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*Original Research*

# Astragalin flavonoid inhibits proliferation in human lung carcinoma cells mediated via induction of caspase-dependent intrinsic pathway, ROS production, cell migration and invasion inhibition and targeting JAK/STAT signalling pathway

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**Abstract:** The aim of the current study was to investigate the anti-lung cancer effects of astragalin. Studies were also undertaken to evaluate its effects on apoptosis induction, ROS production, cellular migration and invasion and JAK/STAT3 signalling pathway. MTT assay was used to evaluate cell viability in NSCLC A549 cells after exposure to astragalin molecule. Apoptosis was investigated using AO/EB staining, comet assay and western blotting assay. Fluorescence microscopy was implemented to estimate ROS production. Cell migration and invasion were measured using transwell chambers assay. Effects of astragalin on JAK/STAT pathway were investigated using western blotting assay. Results showed astragalin molecule induced inhibition of proliferation in A549 cells in a dose-dependent fashion. Further, the antiproliferative effects were found to mediate via apoptosis as suggested by AO/EB staining and western blotting assay. Astragalin modulated the expressions of caspase-3, caspase-9, Bax, Bak, Cyt-c Bcl-2, XIAP and Bcl-xL. Astragalin induced DNA damage in A549 cells which too indicated apoptotic cell death. Astragalin molecule enhanced the production of ROS by A549 cells. It inhibited both cell migration and invasion of A549 cells in a concentration-dependent manner. Finally, astragalin drug was observed with remarkable potential of targeting JAK/STAT pathway in A549 NSCLC cells. These results indicated that astragalin drug could prove helpful in lung cancer treatment and research provided more *in-vivo* studies are performed.

***Key words:*** Lung carcinoma; Flavonoids; Astragalin; Intrinsic pathway; Cell migration.

## Introduction

Natural products, a fathomless pool of biologically active chemical species, continue as leading and infinite resource in the field of drug discovery (1). Nature has gifted us with a valuable gift in the form of medicinal plants which remain as infinite source of potential pharmacological agents (2). Maximum number of drugs discovered either belong to natural products or based on natural products. Natural products bear a huge structural diversity which enables the development of pharmacophores, robust chemotypes and new drugs (3). Flavonoids are a class of low molecular weight active natural product polyphenols showing remarkable structural diversity and molecules that are analogous to biological systems. This class of compounds has been identified with substantial pharmacological and biological activities including antiviral, anti-allergic, anti-inflammatory, anti-diabetic and anticancer activities (4,5). Flavonoids promote tumor suppressive effects against distinct human cancers through blocking of key tumor promoting enzymes and cell cycle arrest (6). Lung cancer is a disastrous respiratory disorder most frequent in many countries. Lung cancer is responsible for causing a huge mortality and morbidity among both the sexes, globally (7). European Union registered 20% deaths and USA registered 27% deaths due lung cancer out of total cancer deaths in the years of 2016 and 2015, respectively (8,9). Across the globe, lung cancer accounts for 19% of deaths due cancer and studies have been reported that approximately 58% of lung cancer cases befall in low-income and meddle-income countries. This lethal malignancy shows higher dominance among men than in women with life time occurrence chances of 1:15 in men and 1:17 in women. The major risk factors contributing to the development of lung cancer includes smoking, air pollution, secondhand smoking, radiation and asbestos exposure (10,11). Tobacco smoking is the chief etiological factor primarily contributing to lung cancer carcinogenesis. The currently available management modalities for curing lung cancer include radiation therapy, chemotherapy, target therapy, surgery and combination therapy (12). Besides advancements in the field of lung cancer management and diagnosis the overall survival remains on the lower side. Therefore, there is an immediate requirement for the development of novel drugs or modalities that can curb this neoplasmic malignancy. Astragalin is a potential flavonoid found in Traditional Medicine plant *Cuscuta chinensis* (13). Astragalin has been reported with strong biological activities and therapeutic potentials including anti-diabetic, antiulcer, anti-osteoporotic, anti-obesity, antioxidant, cardioprotective, anti-inflammatory, neuroprotective and anticancer (14-16). Moreover, astragalin has been shown with proapoptotic and proautophagic against several human cancer cell lines (17,18). Therefore, this current study was designed to evaluate the anticancer effects with underlying mechanism of action of astragalin molecule against lung cancer.

