Original Article

Carthamus tinctorius L. Inhibits Proliferation of Lung Cancer A549 Cells and Tender's Mitochondrial Protection

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ABSTRACT

Background: Plant-based products are well-known as long-lasting chemo preventive and chemotherapeutic medicines against cancer. **Objectives:** The purpose of this research is to identify out how safflower extract affects A549 cells. **Materials and Methods:** The antiproliferative activity was determined using the Trypan blue assay, while the cytotoxicity was determined using the MTT assay. The ethidium bromide/acridine orange (AO/EB) dual staining method was used to observe the apoptotic inducing effect and the morphological assessment of A549 cells using phase contrast microscopy was studied to discern the nuclear changes if any on treatment with safflower. The mitochondrial membrane potential was studied using a cationic lipophilic dye rhodamine 123 under a confocal microscope, and oxidative stress was assessed using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) labelling. **Results:** The trypan blue assay speculated that there was a decrease in cell viability at highest concentration and that the effect was dose dependent. The MTT assay revealed that cytotoxicity increases in cells treated with higher concentration. The LC50 range of the sample was found at 250 μg/ml concentration at 52.2%, whereas the viability of the cell declined to 28.19% at 350μg/ml concentration. The morphological features like shrinkage, detachment, membrane blebbing, and distorted shape were observed on safflower treated A549 cells that supports the antiproliferative activity. The ethidium bromide and acridine orange staining substantiates that safflower extract induces apoptosis wherein the untreated A549 cells showed green fluorescence with intact nuclear morphology and cells treated with safflower extract (200 μg and 250 μg/ml), showed apoptotic bodies, clearly validating the apoptosis inducing effect by safflower on A549 cells. Also, the safflower extract protects the mitochondria by decreasing the oxidative stress and by altering the mitochondrial membrane potential. **Conclusion:** Thus, it is found that safflower extract exerts its antiproliferative activity by inducing apoptosis and protects mitochondria by combating oxidative stress.

Keywords: Safflower, Oxidative stress, Cytotoxicity, Mitochondria, Apoptosis.

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INTRODUCTION

Cancer is leading cause of globally mortality and is a major impediment to life expectancy. Furthermore, lung cancer is the leading cause of cancer death among all malignancies.[1] Although significant progress has been made in treating and controlling cancer progression, significant gaps remain. Many adverse reactions occur during chemotherapy. Natural medicines such as the use of herbal products to treat cancer may reduce side effects. Herbal medicines and their phytocompounds are increasingly recognized as useful complementary therapies against cancer. *Carthamus tinctorius* L., popularly known as safflower or imitation saffron belongs to the family Asteraceae. Flowers of *C. tinctorius* have long been used to treat cardiovascular, cerebrovascular, amenorrhea, and other gynecological issues. Safflower also has excellent digestive, analgesic, and antipyretic qualities, making it beneficial to addiction patients.[2] This plant species, in particular, has shown to be effective in the treatment of dysmenorrhea, postpartum hemorrhage, whooping cough, chronic bronchitis, rheumatism, and sciatica.[3] Safflower is a multipurpose oil seed and is cultivated primarily to produce high quality edible oils that are rich in polyunsaturated acids. This thistle-like species is indigenous to South Asia, China, India, Iran and Egypt and usually breeds in dry climates.[4] The active constituents of safflower species include flavonoids, organic acids, alkaloids, lignanoids, and polyacetylenes, riboflavin, alkanediols, steroids, and quinochalcone C-glycosides.[5] Many research studies have exposed that *C. tinctorius* is effective as an anticoagulant, vasodilator, antihypertensive, antioxidant, neuroprotective agent, anticancer immunosuppressant, and also has the effect of inhibiting the synthesis of melanin.[3] In addition, safflower has also been suggested to be effective in neurotropic, hematopoietic, and diaphoretic systems.[6] In spite of its diverse pharmacological actions like antioxidant, antipyretic and anti-inflammatory activities, the anti-cancer potential or benefit remains unknown. To broaden the prospect of anti-cancer activity of this plant species, this study aims to verify the growth inhibitory potential of safflower extract on A459 cells.

MATERIALS AND METHODS

Plant Material and Preparation of Extract

Safflower was procured from the local market. 20 g of safflower powder were macerated for 24–48 hrs in 10ml of 70% methanol. The extracts were filtered and dried in hot air oven at 40°C. The extract was stored at -4°C until further use.

Cell line and Culture Medium

The Human Lung cancer (A549) cell line was cultured in F-12K medium with 10% Fetal Bovine Serum (FBS) and incubated at 37°C with 5% CO_2.

