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RESEARCH ARTICLE

In Vitro and *In Vivo* Studies Demonstrate Anticancer Property of Root Extract of *Polygala senega*

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Abstract

Polygala senega is extensively used in traditional systems of medicine against various lung diseases including cancer. In the present study we tested the anticancer potentials of ethanolic extract of roots of *P. senega* (generally used as a homeopathic drug) in a mammalian model, where mice, *in vivo*, were treated chronically with benzo[a] pyrene and *in vitro* where lung adenocarcinoma cell line (A549) were used. We deployed various parameters like cell viability assay, chromatin condensation studies with Hoechst 333258 staining, and maintained suitable controls. To understand the possible signal transduction pathways, expression of various signal proteins such as Aryl Hydrocarbon receptor (AhR), cytochrome P450 (CYP1A1), Bcl-2, proliferating cell nuclear antigen (PCNA), Bax and Caspase-3 was studied. Additionally, reverse transcriptase polymerase chain reaction analysis of AhR, p53, PCNA and β-actin (housekeeping) genes was made. Immunohistochemical localization of PCNA proteins was also conducted *in vivo*. Feeding of root extract of *P. senega* to mice (at the rate of 50 mg/kg and 100 mg/kg bw) chronically treated with the carcinogen (50 mg/kg bw dissolved in olive oil) showed positive modulation in expression of signal proteins. Upregulation of apoptotic signals such as p53, Caspase-3 and Bax, and downregulation of AhR, cytochrome P450 (CYP1A1), Bcl-2 and PCNA were observed. Addition of root extract of *Polygala Senega* (at doses of 50 μg and 100 μg) into culture medium containing A549 cells induced recovery of decreased cell viability and increased chromatin fragmentation (apoptosis). Therefore, results of both *in vivo* and *in vitro* studies scientifically validate its potential use as an anticancer agent, particularly against lung cancer, and provide important information potentially helpful in drug designing.

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1. Introduction

Lung cancer is the most common cause of cancer-related death in both men and women, accounting for 29% of all cancer deaths [1]. It is characterized by its poor prognosis and resistance to the apoptosis activity of antineoplastic drugs both *in vivo and in vitro* [2,3]. In light of the very poor survival rates of lung cancer patients, new therapeutic approaches are therefore necessary [4].

At present, surgery and chemotherapy are the main modes of treatment for most cancers but in case of lung cancer treatment outcome by these procedures is limited due to frequent recurrences. Therefore current approaches for control of lung cancer focus mainly on prevention. An alternative way of practical approach could be through chemoprevention by intake of selected food items and beverages. Some plants, vegetables, herbs and spices used in folk and traditional medicines have become subjects of great interest for investigation to see if they really possess anticancer properties that can be scientifically validated and recommended for possible therapeutic use against cancer [5]. *Polygala senega* is used in western herbal medicine as an expectorant to treat cough, sore throat, bronchitis and asthma [6,7]. The antihypoglycemic effect of *P. senega* was investigated in normal and KK-Ay mice [8]. The saponins of *P. senega* are used as vaccine adjuvants to increase specific immune responses [9]. *P. senega* also possesses anti-inflammatory properties when tested in a RAW 264.7 macrophage cell system [10], but its efficacy as an anticancer agent against lung cancer has not been tested. However, we felt that more controlled *in vivo* as well as *in vitro* studies are warranted to verify its reported anticancer effects in model biological systems, which had not been done earlier. Further, it is also necessary to focus on its mechanism of action, if it is found to have anticancer effect.

In the present study, the hypotheses tested were to see (1) if root extract of *P. senega* could induce cytotoxicity and cause cell death in normal primary culture of lung cells of mice; (2) if it did, what could be the safe dose that does not cause any cytotoxicity in normal lung cells; (3) if the treatment of *P. senega* extract could cause cytotoxicity and chromatin fragmentation (apoptosis) in cultured adenoma cell line A549; (4) if it did, what could be the optimum dose that caused the maximum effect; (5) if tested positive in *in vitro* studies, whether extract of *P. senega* can also be effective *in vivo*, in mice chronically treated with benzo[a]pyrene to develop hyperplasia in lung; (6) whether signal transduction pathways can be modulated; and (7) whether modulation in regulatory signal proteins is also reflected in DNA ladder.