## Materials and Methods

### Chemicals, cell culture and conditions

Astragalin molecule was procured from Sigma-Aldrich (St. Louis, MO, United States). Human non-small cell lung carcinoma (NSCLC) cells were provided by China Center for Type Culture Collection (Wuhan, China). Cells were cultured and maintained in Dulbecco's modified Eagle's media (DMEM) (Lonza Biologics, Singapore) containing fetal bovine serum (10%), 100 µg/mL of streptomycin and 100 U/mL of penicillin (Vega Pharma Limited, Zhejiang, China). Cultural conditions were set at humid with 37°C of temperature and 5% $CO_2$.

### Evaluation of cell growth

The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to screen the cellular growth in astragalin treated NSCLC A549 and normal lung NHBE cells. A549 and NHBE cells were seeded at a density of $2.6 \times 10^4$ cells/well for 24 h within 96-well plates. Post seeding, cells were treated with astragalin molecule for 24 h at altering doses viz 0, 5, 25, 75 and 150 µM. Afterwards, MTT solutions was supplied to each well of 96-well plates followed by incubated for 3 h. After completion of incubation period, formazan crystals formed were solubilized within dimethyl sulfoxide (100 µM). Finally, cells were placed in a microplate reader (Bio-Rad) for absorbance recording at a wavelength of 492 nm.

### Acridine orange/ethidium bromide (AO/EB) staining assay

To assess the effects of astragalin drug on cellular apoptosis in A549 cells, AO/EB staining assay was executed. A549 cells were seeded with a density of $0.7 \times 10^5$ cells/well of 6-well plates followed by astragalin treatment at different doses of 0, 25, 75 and 150 µM, for 24 h. Cells were let to slough off followed by the addition of AO/EB staining (1µl) after loading to glass slides. Afterwards, cells were covered using coverslips and subsequently loaded over a fluorescent microscope for apoptotic studies.

### Comet assay

The degree of DNA damage in A549 cells was evaluated via comet assay (alkaline single cell gel electrophoresis) after being exposed to astragalin drug. In brief, A549 cells were exposed to astragalin drug at changing concentrations viz 0, 25, 75 and 150 µM for 24 h over 6-well plates. Then, cell suspensions were placed onto the frosted microscopic slides bearing a layer of normal melting agarose (1%) followed by dipping in a lysing solution and left untouched overnight at 4°C. Thereafter, lysed cells were subjected to electrophoresis (280 mA, 20 V) for 30 min. The slides were neutralized within a neutralizing buffer bearing Tris 0.4 M with pH 7.5. As a final point, slides were stained using EB and subjected to fluorescence investigations under a fluorescence microscope (Leica DM3000, Germany).

### Estimation of reactive oxygen species (ROS) levels

The intracellular ROS levels in astragalin treated A549 cells were evaluated through fluorescence microscopy using 2′,7′- dichlorodihydrofluorescin diacetate (DCFH2-DA) dye. In brief, A549 cells at a density of $1 \times 10^4$ cells/well of 6-well plates were cultured for 24h at 37°C. Afterwards, cells were subjected to astragalin drug treatment at changing concentrations viz 0, 25, 75 and 150 µM for 12 h. Thereafter, cells were collected for centrifugation followed by washing with PBS and staining with 20 µM of DCFH2-DA fluorescence dye. Stained cells were then placed in dark for half an hour. Finally, cells were again washed using PBS followed by investigations under fluorescence microscopy (Olympus Corporation, Japan) at 100x of magnification.

