

Evaluation of Antioxidant and Oxidative Stress Activity of *Carthamus tinctorius* L. Extract in Lung Cancer A549 Cells

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ABSTRACT

Background: *C. tinctorius* (safflower) natural food colorant and has been used to control high blood pressure, suppress oxidation, cancer immunosuppressive drug, inhibits blood clots, dilate blood vessels, and as neuroprotective agents. Several investigations indicate the relationship between oxidative processes and emergence of cancer. The current investigation data confirms the antiproliferative activity of Safflower against A549 cancer cell line but little is known about their antioxidant mechanisms. **Materials and Methods:** Nitric Oxide (NO) and Lipid Peroxidation (LPO) levels were measured in order to explore the antioxidant properties of safflower extract. Lactate Dehydrogenase (LDH), reduced Glutathione (GSH), Superoxide Dismutase (SOD) and Catalase (CAT) were measured by UV-spectrophotometric analysis. Further, apoptotic key regulator BAX and SMAC gene expression were also analysed. **Results:** The dose-dependent antioxidant activity of Safflower extract induced apoptosis in A549 cells. There was significant increase in LPO, NO, LDH, SOD and CAT (* $p < 0.0001$) activities except GSH. Our data confirmed that safflower treated A549 cells inhibits the oxidation by scavenging free radicals and induced apoptosis by increased gene expression BAX and SMAC. **Conclusion:** The current findings indicates safflower extract might be potential alternative medicine for the treatment of lung cancer.

Keywords: Safflower, Antioxidant, Anti-cancer, Lipid Peroxidase, Nitric Oxide, Polymerase Chain Reaction.

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INTRODUCTION

Extensive research on Oxidative stress reveals a disparity between production of free radical and reactive metabolites leads to damage of important biomolecules and cells which in turn progress into number of metabolic, degenerative conditions, or malignancies.¹⁻⁴ Cancer as a second leading cause of death in developing countries has received worldwide attention in the past decades. Compared to normal cells, cancer cells have lower levels of antioxidant enzymes, which accumulates more Reactive Oxygen Species (ROS), and aid in regulating variety of signalling pathways for cell proliferation and survival.⁵

Cancer cells can be dragged towards programmed cell death, when the levels of ROS were above the sublethal threshold, therefore the cancer cells maintain a substantially higher degree of oxidative stress which are below the lethal limits. Owing to

this characteristic property, controlling oxidative stress in cancer cells may play a beneficial role for both cancer treatment and prevention.⁶

Oxidants are produced by some cytosolic enzyme systems, as well as by normal intracellular metabolism in mitochondria and peroxisomes. To maintain physiological homeostasis, a complex, enzymatic and non-enzymatic antioxidant defence system, combats and controls overall ROS levels. Based on this knowledge, there has been significant interest in the role of antioxidants as potential chemoprevention of lung cancer. Though recent treatments can decrease disease recurrence and improve patients' survival, these therapies have limited benefits for lung cancer patients at the early stage and they have detrimental side effects and problems and do not increase the prognosis or survival of people with early-stage lung cancer.⁷⁻¹⁰

Natural medicines and their compounds are familiar as a useful complementary therapy against the adverse reactions occurring during chemotherapy. Even though numerous studies have demonstrated the benefits of *C. tinctorius* (safflower) as a cancer immunosuppressive drug, which can also prevent blood clots, dilate blood vessels, treat high blood pressure, inhibit oxidation



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and as a neuroprotective agent, our earlier publications have demonstrated the benefits of *C. tinctorius* and its inhibitory effect on A549 cell viability and growth by inducing apoptosis and protects mitochondria.¹¹ Furthering, the current study aims to identify the antioxidant properties of *C. tinctorius* on Lung cancer cell lines.

MATERIALS AND METHODS

Plant Materials: Collection and Extraction

Carthamus tinctorius L. (Safflower) powder was obtained from Saudi market. About 20 g of the powder were soaked in 10 mL of 70% methanol for 2 days. The extract was then filtered through a filter paper (Whatman filter paper No.1) and dried at 40°C in hot air oven and was stored at -4°C until further process.

