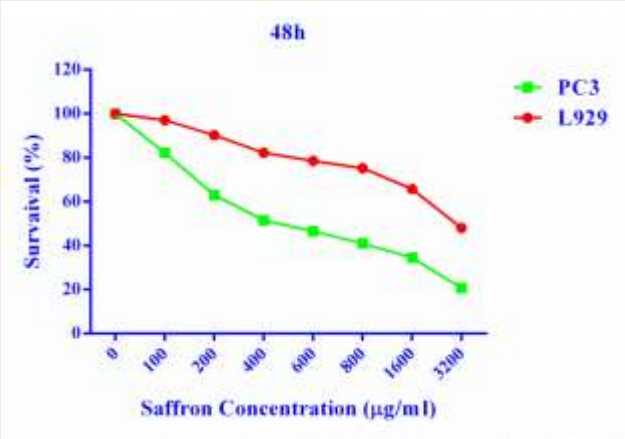

Figure 2. After 48 hours of incubation with the concentration of 400 µg/mL**,** PC3 cells were granulated and spheroid (left picture) in comparison with the cells which were not incubated with saffron extract, as a control group (right picture). The absorbance of the dye was read at Ex: 488 nm, Em: 585 nm.

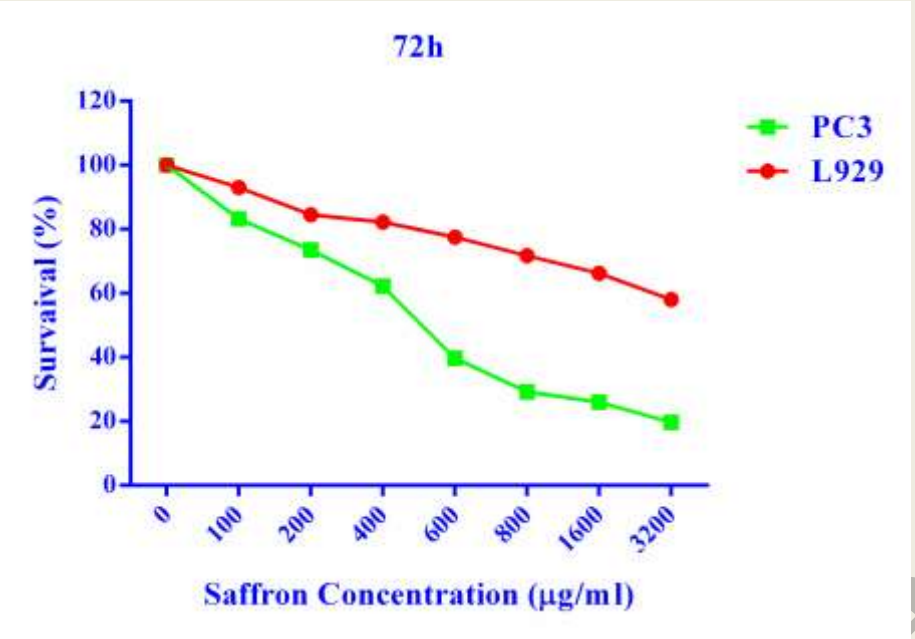

Figure 3. Viability percent for PC3 and L929 cells after 24, 48, 72, 96 and 144 h incubation with different concentrations of saffron extract.