### Transwell chambers migration and invasion assays

Transwell chambers assay were executed to evaluate the effects of astragalin drug on A549 cells tendency of migration and invasion. Briefly, $3 \times 10^4$ cells were seeded to upper transwell chambers maintaining different astragalin drug concentrations viz 0, 25, 75 and 150 µM and RMPI medium (600 µL). The lower chambers were only supplied with RMPI-1640 medium bearing fetal bovine serum (10%). Afterwards, all the chambers were incubated at 37°C for 36 h and non-migrated cells were cleared with a cotton swab. Migrated cells were fixed for 10 min using 4% formalin following by staining for 15 min using crystal violet (0.1%) dye. Finally, a light microscope was used to capture 5 random fields under a magnification 100X and ultimately cells were numbered. A similar mechanism was followed for determination of invasion except transwell chambers were fixed with Matrigel.

### Western blotting assay

After preculturing of the cells for 24 h, astragalin treatment was instigated with altering doses viz 0, 25, 75 and 150 µM for 24 h. treated A549 cells were then placed within lysis buffer at 4°C and then at 95°C. The quantification of protein content from within each lysate was monitored using Bradford assay. About 35 µg of proteins from each sample were placed over SDS-PAGE for separation and then electrophoretically moved to polyvinylidene fluoride (PVDF) membranes. Prior the primary antibody treatment, membranes were subjected to TBS (tris buffered saline) treatment. The primary antibodies used were against caspase-3, cleaved caspase-3 caspase-9, Bax, Bak, Cyt-c, XIAP, Bcl-2, Bcl-xL, JAK1, STAT1 and STAT3. Following primary antibody treatment at 4°C overnight, membranes were treated with appropriate secondary antibodies for 1 h. Finally, the protein signals were recorded and visualized using enhanced chemiluminescence reagent (Sigma).

### Statistical analysis

Statistical analysis was performed using GraphPad prism 7 software and the overall experimental data was represented as mean ± SEM (standard error of mean). Every individual procedure was experimented in triplicates. Statistical analyses were employed using one-way ANOVA followed by Dunnett's multiple comparison test against the control group or by Student's t-test between two groups; $*p < 0.05$ and $**p < 0.01$ were used to indicate statistical significance.

## Results

### Cytotoxic effects of astragalin against A549 cells

The cytotoxicity of astragalin (Fig. 1A) was investigated against normal NHBE and cancerous A549 lung cells through MTT assay. It was observed that astragalin triggered cytotoxic effects in A549 cells. Control cells were considered as 100% viable cells. Post A549 cells were exposed to variant concentration of astragalin (0-150 µM), the cellular viability reduced considerably from 100% to nearly 15% (Fig. 1B). Additionally, astragalin showed miniscule toxicity against normal lung NHBE cells. Therefore, astragalin showed outstanding antiproliferative propensity selectively against A549 cells in a concentration-dependent manner.

### Apoptotic effects of astragalin in A549 cells

The apoptotic effects of astragalin in A549 cells were determined by using AO/EB staining and comet assay. AO/EB staining showed that the percentage of apoptotic (early and late) and necrotic cells increased in astragalin treated groups in comparison to controls. Control cells showed high frequency of green florescence representative of normal cell morphology while treated cells showed yellow-green, orange-red and red fluorescence indicative of early apoptotic, late apoptotic and necrotic cells, respectively (Fig. 2A). The early and late apoptotic cell percentage at 150 µM of astragalin concentration was nearly 20 % and 25%, respectively (Fig. 2B). Further, astragalin remarkably induced damage to DNA of A549 cells. On analysis through comet assay, it was observed that the comets originated in astragalin treated cells bear broad and long tails and higher DNA intensity