Preparation of Cells

The pelleted cells were rinsed in phosphate buffered saline (PBS) and embedded in 96 well plates for the assays, which were incubated for 24 hr with a 95% air and 5% CO_2 environment. Plant extracts with concentrations of 50, 100, 150, 200, 250, 300, and 350µg/ml were used to treat cells. Identical concentrations of the plant extract were used 3 times of the same batch for subsequent analysis.

Effect on Cell Viability -Trypan Blue Exclusion Assay

0.25 percent trypsin-EDTA solution was used to trypsinized A549 cells. After this, the cells were resuspended in phosphate buffered saline (PBS) and treated with 0.4% trypan blue and counted using hemocytometer,[7] Dead cells will be stained if the membrane's translucency is lost and the blue pigment is retained, but live cells will not be stained. The number of cells per ml was calculated by total cell number x dilution factor x 10^4 cells / mL.

The Percentage viability of cells was calculated as

$$\text{live cells} / (\text{dead cells} + \text{live cells}) \times 100.$$

Inhibition of growth was expressed as {cell viability (control)-cell viability (including extract)}.

Cytotoxicity Assay

The cell growth was quantified based on the potential of viable cells to convert MTT violet color formazan crystal. The culture medium was removed and 5 mg / ml MTT was added to each well and incubated for 4 hr at 37°C in 5% CO_2. The formazan crystals were dissolved in DMSO and subjected to gentle mixing of plate. The absorbance was observed at 492nm Using microplate reader.[8] The proportion of growth was calculated by the following equation.

$$\%\ \text{cell inhibition} = \left\{1 - \left[\frac{A_{490} - A_{630}(\text{treated})}{A_{490} - A_{630}(\text{control})}\right] \times 100\right\}$$

The effects of safflower extracts were expressed in terms of IC_{50} values.

Morphological Assessment of Cancerous Cells

To examine the morphology, the cells were seeded on a plate, and incubated for 24 hr at 37°C. The next day, cells were treated with 30 µg / ml plant leaf extract and reincubated under the same conditions. The cells treated with DMSO alone, served as a control. Using an inverted phase contrast microscope (Olympus, CK40SLP) at 200x magnification, morphological alterations in cancer cells with and without safflower extracts were studied.[9] After 8, 16, and 24 hr of incubation, photos were taken.

Study on apoptosis- Acridine Orange and ethidium Bromide Dual Staining

Cells were seeded in 24 well plates and incubated at 37°C for 24 hr under 5% CO_2.cells were washed with cold PBS after the end of treatment with safflower extracts. 20 µg / ml of acridine orange/ ethidium bromide were added to cells to observe under fluorescence microscope and photographed with digital camera (OPTIKA). At each data point, 300 cells from a randomly selected field were duplicated, numbered, and quantified for every concentration. Cells were classified as alive or dead, and if they were dead, they were classified as apoptotic or necrotic based on nuclear morphology and cytoplasmic tissue.[10] AO staining was utilized to distinguish live neurons from neurons undergoing apoptosis, using the AO property to shift its fluorescence from green at normal pH toward brilliant orange-red in the process of acidification. Further EB application labels nuclei of necrotic neurons in red. Sequential treatment by AO and EB can be employed as an express vitality test to count fractions of live and dead cell via apoptosis and necrosis, respectively. An algorithm of automatic quantification of cell types is based on the image correlation analysis. Our conclusion is validated by experiments with the vital dye trypan blue and the pharmacological study of receptor subtypes involved in the excitotoxicity. The approach described here, therefore, offers an express, easy, sensitive and reproducible method by which necrosis and apoptosis can be recognized and quantified in a population of living neurons. Because this assay does not require any preliminary tissue treatment, fixation or dissociation in a cell suspension its utility is likely to be extended for measuring cell viability and cytotoxicity on a variety of living preparations (tissues, brain slices and cell cultures

Mitochondrial Membrane Potential (Δψm)

After 24 hr of treatment, A549 cells were collected, washed twice with PBS, and about 1 x 10^7 cells were mixed with the cationic lipophilic dye rhodamine 123 (1µM, final concentration) and incubated for 10 min and later photographed using a confocal microscope.