Cell culture and sample preparation

In DMEM media complemented with 10% Foetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (PenStrep), human lung cancer cell lines (A549 cells) purchased from NCCS were cultured. The cells were then kept at 37°C in a humidified environment of 5% CO₂. When confluent (80% confluence), cells were passaged and used for further experiments in an exponential growth stage.

Confluent cells were pelleted, rinsed in Phosphate Buffered Saline (PBS) and seeded (1 x 10⁵ cells/mL) on 24 well plates, incubated with 5% CO₂ for 24 hr. Based on our earlier publication, plant extracts with concentrations of 200, 250 and 300 µg/mL¹¹ were used to treat cells.

LPO (Lipid Peroxidase) Assay

Lipid peroxidation was evaluated by modified Ohkawa *et al.*,¹² method. When exposed to air, lipid peroxides break down into a complex series of molecules, such as reactive carbonyl compounds. Briefly, A549 cells were seeded at 1 x 10⁵ cells/mL (duplicates) and treated with safflower extract.

The MDA standard was carried out using samples of MDA that had been treated and untreated. Re-dispersed as mentioned by Ohkawa, the cell pellet was then incubated at room temperature for 1 hr at 95°C.

NO (Nitric Oxide) Assay

The impact of the safflower extract on nitric oxide scavenging potential of A549 lung cancer cells was estimated using the Dirsch *et al.*¹³ method. Griess-Ilosvay reaction quantifies the nitric oxide scavenging potential of extract in treated and untreated cells. A549 cells were seeded, treated and the potential to quench NO was evaluated. Griess reagent was added in equal parts to the spent medium, which was then incubated for 10 min at room temperature in the dark. At 540 nm, the reaction mixture is read.

$$\text{NO quenching effect (\%)} = ((A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}) \times 100$$

where A is the absorbance at 540 nm.

Determination of intracellular antioxidants

Glutathione Reduced (GSH) Assay

The GSH-DTNB reaction, results in the production of the TNB chromophore, which has a maximum absorbance at 412 nm, and the oxidised glutathione-TNB adduct, which is the basis for the assay (GS-TNB). The amount of GSH in the sample directly correlates with the rate of TNB production as measured at 412 nm. A549 cells were seeded, treated, harvested and pelleted and processed for GSH assay as per Ellman's method.¹⁴ Standards were prepared. The enzymatic reaction was analysed for standard, samples and controls at 415 nm.

Catalase Activity (CAT)

The antioxidant production was measured based on Goth method¹⁵ with minor variations. A549 cells were seeded and treated for 24 hr, harvested, washed (3X in PBS), sonicated and centrifuged. The reaction of molybdate with hydrogen peroxide and sodium-potassium phosphate buffer was stopped with ammonium moly bicarbonate and measured at 405 nm.

$$\text{Catalase activity (kU/l)} = (A(\text{sample}) - A(\text{blank 1}) / A(\text{blank 2}) - A(\text{blank 3}) \times 271$$

Blank 1 constitute substrate, molybdate and cell supernatant; blank 2 has substrate, molybdate and buffer; blank 3 has buffer, molybdate and buffer. Untreated cells were used as control.

Superoxide Radical Scavenging Assay

The potential of the Safflower extract was determined by the method defined by Luo *et al.*¹⁶ A549 cells were treated with safflower extract, harvested, homogenized with Tris HCl and mixed with NBT, NADH and PMS. After being incubated at room temperature for 10 min in complete darkness, the reaction solution was detected at 560 nm and calculated as follows:

$$\text{Scavenging ability (\%)} = (1 - A1/A0) \times 100$$

A1 - absorbance of the reaction solution with the sample and A0 - untreated group.

Detection of Lactate Dehydrogenase (LDH) activity

LDH was detected as per Ahamed *et al.*¹⁷ protocol with slight variation. When the integrity of the plasma membrane is damaged, LDH, present in cytosol, seeps into the culture medium. Seeded cells were exposed to different concentrations of safflower extract for 24 hr and then centrifuged at 2,300 g for 10 min. Using a spectrophotometer, the rate of NADH oxidation was calculated by monitoring the decline in absorbance at 340 nm for 3 min at intervals of 30 sec.