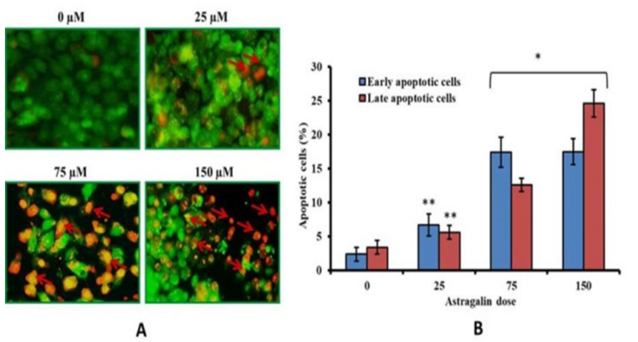


**Figure 2.** (A) The AO/EB staining assay was used differentiate normal and apoptotic A549 cells after astragalin treatment. In figure, green, yellow-green, orange-red and red fluorescence represents normal, early apoptotic, late apoptotic and necrotic cells, respectively. (B) The graph represents the percentage early and late apoptotic stage A549 cells after astragalin exposure. The number of both early as well as late apoptotic cells increased with increase in drug concentration as revealed. Statistical analysis was carried out through one-way ANOVA followed by Student's t-test between two groups or by Dunnett's multiple comparison test against the control group; * and ** represents $p<0.05$, and $p<0.01$. Individual experiments were carried out in triplicates.

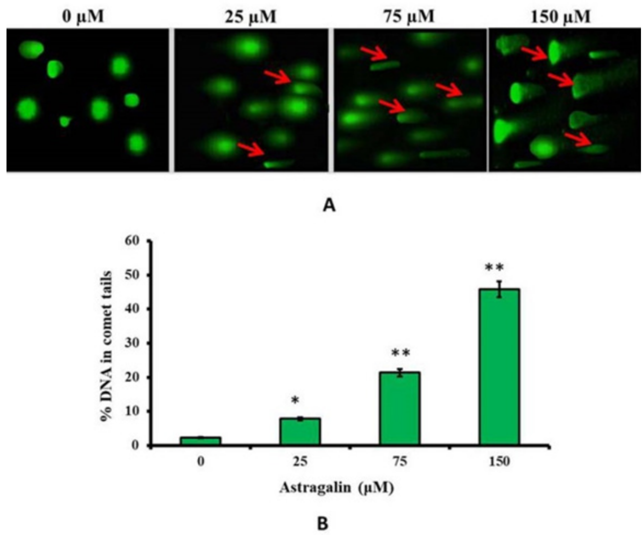


**Figure 3.** (A) The extent of DNA damage caused by astragalin drug exposure to A549 cells was evaluated by comet assay. The tail lengths of comets formed are directly proportional to the damage done to cellular DNA. (B) The graph represents the percentage DNA present in comet tail. It was observed that with increasing doses of astragalin drug more DNA damage was induced in A549 cells. Statistical analysis was carried out through one-way ANOVA followed by Student's t-test between two groups or by Dunnett's multiple comparison test against the control group; * and ** represents $p<0.05$, and $p<0.01$. Individual experiments were carried out in triplicates.