Reactive Oxygen Species (ROS) Measurement

2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining was used to assess oxidative stress.[11] To create a 10 mM stock solution, 4.85 mg of DCFH-DA was diluted in 1 mL dimethyl sulfoxide (DMSO). The stock solution was diluted into 10 mM with DMEM and vortex for 10 sec using vortex mixer. The safflower-treated and untreated cells were rinsed again in DMEM and 500 µL of the DCFH-DA was added and incubated at 37°C. The DCFH-DA working solution was removed after a single wash with DMEM and each well received 500µL of 1X PBS. The fluorescence microscope was then used to capture the digital images (Labomed, USA).

RESULTS

Effect of Safflower Extract on Cell Viability

The effect of safflower extract on the viability of A549 cells is shown in Figure 1. The safflower treated cells showed a decrease in cell viability and that the decrease was found to be dose dependent. The cell viability was recorded as 95.23% at 50 µg concentration and 28.19% at 350 µg concentration.

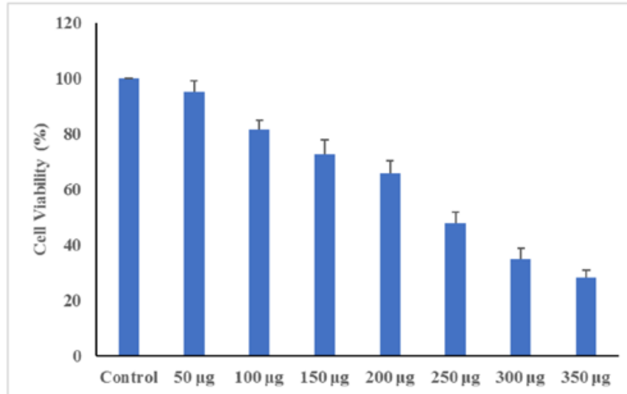

Figure 1: Effect of safflower on cell proliferation.

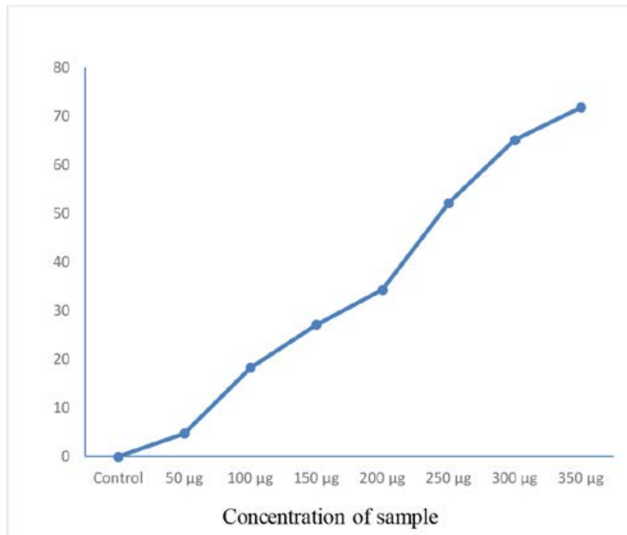

Figure 2: Cytotoxic effect of safflower on A549 cell line.

Cytotoxic Effect of Safflower Extract on A549 Cells

Figure 2 shows the MTT assay results for safflower extract cytotoxicity in lung cancer cells (A549). The effects of various safflower extract concentrations viz 50, 100, 150, 200, 250,300 and 350 µg/ml are shown graphically in Figure 2. There is a concentration dependent cytotoxic effect. Maximum inhibition was found at 350µg/ml. The LC_{50} range of the sample was found at 250 µg/ml concentration at 52.2%, whereas the viability of the cell declined to 28.19% at 350µg/ml concentration.

Morphological Assessment of Cancerous A549 cells

Figure 3 represents the photomicrograph (10X) of morphological changes in lung cancer cells. The safflower extract treatment caused morphological alterations such as shrinkage, detachment, membrane blebbing, and

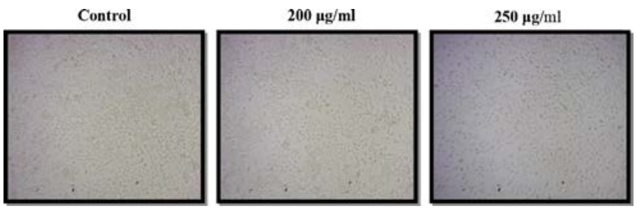

Figure 3: Morphological changes in control and sample safflower extract treated Lung cancer (A549) cells for 24 hr.

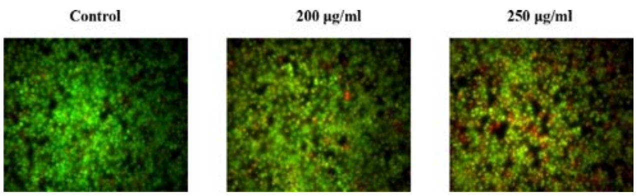

Figure 4: Effect of Safflower extract on the apoptotic incidence in Lung cancer (A549) cells.