Gene Expression Analysis

A549 cells were cultured and exposed to different concentration of safflower extract for 24 hr. At the end of the exposure time, cells were trypsinized, cellular DNA extracted, and then processed for PCR-based gene expression profiling.

Reverse Transcriptase PCR (RT-PCR) was performed for Beta Actin (F-5' TCAAGGTGGGTGTCTTTCCTG3' and R-5' TTTGCGGTGGACGATGGAG 3', BAX (F - 5' CGTGTCTGATCAATCCCCGA 3' and R - 5' GAGGCCAGAAGGCAGGATTG 3') and SMAC (F-5' ACAAGCTACCTTTGGGCACC and R-5' CATCAATCCGATCCTGGGCT) primers. The denaturation step was set 95°C for 2 min. Annealing temperature was set 54°C for 30 sec for the β Actin, 54.4°C for 30 sec for BAX and 53.8°C for 30 sec for SMAC. The products of PCR were visualised under UV illumination.

Statistical Analysis

The statistical evaluation was done using GraphPad Prism (Prism 5.1). Following a Tukey multiple comparison test across groups, a one-way ANOVA was employed to analyse the data's means. Statistics were considered significant if the p value was less than 0.05. The findings are represented as the averages and standard deviations from three different experiments.

RESULTS

LPO (Lipid Peroxidase) Assay

The effect of *Carthamus tinctorius* L. (Safflower) on lipid peroxidation of A549 lung cancer cells was estimated. Lipid peroxidation increased as a result of the extract in a dose-dependent manner. At 250 and 300 g/mL, the increase was statistically significant (Figure 1). The lipid peroxidation and NO assay showed a positive association. Cells treated at 300 g/mL showed a nearly 3- to 4-fold increase in lipid peroxidation, which

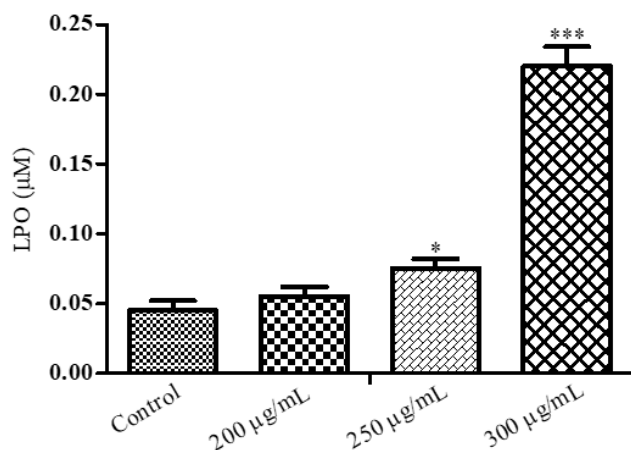


Figure 1: Effect of *Carthamus tinctorius* L. (Safflower) on LPO in A549 lung cancer cells.

was also statistically significant ($***p<0.0001$) with respect to the control.

NO (Nitric Oxide) Assay

Nitric oxide quenching by *Carthamus tinctorius* L. (Safflower) is enhanced with dose (Figure 2). The quenching activity was significantly higher than the control at 250 and 300 g/mL, with a corresponding increase in NO level of 4 to 5 fold that was statistically significant ($***p<0.0001$).

Determination of intracellular antioxidants

Glutathione Reduced (GSH) Assay

Figure 3 depicts the levels of GSH in control as well as treated cells. The levels of GSH in treated cells were reduced significantly in a dose dependent manner. After 24 hr, GSH level in cells treated at higher concentration of safflower extract reduced one-fold compared to the control.