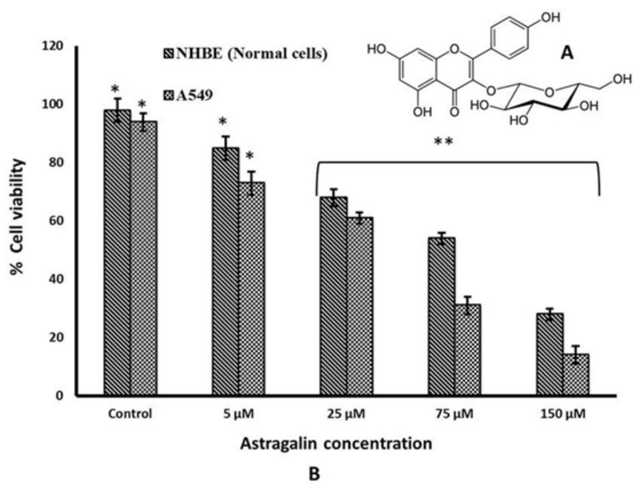


**Figure 1.** (A) Chemical Structure of astragalin molecule. (B) Cytotoxicity of astragalin molecule evaluated against cancerous A549 and normal NHBE lung cells using MTT assay. After the completion of astragalin treatment for 24 h, both cell lines were stained with MTT and viability assessments were made using a microplate reader. The graph displays the decreasing viability of astragalin treated-A549 cells in comparison to normal NHBE cells and controls. Statistical analysis was carried out through one-way ANOVA followed by Student's t-test between two groups or by Dunnett's multiple comparison test against the control group; * and ** represents $p<0.05$, and $p<0.01$. Individual experiments were carried out in triplicates.

(Fig. 3A). Thus, suggesting a generous accumulation of DNA fragments in comet tails due astragalin treatment. The percentage DNA in comet tails was found to be almost 0% in controls while it enhanced to almost 45% in astragalin treated group (Fig 3B).

### Effects of astragalin drug on caspase activity and intrinsic proapoptotic proteins

The intrinsic apoptosis pathway initiates on the release of cytochrome-C into cytoplasm were it goes through several reaction cascades that ultimately result in apoptosis. Caspases play crucial role in deciding the fate of intrinsic apoptosis. Caspase-9 is a member of apoptosis initiator caspases which results in the cleavage and activation of caspase-3 (an executioner caspase) and complex formation with cytochrome-C in cytoplasm. Herein, astragalin was reported to induce remarkable modulatory effects against initiator and executioner caspases (-3 and -9), proapoptotic Bax and Bak, antiapoptotic Bcl-2, Bcl-xL and XIAP and Cyt-C activity. After the astragalin treatment A549 cells the expressions of caspases (-3 and -9), cleaved caspase-3, Bax, Bak and Cyt-C were all observed to increase while the expressions of Bcl-2, Bcl-xL and XIAP all decreased in concentration-dependent manner (Fig 4). Therefore, it was concluded that astragalin could induce cytotoxic effects in A549 cells via mediation of caspase-dependent intrinsic apoptosis.

**Effects on ROS production by astragalin drug in A549 cells**

The effects of astragalin drug on intracellular ROS production were monitored by DCFH2-DA fluorescent dye using fluorescence microscopy. Results showed enhanced fluorescence intensity in treated groups than in control groups indicative of amplifying ROS production by astragalin drug (Fig 5A). The ROS production in astragalin-treated A549 cells enhanced from nearly 5% to about 55% (0-150 µM) (Fig 5B).

**Effects on cell migration and invasion of A549 cells by astragalin**

Cell migration and invasion in A549 cells after subjecting to astragalin drug were evaluated using transwell chambers assay. It was found that astragalin inhibited the cell migration in A549 cells in a concentration-dependent manner (Fig 6A). The number of migrated cells decreased from about 275 cells to only 50 cells on enhancing the astragalin doses from 0-150 µM (Fig 6B). The percentage of invasive A549 cells also decreased on the application of astragalin drug. The blue stained cells in Fig. 7A represent the invasive cells and their number is observed to decrease with increase in drug concentration. The number of invaded cells diminished from 350 cells to almost 50 cells (Fig 7B).

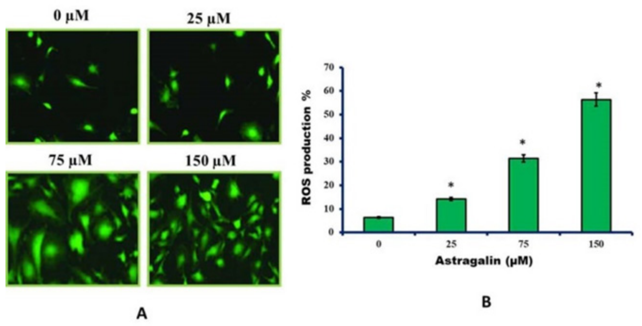


**Figure 5.** (A) The ROS production in astragalin treated A549 cells were assessed using fluorescence microscopy. It was observed that ROS production enhanced remarkably on astragalin drug exposure as indicated. (B) The graph represents increasing ROS percentage with increasing doses of astragalin drug. Statistical analysis was carried out through one-way ANOVA followed by Student's t-test between two groups or by Dunnett's multiple comparison test against the control group; * and ** represents $p<0.05$, and $p<0.01$. Individual experiments were carried out in triplicates.