Both *in vitro* and *in vivo* models were chosen in the present study; experiments were so designed to ascertain if root extract of *P. senega* has anticancer properties that could be of therapeutic value as a chemopreventive nontoxic drug.

2. Materials and Methods

2.1. Reagents

Benzo[a]pyrene was purchased from Sigma-Aldrich Inc. (St-Louis, MO, USA); Aryl hydrocarbon receptor (AhR) and proliferating cell nuclear antigen (PCNA) mouse monoclonal antibody (primary antibody) were purchased from Abcam Inc. (Cambridge, MA, USA). Caspase-3 and bax (Primary antibody) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Cytochrome P450 (CYP1A1), p53, Bcl-2 (primary antibody) and anti-mouse (secondary antibody) were purchased from Santa Cruz Biotechnology Inc. (San Francisco, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, streptomycin, neomycin (PSN) antibiotic, trypsin and ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were obtained from BD Biosciences. All organic solvents used were of high performance liquid chromatography grade. MTT [3-(4,5-Dimethyl-thiazol-2-yl)-2, S-diphenyltetrazolium bromide] and all other chemicals used were purchased from Sigma-Aldrich Inc.

2.2. Preparation of crude extract

The extract of root of *P. senega* was procured from Schwabe GmbH, Karlsruhe, Germany. During the process of preparation, dried plant roots were macerated with 48% alcohol and filtered. Filtrate was evaporated to a thick residue at 50ºC. The yield of the extract was about 60%. The extract was re-suspended in 48% alcohol and used for in *vitro* and *vivo* experiments.

2.3. Cell culture

The cell line A549 (human nonsmall lung carcinoma) cells were obtained from National Centre for Cell Science, Pune, India. Cells were cultured in DMEM supplemented with 10% fetal bovine serum, and 1% PSN antibiotic at 37ºC in a humidified incubator with 5% CO_2. Cells were harvested with 0.025% trypsin and 0.52 mM EDTA in phosphate buffered saline and plated at required cell numbers and allowed to adhere for 24 hours before treatment. Cultures of $0.5–1.0 \times 10^5$ cells/mL were used for the experiments.

2.4. Dose for cultured cells

Cells (A549 and isolated lung) were treated with different doses of root extract of *P. senega* (50 μg, 100 μg, 200 μg and 400 μg) for different periods of time, that is, 24 hours, 48 hours and 72 hours.

2.5. A549 cell culture

The human lung A549 cells were obtained from the National Centre for Cell Science, Pune, India. A549 cells from one vial (containing approximately 10^5 cells) were thawed rapidly by immersing in a 37°C water bath. The cells were transferred to a 15 mL centrifuge tube containing 10 mL DMEM and re-suspended by gentle aspiration with a pipette. After centrifugation for 10 minutes at 1000 rpm, the supernatant was removed and the cells were re-suspended in complete medium supplemented with 10% heat-inactivated fetal calf serum, 1% nonessential amino acids with a pH of 7.4. The well-grown cells were harvested and seeded into 96-well plates at a density of 2×10^5 cells/mL for experiments.

2.6. Isolation and primary culture of lung cell

Lung was removed from mice aseptically followed by addition of collagenase. Single cell suspensions were made in DMEM by passing the cell population through a nylon mesh with 50-μm pore size. After the preparation of single cell suspension by passing through nylon mesh, the cell suspension was incubated at 37°C. Then MTT assay was performed.