Catalase Activity (CAT)

The effect of *Carthamus tinctorius* L. (Safflower) on catalase activity in lung cancer cells was estimated. The extract caused a dose-dependent increase in catalase activity. Catalase activity was significantly increased in safflower treated cells compared to catalase activity measured in untreated A549 cell lines. The increase was statistically significant in all concentrations including 200, 250 and 300 μg/mL (Figure 4).

Superoxide Radical Scavenging Assay

In comparison to untreated control cells, Safflower-treated A549 cells at 250 μg/mL and 300 μg/mL doses significantly ($*p<0.0001$) enhanced superoxide dismutase activity (Figure 5). This increase is similar to LPO, NO and catalase activity.

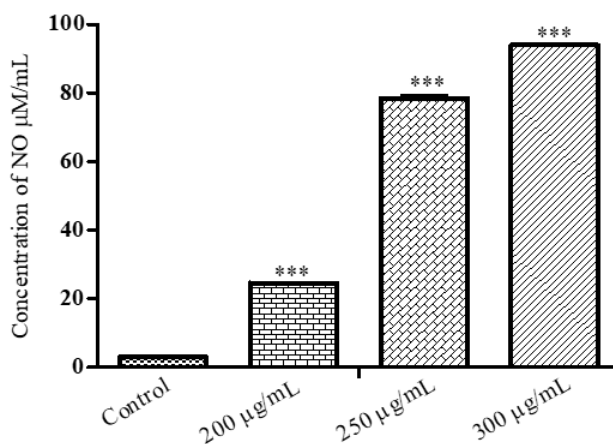


Figure 2: Effect of *Carthamus tinctorius* L. (Safflower) on Nitric Oxide in A549 lung cancer cells.

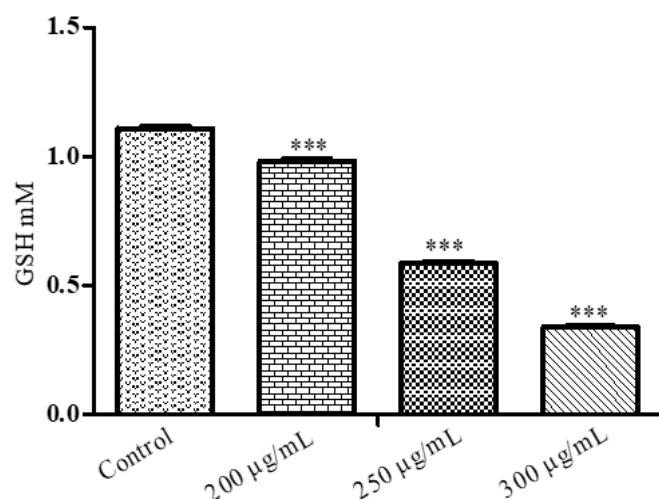


Figure 3: GSH activity of *Carthamus tinctorius* L. (Safflower) on A549 lung cancer cells.

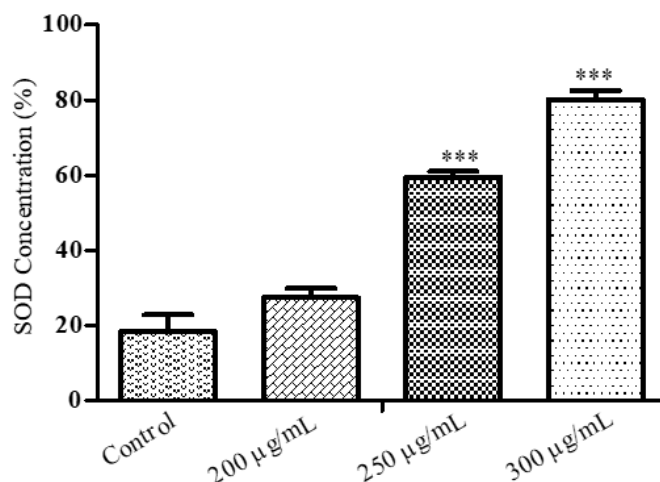


Figure 5: Superoxide dismutase activity of *Carthamus tinctorius* L. (Safflower) on A549 lung cancer cells.