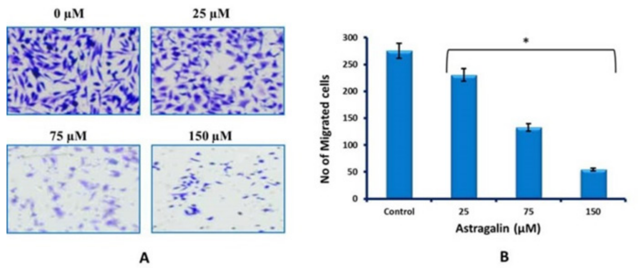


**Figure 6.** (A) Transwell migration analysis indicating decreasing number of migrated A549 cells after astragalin exposure. (B) The graph represents the extent of decreasing migratory efficacy of A549 cells at indicated doses astragalin. Statistical analysis was carried out through one-way ANOVA followed by Student's t-test between two groups or by Dunnett's multiple comparison test against the control group; * and ** represents $p<0.05$, and $p<0.01$. Individual experiments were carried out in triplicates.

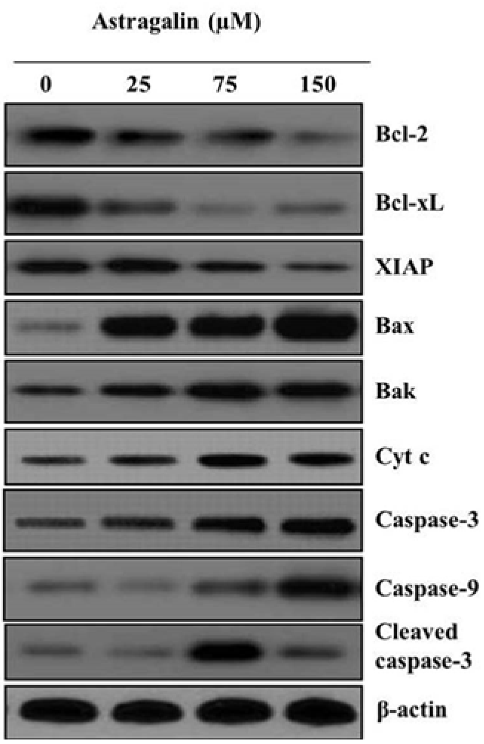


**Figure 4.** The expressions of proapoptotic proteins including Bax, Bak, caspases (-3 and 9) and cyt-C and antiapoptotic BCL-xL, BCL-2 and XIAP proteins were determined using western blotting. It was observed that expressions of proapoptotic proteins enhanced and antiapoptotic proteins reduced on astragalin exposure. Individual experiments were carried out in triplicates.

**Effects on JAK/STAT pathway in A549 cells by astragalin**

Furthermore, the effects of astragalin drug on JAK/STAT pathway in A549 cells were studied by western blotting. The astragalin drug was found to inhibit the phosphorylation of JAK2, STAT1 and STAT3 proteins in A549 cells. The expressions of JAK1, p-JAK1, STAT1, p-STAT1, STAT3 and p-STAT3 proteins were all reduced remarkably in A549 cells by astragalin drug (Fig 8). This indicated that astragalin drug targeted JAK/STAT pathway and blocked its expressions in A549 cells.