2.7. MTT assay

Cell viability was assessed using MTT assay as described by Mosmann [11]. A549 and isolated lung cells (10^5 cells/mL) were cultured for 24 hours on 96-well microplates. After that cells were incubated for 72 hours with or without the drug. The control received no drug. Absorbance was measured at 595 nm by enzyme-linked immunoabsorbant assay. Cell survival rate was calculated as the percentage of MTT inhibition as follows: percentage of survival = (mean experimental absorbance/mean control absorbance) × 100%.

2.8. Hoechst 33258 staining

Apoptotic nuclear morphology was assessed using Hoechst 33258 staining as described by Chen et al. [12] with slight modification. Hoechst 333258 (1 μg/mL) was added for 5 minutes, and cells were examined by fluorescence microscope (Axiscope plus 2; Carl Zeiss, Oberkochen, Germany).

2.9. DNA ladder analysis

For DNA laddering assay, cells were harvested into extraction buffer (10 mM Tris-HCL pH 7.4, containing 10 mM NaCl, 20 mM EDTA and 1% Triton X-100). Genomic DNA was isolated by digesting the cell extract with 10 μg/mL of proteinase K at 56°C for 8–12 hours. DNA was purified by phenol/chloroform precipitated with ethanol and dissolved in Tris-EDTA. Integrity of DNA was analyzed by gel electrophoresis on 1% agarose gels followed by ethidium bromide staining.

2.10. Feeding procedure for mice

Each mouse was fed 100 μL of either root extract of *P. senega* or double distilled water with the aid of a fine pipette as per requirement of a particular series.

2.11. Animals

Healthy Swiss albino mice weighing 20–25 g (8–10 weeks old) were reared in the animal house of the Department of Zoology, University of Kalyani. The animals were kept in plastic cages under hygienic conditions and were provided standard animal feed. Necessary approvals were obtained from the Institutional Ethical Committee.

2.12. Experimental protocol

The experimental animals were divided into five groups, each group comprising six animals:

Group 1 served as normal olive oil control. Group 2 animals were administered with benzo[a]pyrene (50 mg/kg bw dissolved in olive oil, orally) twice a week for 4 successive weeks to induce lung cancer by 16th week [13]. Group 3 was fed benzo[a]pyrene as in group 2 and 48% alcohol as the drug (root extract of *P. senega*) was dissolved in 48% alcohol; this served as the alcohol control. Group 4 animals were fed 50 mg/kg bw of root extract of *P. senega* 1 day before the first dose of benzo[a]pyrene administration and continued for 16 weeks. The treatment was used to study the chemopreventive effects of root extract of *P. senega*, if any, in the experimental animals. Group 5 animals were treated with 100 mg/kg bw of extract for 16 weeks as in group 4.

2.13. Immunohistochemistry of PCNA

After having sacrificed the mice from all groups, lungs from each mouse were collected, washed in phosphate buffered saline and soaked in blotting paper to remove the blood. Tissues were then fixed in 10% neutral buffered formalin for 24 hours. The tissue

samples were dehydrated in ascending concentrations of ethanol, cleared in xylene, and embedded in paraffin to prepare the block. The 6 μm serial sections were used in such a way that the corresponding sections were used for immunohistological evaluation. Immunohistochemistry was performed following the methods used by Ramakrishnan et al [14].

2.14. Protein extraction and western blot analysis

Protein was isolated for the analysis by western blot. For protein isolation, lungs were collected from all groups, homogenized in lysis buffer [15]. The homogenized material was centrifuged at 13,000*g* for 15 minutes at 4°C; the supernatant known as cytoplasmic extract was removed. This cytosolic supernatant was analyzed with western blot and the amount of protein was quantified with the Lowry method. Western blot analysis was performed as described previously [16]. For quantitative analysis of each band, density was determined using Gel Doc System (UltraLum Inc., Claremont, CA, USA).