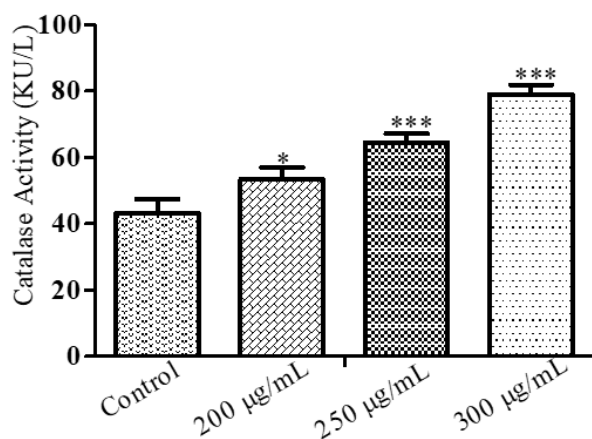


Figure 4: Catalase activity of *Carthamus tinctorius* L. (Safflower) on A549 lung cancer cells.

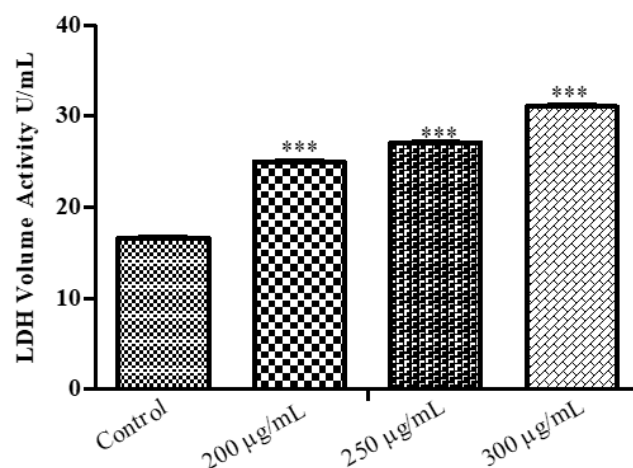


Figure 6: Effect of Safflower extract on LDH activity in A549 lung cancer cells. with increasing concentration. This pattern of gene regulation was shown to be dose-dependent (Figure 7).

Detection of Lactate Dehydrogenase (LDH) activity

LDH leakage caused by safflower extract was discovered to be dose-dependent (Figure 6). In every concentration, the rise was statistically significant. Catalase and superoxide dismutase activity showed a favourable connection with LDH activity. Cells treated with 300 µg/mL showed a statistically significant increase in LDH (** $p < 0.0001$) over the control of over a 2-fold.

Gene Expression Analysis

Using PCR-based gene analysis, the effect of safflower extract on the molecular level was evaluated. Apoptotic stimuli cause the mitochondrial protein SMAC to be released into the cytosol. The BAX play a major role in the signalling of apoptotic genes. After 24 hr of treatment, gene analysis was carried out on A549 cells that had been exposed to safflower extract. Similar to the pro-apoptotic gene SMAC, the apoptotic gene BAX was elevated

DISCUSSION

Eliminating uninhibited cell proliferation or metastatic spread in cancer events is the purpose of many complementary and alternative treatments. Owing to the inadequate or adverse impact of existing tactics, development of natural therapies has been expedited.¹⁸⁻²⁰ Although *Carthamus tinctorius* L. (Safflower, Compositae) have proved as a promising therapeutic agent in numerous laboratory studies^{21,22} uncertainty exists regarding the mechanism underlying this impact. Suppression of cell viability, levels of antioxidants and triggering of pro-apoptotic genes are the most important strategies in cancer treatment.

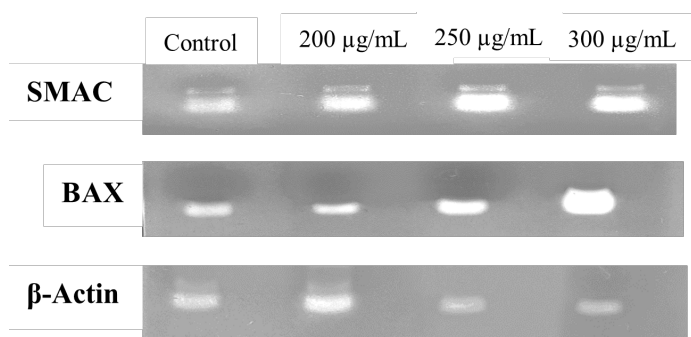


Figure 7: Analysis of BAX and SMAC genes by PCR.