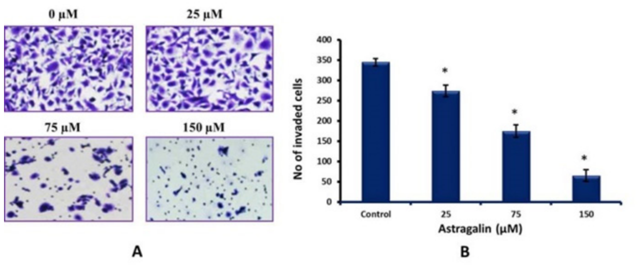


**Figure 7.** (A) Transwell invasion analysis indicating decreasing number of invasive A549 cells after astragalin exposure. (B) The graph represents the extent of decreasing invasive efficacy of A549 cells at indicated doses astragalin. Statistical analysis was carried out through one-way ANOVA followed by Student's t-test between two groups or by Dunnett's multiple comparison test against the control group; * and ** represents $p < 0.05$, and $p < 0.01$. Individual experiments were carried out in triplicates.

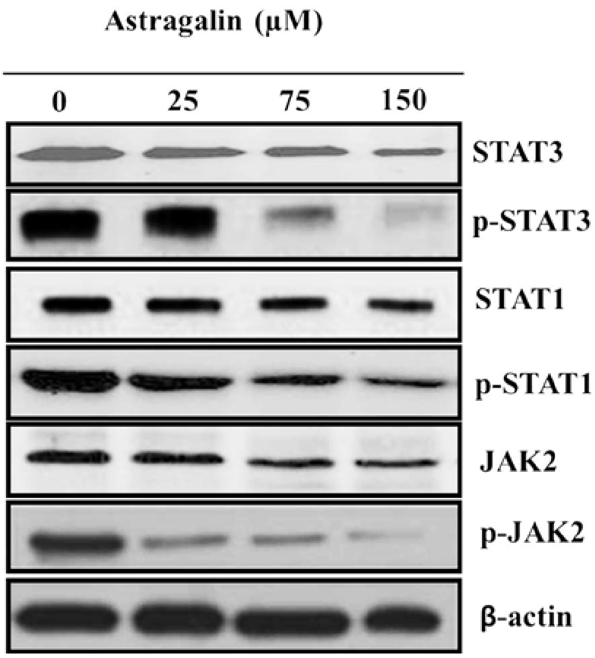


**Figure 8.** Western blotting analysis was used to study the effects of astragalin drug on JAK/STAT signalling pathway. Results suggesting that astragalin drug inhibited the phosphorylation of JAK1, STAT1 and STAT3 proteins as well as inhibited their expressions in A549 cells.

## Discussion

The occurrence of lung cancer is amplifying with an alarming pace throughout the world. The non-small cell lung carcinoma (NSCLC) is a major subtype of lung cancer prevailing in 85% of total lung cancer patients (19). The clinical results of presently accessible treatment modalities are very poor due to the flaws in chemotherapy, frequent relapse of the disease and diagnosis at advanced stages. Unfortunately, the appearance of drug-resistance in cancer cells makes it more problematic to manage lung cancer. Herein, the present investigation was undertaken to estimate anticancer effects of naturally occurring astragalin flavonoid against lung cancer.

Previous investigations have reported outstanding anticancer potency of astragalin drug against different human cancer cell lines including SK-MEL-2, A375P, and HaCaT skin cancer cells, HepG2, H22 and Huh-7 hepatocellular cancer cells and HL-60 leukemia cells (16,20,21). Astragalin induced anticancer effects were found to arbitrate via promotion of apoptosis and targeting of different survival signalling pathways. In a similar study, astragalin has been shown to inhibit proliferation of NSCLC cells both *in vitro* and *in vivo*. It was reported that astragalin inhibited MAPK/PI3K/Akt signalling, reduced TNFα-induced nuclear translocation of NF-κB, modulated Bax/Bcl-2 ratio and activated caspase cascade (22). Herein, astragalin induced significant proliferation inhibitory and dose-dependent effects against NSCLC A549 cells.