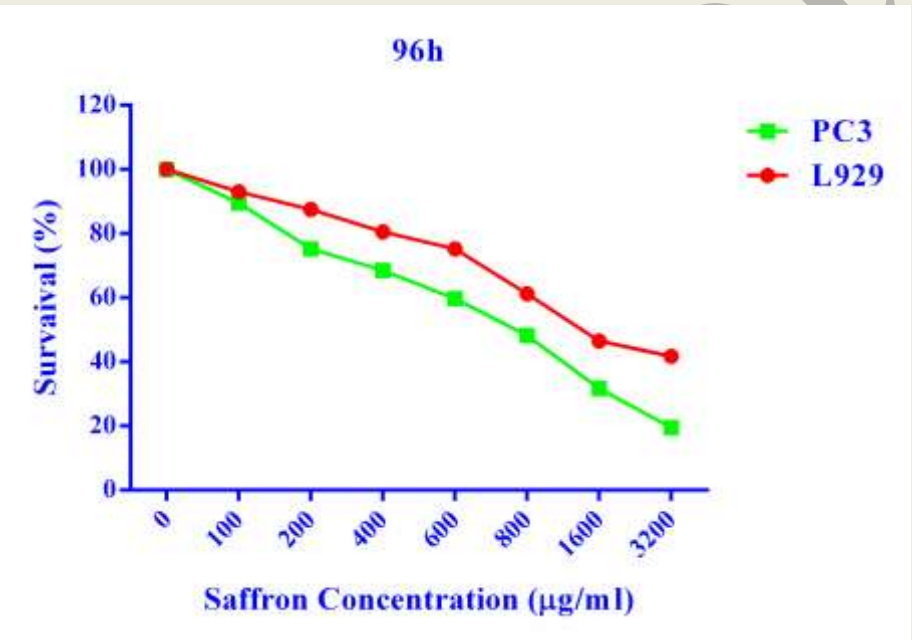

Figure 4. After 72 h incubation, the PC3cells incubated with 400 µg/mL of saffron extract were granulated and spheroid with reduction in cell counts (left picture) in comparison with the cells which were not incubated with saffron extract as a control group (right picture). The absorbance of the dye was read at Ex: 488 nm, Em: 585 nm.

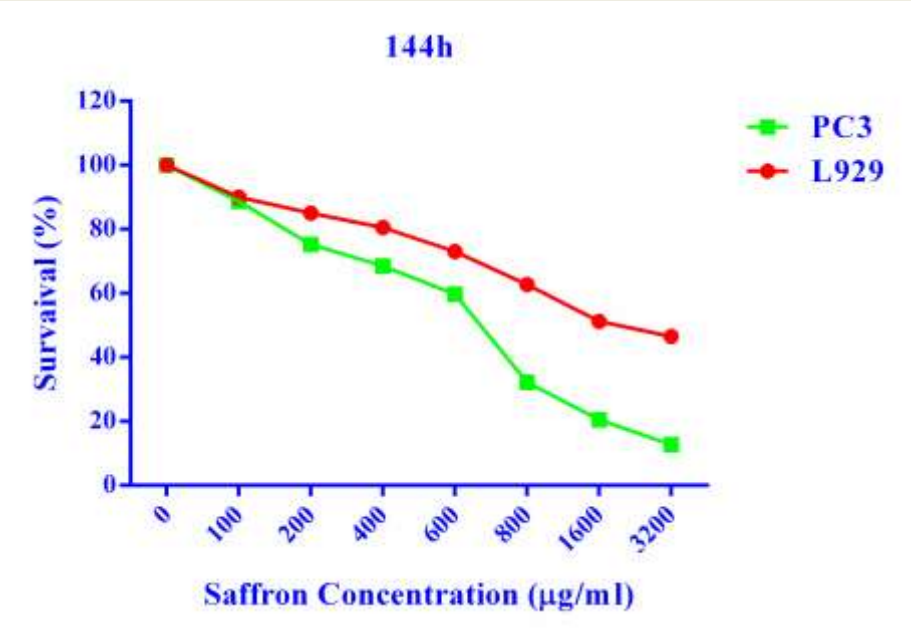

Figure 5. After 144 h, the PC3 cells incubated with 400 µg/mL of saffron extract were granulated and spheroid with reduction in cell counts, presenting the cellular death (left picture) in comparison with the cells which were not incubated with saffron extract as control group (right picture). The absorbance of the dye was read at Ex: 488 nm, Em: 585 nm.