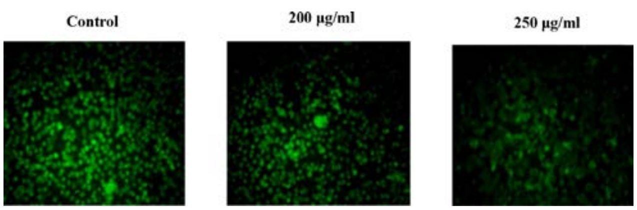

Figure 5: Effects of safflower extract on the mitochondrial membrane potential of Lung cancer (A549) cells.

distorted shape at 200 µg and 250µg/ml for 24 hr as compared with control. The cell morphology of control cells was normal.

Effect of Safflower Extract on Apoptosis

Figure 4 shows the apoptotic effect by safflower extract on A549 cells. If the membrane is intact, acridine orange nuclear staining shows a crescent-shaped or granular yellow-green in apoptotic cells at an early stage, while late apoptotic cells show orange fluorescence with ethidium bromide staining since it enters only in cells with damaged membranes. Here in this study, the untreated A549 cells shows green fluorescence with intact nuclear morphology whereas the lung cancer cells treated with safflower extract (200 µg and 250 µg/ml) for 24 hr, showed apoptotic bodies, clearly validating the apoptosis inducing effect by safflower on A 549 cells.

Effect on Mitochondrial Membrane Potential (MMP)

The MMP was measured by Rhodamine staining. Figure 5 shows the effect of safflower extract on MMP. There is gradual decrease of green fluorescence in A549 treated cells indicating a decrease in MMP by safflower extract (200 µg and 250µg/ml).

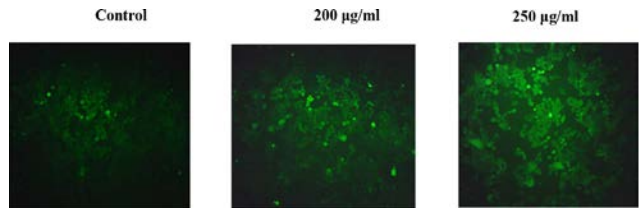

Figure 6: Effect of safflower extract on the intracellular ROS generation in Lung cancer (A549) cells by DCFH-DA staining assay.

Effect on Reactive Oxygen Species (ROS)

DCFH-DA staining was used to detect ROS levels. Lung cancer cells were treated with safflower extract (200 µg and 250 µg/ml) for 24 hr, stained with DCFH-DA staining (Figure 6). Control sample portrayed dull green fluorescence whereas safflower extract (200 µg and 250 µg/ml) treatment depicted bright DCF stained green fluorescence in A549 cells.

DISCUSSION

The results of this investigation revealed that safflower extract has an inhibitory effect on viability and growth of A549 cancer cell lines. The trypan blue dye exclusion assay is used to discriminate the dead and live cells. The results are presented in Figure 1. The proliferation of the safflower-treated A549 cells was shown to be dose-dependently decreased. The uptake of dye will be expelled by live cells. Because trypan blue is a weak acid, its affinity for basic proteins is increased, the uptake of dye in the nucleus is often stronger due to the presence of histones, resulting in a visible blue intensity, whilst the cytoplasm is only slightly stained.