2.15. Real-time polymerase chain reaction (RT-PCR) for the detection of mRNA expression of different genes

Total RNA was extracted from the lung tissue using Trizol reagent according to the manufacturer's instructions (Bangalore GeNei P Ltd., Karnataka, India) Following PCR, 5-μL sample aliquots were subjected to electrophoresis on 1% (w/v) agarose gel [17] for 20–30 min and then stained with ethidium bromide and photographed. The primer sequences of amplified genes are shown in Table 1. Densitometry was performed using Total Lab software (TotalLab Ltd., Newcastle, UK). β-actin mRNA was amplified as loading control.

2.16. Statistical analysis

Statistical analysis of the data was performed by student *t* test. A $p<0.05$ was considered significant.

Table 1 Primer sequences of cancer-related genes used

Primer name	Primer sequences
β-actin*	Catalogue number-117816,GeNei
AhR	F: AAGCATGCAGAACGAGGAGT
	R: CCAACCCTCACAGTTCTGGT
PCNA*	Catalogue number-117813,GeNei
p53*	Catalogue number-117810,GeNei

*Purchased from Bangalore GeNei P Ltd. AhR = Aryl hydrocarbon receptor; PCNA = proliferating cell nuclear antigen.

For all quantitative analyses, data were expressed as mean values obtained from triplicate experiments (on blot and immunohistochemistry studies) showing SE and levels of significance.

3. Results

3.1. Cell-viability studies in primary mice lung cells

In order to investigate the cell cytotoxicity in long-term-cultured lung cells, medium described above was supplemented with various doses of root extract of *P. senega*. After 24 hours, 48 hours and 72 hours of incubation at various doses as shown in Table 2, a dose-dependent cell viability by MTT assay were also found in cultured primary mice lung cells, where cells viability dropped slightly with increasing doses of *P. senega* extract.

3.2. Cell-viability studies in A549 cells

After about 24 hours of culture when cells reached 60–70% confluence, unattached cells were removed by gentle agitation and the medium was changed to medium containing various doses of root extract of *P. senega* (50 μg, 100 μg, 200 μg and 400 μg) or vehicle (alcohol) for control. As shown in Table 2, the addition of root extract of *P. senega* to A549 cell culture resulted in dose-dependent cell viability after 24 hours, 48 hours and 72 hours of exposure. Although no apparent cytotoxic effect on cell viability was observed at lower doses (50 μg, 100 μg and 200 μg), at higher dose (400 μg), root extract of *P. senega* was more toxic to A549 cells than to mice lung cells (normal) with respect to time.

3.3. Hoechst 333258 staining

To examine the biological findings of apoptosis in A549 cells induced by root extract of *P. senega*, Hoechst staining was performed. As shown from Hoechst staining, some nuclei of the root extract treated cells (Figure 1) exhibited typical features of apoptosis such as nuclear condensation.

3.4. DNA damage by DNA laddering

DNA fragmentation data as revealed from the DNA ladders in agarose gel electrophoresis are presented in Figure 2. As compared to the control, the fragmentation in 50 μg, 100 μg and 200 μg treated cells appeared to be more smeared, indicating greater DNA fragmentation suggestive of greater degree of apoptosis.

Table 2 Effect of different doses of root extract of *Polygala senega* on cell viability of isolated lung cells and A549 cells by MTT assay in a dose-dependent and time-dependent manner