Our earlier pilot investigation confirmed that safflower extract had the ability to suppress the development of A549 cells.¹¹ Based on the above results, we speculated that Safflower extract can exert antioxidant potential and induce pro-apoptotic signalling pathways in cancer cells. Therefore, we studied the antioxidant and molecular potential of Safflower extract on A549 cells. The results showed that Safflower extract significantly inhibited the free radicals in A549 cells, arrested their proliferation and up-regulated the expression of BAX and SMAC levels. These results support earlier studies' conclusions that safflower extract has antioxidant properties.^{23,24} Most experts agree that the polysaccharides in safflower primarily target the NF- κ B and Nrf2/Keap1 ARE signalling pathways to exert their antioxidant effects.^{24,25} Consuming polysaccharides can initiate pathways and up-regulate the expression of defensive genes, which protects cells from oxidative stress by increasing the concentrations of antioxidant molecules and enzymes like Superoxide Dismutase (SOD), Glutathione Peroxidase (GSH-Px), Catalase (CAT), Glutathione (GSH), and Glutathione Reductase (GR)^{25,26} which were similar to the result expressed in our present study.

In cells or across cell membranes, ROS can initiate a cascade of reduction-oxidation cycles that deplete cellular antioxidants and cause irreversible oxidative damage to cells. Lipid peroxidation is a result of oxidative stress, and several types of aldehydes are created during the reaction that breaks down these lipid hydroperoxides. Few of these aldehydes have a higher level of reactivity, and they operate as secondary messengers that propagate and support early free radical processes. To evaluate the function of oxidative damage in cells, lipid peroxidation must be quantified.²⁶ In the current investigation, cells treated with 300 g/mL of safflower extract produced noticeably higher membrane lipid peroxidation than untreated control cells. The concentration dependent effect of safflower extract showed that it may control how susceptible cells were to lipid peroxidation. Due to their capacity to act as "radical scavengers" or exert a chelating action, safflower polyphenols may be responsible for the antioxidant activity against lipid peroxidation.²⁷

A relationship between antioxidant activity and structural characteristics of polyphenols has been reported by several

authors.^{27,28} According to published evidence, the presence of hydroxyl groups directly correlates with the scavenging activity of phenolic compound.²⁷ Therefore, it is plausible to hypothesise that the structural properties of the bioactive components in safflower extract are probably related to the antioxidant activity found in this study.

In addition to ROS, nitric oxide has also been associated with inflammation, cancer, and other pathological conditions. Nitric oxide is a highly reactive hazardous gas that can freely enter any subcellular space and can cross any kind of membrane system. We examined the Safflower extract's inhibitory effect on A549 cells due to its harmful effects on cellular function. A dose-related increase in NO generation was seen with increasing sample concentration. This may be because safflower extract can counteract the effects of NO synthesis, which may be helpful in reducing the negative consequences. In a similar study by Wang *et al.*, dried Safflower petals' Aqueous extracts (SFA) were used to treat LPS-induced inflammation in RAW264.7 macrophages, and the results showed that SFA reduced the expression of the proteins inducible Nitric Oxide Synthase (iNOS) and cyclooxygenase-2 to reduce the release of nitric oxide, prostaglandin E2, and interleukin 1.²⁹