Apoptosis is one of the key cellular processes regulating cell death through internal (intrinsic) as well as external (extrinsic) signals (6,23). The intrinsic pathway involves functional outcome of proapoptotic signalling is release of cytochrome-c into cytoplasm via perturbation in mitochondrial membrane potential. Cytochrome-c presence in cytoplasm undergoes complex formation with caspase-9 (initiator) and protease activating factor 1 (APAF1). Apoptosome helps in cleavage and activation of caspase-9 via hydrolyzing the adenosine triphosphate. The activation of caspase-9 then activates executioner caspase-3 or -6 or -7 through cleavage, which ultimately results in cellular apoptosis (24). It serves as a major target and often primary target for chemopreventive drugs (25). Astragalin has been previously recognized with remarkable proapoptotic effects against a wide range of cancer cells. It possesses a higher tendency to alter expressions of key apoptotic regulatory proteins in cancer cells including caspases (-3, -8 and -9), LC3A/B, cytochrome-C, IgE, Beclin-1, Bax, Bcl-2 and Bcl-xL (22). Herein, astragalin showed remarkable proapoptotic effects against A549 cells. The number of apoptotic and necrotic cells were seen enhancing in treated groups. Astragalin induced modulatory effects on the expressions levels of caspase-3 (up), caspase-8 (up), caspase-9 (up), Bax (up), Bad (up), Cyt-c (up), XIAP (down) Bcl-2 (down) and Bcl-xL (down). Further, comet assay results revealed DNA damage to a higher extent in astragalin treated A549 cells. Thus, these results were suggestive that antiproliferative effects of astragalin drug against A549 cells could mediate via its proapoptotic potency.

Further, astragalin drug enhanced the ROS production in A549 cells as showed by the results of fluorescence microscopy. The astragalin molecule has been previously reported of potential modulatory effects on ROS production. Astragalin enhanced the production of ROS in Nrf2 knocked down BEAS-2B cells (26).

Cell migration and invasion are the two key mechanisms in cancer metastasis. Metastasis involves the migration of cells from primary tumor cite to a distant cite where it invades and establishes new cancer colonies. Herein, the astragalin drug showed remarkable suppression on cell migration and invasion propensity of NSCLC A549 cells in a concentration-reliant-manner.

The JAK-STAT pathway is an important signalling transduction pathway involved in cell survival, proliferation, differentiation and embryological processes (27,28). Anomalous activation of this pathway due different acquired genetic polymorphisms, mutations or amplifications leads to persistent or constitutive stimulation of the pathway and hence affects the development of certain cancers (29). Burmistrova *et al.*

have reported that astragalin derivative promoted cell death in human leukemia cells via abrogation of free radical scavenging, suppression of extracellular signal-regulated kinases (ERKs) 1/2 and c-jun NH2-terminal kinases/stress activated protein kinases (JNK/SAPK) signaling, downregulation of Bcl-xL and discharge of cytochrome c (16). Furthermore, astragalin has been shown to suppress MAPK/PI3K/Akt signalling in lung cancer cells (22). Herein, effects of astragalin on JAK/STAT signalling pathway were evaluated for the first time against lung cancer cells. Our findings showed that astragalin induced significant effects on JAK/STAT signalling pathway and blocked its expressions through phosphorylation inhibition of JAK1, STAT1 and STAT3. Provided further in vitro and in vivo studies are carried out, astragalin molecule can be a potential lead molecule in clinical applications to treat lung cancer patients. For this purpose, toxicology studies involving human subjects need to be performed.

Taking altogether, this investigation indicated that astragalin drug holds remarkable anticancer affinity against lung cancer. The results suggested that astragalin induced caspase-dependent intrinsic apoptosis, enhanced ROS generation, inhibition of cell migration and invasion and blocking of JAK/STAT signalling pathway. These results collectively indicate that astragalin drug could prove a lead molecule in lung cancer drug discovery and treatment.

## Conflict of interest

The authors declare that there is no conflict of interest to indicate.

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