Cell viability of isolated lung cells by MTT assay		
Series	24-hr	48-hr	72-hr
Control	100 ± 0.00	100 ± 0.00	100 ± 0.00
Control+alcohol	94.66 ± 1.20	90.0 ± 1.73	88.33 ± 0.882
50 µg	$91.0\pm1.73^*$	$87.33\pm1.85^*$	$80.6\pm2.7^*$
100 µg	$85.0\pm2.1^†$	$83.0\pm1.52^†$	$74.66\pm2.4^‡$
200 µg	$84.0\pm1.52^‡$	$79.0\pm1.8^†$	$70.66\pm1.76^§$
400 µg	$82.0\pm1.15^‡$	$72.66\pm1.45^‡$	$50.33\pm1.5^§$
Cell viability of A549 cells by MTT assay		
Series	24-hr	48-hr	72-hr
Control	100 ± 0.00	100 ± 0.00	100 ± 0.00
Control+alcohol	97.0 ± 2.1	95.33 ± 1.85	93.66 ± 0.882
50 µg	$81.0\pm2.8^†$	$70.66\pm2.8^‡$	$65.0\pm1.15^§$
100 µg	$75.33\pm2.4^‡$	$63.33\pm1.5^§$	$47.33\pm1.45^‡$
200 µg	$70.33\pm2.5^‡$	$52.0\pm1.73^§$	$39.66\pm3.18^§$
400 µg	$50.33\pm2.5^§$	$37.33\pm1.45^§$	$18.33\pm2.5^§$

*Not significant; $^†p<0.05$; $^‡p<0.01$; $^§p<0.001$, significance represents significant difference from alcohol control treated series.
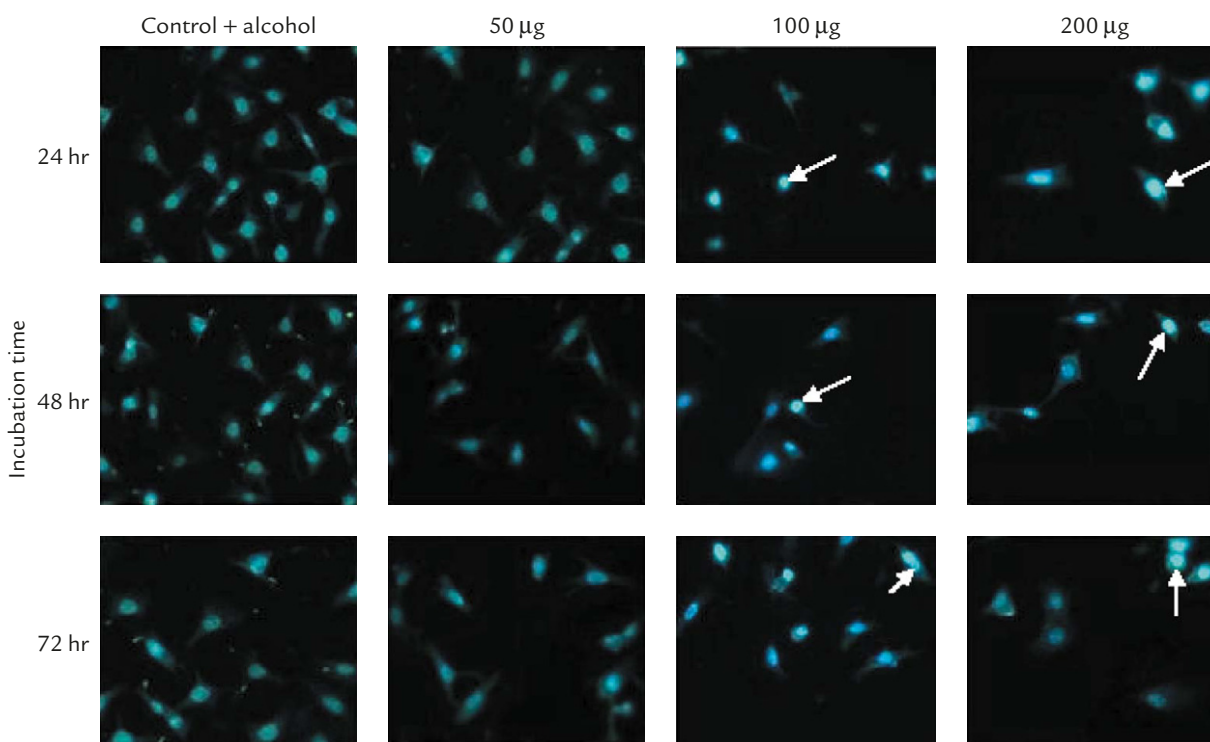

Figure 1 Fluorescence photomicrographs of A549 cells with Hoechst 33258 staining. The assessment of nuclear morphology of the cell was performed after A549 cells were incubated for 24 hours, 48 hours and 72 hours with vehicle (alcohol) and with root extract of *Polygala senega* (50 µg, 100 µg and 200 µg). Arrows indicate nuclear fragmentation.