GSH (γ -glutamyl-cysteinyl-glycine) is a tripeptide, is an endogenous, non-enzymatic antioxidant, mostly dispersed in cytosol, but also in nuclei, peroxisomes and mitochondria can act as a potent ROS scavenger directly, without enzymatic help and its intracellular concentration is an indicator of oxidative stress.³⁰ In this study, the levels of GSH in treated cells were reduced significantly in a dose dependent manner. After 24 hr, GSH level in cells treated at higher concentration of safflower extract reduced one-fold compared to the control. Since the GSH concentration is reduced than control, it is safe to say that Safflower extract maintains the oxidative stress level in A549 cells. The effect of catalase upregulation on lung cancer cells' receptivity to herbal therapy was assessed. Our findings shown that overexpressing catalase in A549 cells increases twice while causing a onefold reduction in the expression of reduced glutathione.³¹ In the current work, we also demonstrated that treatment of A549 lung cancer cells with safflower extract dramatically lowered the dehydrogenase activity, causing a reduction in NADH/NAD⁺, which then in turn triggered cell death due to decreased mitochondrial membrane potential. Lysosomal membrane damage has been shown to release lysosome protease into intracellular regions, which damages neighbouring cells and causes apoptosis, or cell death. LDH leaking from cells is more proof that nanoparticles can enter cells and disrupt cell membranes.³²⁻³⁴ Additionally, compared to untreated A549 cells, the Safflower extract treatment boosted superoxide dismutase activity ($p < 0.0001$). This may cause a redox imbalance, which may lead to a redox imbalance and an increase in SOD activity, which may ultimately lead to cell death.³⁵

Using PCR-based gene analysis, the effect of safflower extract on the molecular level was evaluated. SMAC and BAX were upregulated in dose-dependent manner. These findings are consistent with earlier studies that were conducted on several malignant tumour types. One such study³⁶ shown that over-expression of SMAC might cause apoptosis in ovarian cancer cell lines, suggesting that increasing the cytosolic SMAC protein may be a feasible strategy for cancer therapy.³⁷ The protein BAX, on the other hand, affects the mitochondria's membrane potential and promotes apoptosis. Studies show that ROS triggers the phosphorylation of BAX, which kills cancer cells.³⁷ This is in line with our findings, which demonstrate that the quantity of PCR products expressing SMAC and BAX increased as Safflower concentrations increased, the release of SMAC from mitochondria into the cytosol, which may increase the activities of Caspase-3 and Caspase-9. Our findings indicate that Safflower extract treatment inhibited A549 cell growth and promoted apoptosis.

CONCLUSION

In this study, it was obvious that the Safflower extract induces good antioxidant ability and ROS-dependent apoptosis in lung cancer cell lines. Furthermore, we affirmed both BAX and SMAC regulate the time-delay and activation threshold of the apoptotic switch. These results provide an insight into the antioxidant activities of Safflower extract and provide the basis for its clinical application. *C. tinctorius* (Safflower) may also be used as a novel bioresource to fight against human lung cancers, especially by enhancing the antioxidant property of normal cells.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ROS: Reactive oxygen species; **TBARS:** Thiobarbituric acid reactive substances; **SOD:** Superoxide Dismutase; **CAT:** Catalase; **GSH:** Glutathione; **DMSO:** Dimethyl sulfoxide; **DTNB:** 5,5'-dithiobis-(2-nitrobenzoic acid); **KCL:** Potassium chloride; **SDS:** Sodium dodecylsulfate; **NBT:** Nitro blue tetrazolium; **NED:** N-(1-naphthyl)-ethylenediamine dihydrochloride; **RT-PCR:** Real time-Polymerase chain reaction.

SUMMARY

- The current study intended to evaluate safflower's antioxidant effect on A549 cells.

- Carthamus tinctorius*, has been utilised as an anti-coagulant, vasodilator, anti-hypertensive, antioxidant, neuroprotective agent, and immunosuppressant.
- On treating the A549 cells with safflower, the oxidative stress enzymes such as Reactive oxygen species, Lipid peroxidation, and Nitric oxide were significantly elevated.
- Antioxidants such as Superoxide dismutase, Catalase were increased in dose dependent manner and GSH were significantly suppressed.
- The pro-apoptotic gene SMAC and the apoptotic gene BAX both showed upregulation with increasing concentration.
- Overall, Safflower treated A549 cells enhances the antioxidant activity by scavenging the free radicals.

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