The MTT (dimethylimidazole diphenyltetrazolium bromide) assay determines the functional status of mitochondria and also indicates a measure of cell viability. Living cell mitochondrial dehydrogenase enzymes reduce the yellow tetrazolium salt to the blue formazan. It precipitates on intact cells.[12]\”water\”, and \”oil\” (where \”water\” and \”oil\” are selective solvents for the different blocks The crystal formed is directly related to number of viable cells. Here in this study, dose depended inhibition of cell growth was observed. The safflower species has antioxidant and lipid peroxidative effects which might be responsible for the cytotoxic effect.[13-14] In a skin and breast cancer animal model, oil extracts from safflower seeds have anticancer properties and the same have also been observed in a melanoma cell assay system.[15-17] The results of this study are comparable to those of the previous one, which found that polysaccharides from safflower flowers had anticancer properties via the toll-like receptor/NFkappa B pathway.[18] The cytotoxic action of safflower extract is substantiated with the morphological changes observed using light microscopy (Figure 3). The process of cell death process is accomplished by apoptotic changes like shrinkage and membrane blebbing. Here in this study, Safflower extract brings apoptosis in A549 cells (Figure 4)[19] had similarly observed that carthamin, the active constituent of safflower induces apoptosis by stimulating pro apoptotic effect on hepatic stellate cells. Reactive oxygen species are a universal product of aerobic metabolism and are produced progressively more under stressful conditions. Mitochondria are the main intracellular source of ROS generation in eukaryotic cells. Increased intracellular ROS levels alter mitochondrial membrane permeability, reduce MMP, and promote Cyto-c release into the cytoplasm, resulting in apoptosis.[20]or mitochondrial, apoptotic pathway is permeabilization of the mitochondrial outer membrane. Permeabilization triggers release of apoptogenic factors, such as cytochrome c, from the mitochondrial intermembrane space into the cytosol where these factors ensure propagation of the apoptotic cascade and execution of cell death. However, the mechanism(s Lin K *et al*., 2021[21] proposed that hydroxy safflower B reduces mitochondrial membrane potential in breast cancer MCF7 cells via an independent mechanism and also that hydroxyl safflower B when coupled with doxorubicin suppresses the growth of cancer cells. Cancer cells undergo myriad of profound changes culminating in hyperpolarization of the mitochondrial membrane. Disruption of the MMP has been considered as the end point in apoptotic signaling. This study also observed that the control cells exhibit significant fluorescence due to accumulation of rhodamine whereas the safflower extract 200 and 250 μg/ml treated cells did not show any rhodamine accumulation. Thus, it is found that safflower is offering protection to mitochondria by suppressing oxidative stress, protecting mitochondrial membrane potential and regulating apoptosis.

CONCLUSION

The study found that safflower extract is efficient in altering the morphology of nucleus and protects the mitochondria by regulating ROS and membrane potential in cancer cells. Further studies are required on the isolation of active compounds responsible for mitochondrial protection and also studies on the signaling pathways to identify how safflower regulates ROS release.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

ABBREVIATIONS

DCFH-DA: 2',7'-dichlorodihydrofluorescein diacetate; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **ROS:** Reactive Oxygen Species; **AO/EB:** Acridine orange /Ethidium bromide; **EDTA:** Ethylenediamine tetra acetic acid; **PBS:** Phosphate buffered saline; **FBS:** Fetal Bovine Serum; **ELISA:** Enzyme-linked Immunosorbent Assay; **DMSO:** Dimethyl sulfoxide; **DMEM:** Dulbecco's Modified Eagle Medium; **MMP:** Mitochondrial Membrane Potential.

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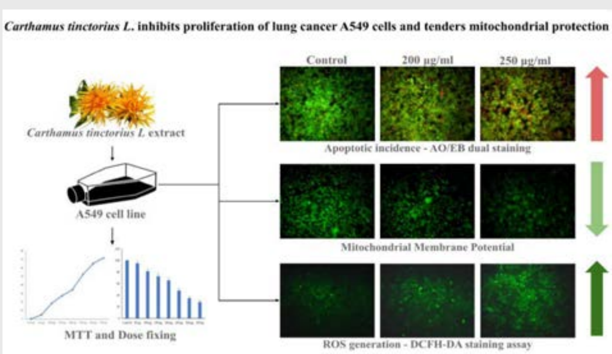
PICTORIAL ABSTRACT



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SUMMARY

- The current study intended to evaluate safflower's antiproliferative effect on A 549 cells.
- The trypan blue assay predicted that at the maximum dosage, cell viability is considerably reduced, and that the effect is found to be dose dependent.
- The MTT assay revealed that cytotoxicity increased in a dose-dependent manner. The sample's LC50 range was determined to be 52.2 percent at 250 g/ml concentration, while the cell's viability dropped to 28.19 percent at 350 g/ml concentration.
- Cells shrinkage, cell detachment and membrane blebbing were observed in safflower-treated A549 cells, indicating antiproliferative activity.
- The ethidium bromide and acridine orange staining substantiates that safflower extract induces apoptosis, as untreated A549 cells showed green fluorescence with intact nuclear morphology, while cells treated with safflower extract (200 g and 250 g/ml) showed apoptotic bodies, demonstrating that safflower extract induces apoptosis in A549 cells.
- Safflower extract also protects mitochondria by reducing oxidative stress and changing mitochondrial membrane potential.
- In conclusion, it is found that safflower extract has antiproliferative properties by triggering apoptosis and protects mitochondria by inhibiting oxidative stress.

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