3.5. Immunohistochemical analysis of PCNA protein on lung

Our next attempt was to determine the impact of root extract of *P. senega* on the expression of genes associated with cell proliferation. Immunohistochemical staining for PCNA in the lung of control and experimental group of animals (Figure 3). Benzo[a] pyrene and alcohol fed group 3 animals showed a significant increase in the number on PCNA positive

Lane 1   2   3   4   5
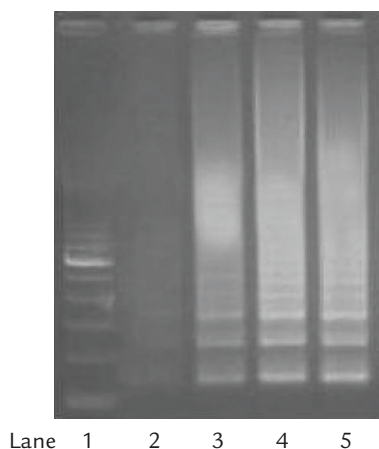
Figure 2 Effect of root extract of *Polygala senega* on A549. A549 cells were treated with different doses of drugs for 24 hours. Genomic DNA was extracted and DNA was separated on 1% agarose gel and stained with ethidium bromide. Lane 1 contains a standard DNA ladder, lane 2 contains DNA from untreated cells, lane 3 contains DNA from drug (50 μg), lane 4 contains DNA from drug (100 μg) and lane 5 from drug-treated (200 μg) cells.

nuclei when compared with group 1 normal control animals, while root extract of *P. senega* treatment significantly decreased the number of PCNA positive nuclei when compared with benzo[a]pyrene and alcohol fed animals.

3.6. Expression of AhR and CYP1A1 on lung cancer

AhR and CYP1A1 gene expression were measured in the lungs of mice after induction lung cancer by 16th week. The expression level of AhR and CYP1A1 was found to be downregulated in the drug-treated group, whereas expression of AhR and CYP1A1 protein from lung tissues of tumor-bearing mice (benzo[a] pyrene fed) are upregulated (Figure 4A).

3.7. Effect of root extract of *P. senega* on expression of proapoptotic and antiapoptotic proteins

After confirming that root extract of *P. senega* induces apoptosis during lung carcinogenesis, our next attempt was to determine the impact of the extract on the expression of genes associated with apoptosis. As it is well established that various proapoptotic and antiapoptotic proteins play a crucial role in programmed cell death, we examined whether or not the root extract of *P. senega* can affect the expression of proapoptotic protein, Bax as well as antiapoptotic protein, Bcl-2 in our mice model. Interestingly, it was observed that the extract could elevate the expression of Bax in the

benzo[a]pyrene-fed mice and as shown in Figure 4A. Bcl-2 expression was found to be downregulated by the root extract at the same stage of carcinogenesis (on the 16th weeks).

3.8. Effect of root extract of *P. senega* on Caspase-3 expression

After assessing that root extract of *P. senega* increased the Bax expression, our next interest was to see whether it increased the levels of Caspase-3 expression via upregulation of Bax expression or not. Interestingly it was found that it increased Caspase-3 expression on the 16th weeks (Figure 4A).

3.9. Gene expression by root extract of *P. senega* in benzo[a]pyrene treated mice

mRNA expression of different gene such as AhR, p53 and PCNA, have been shown in (Figure 4B). The data also normalized with β-actin. The expression of AhR and PCNA have been downregulated by the root extract at a dosage of 50 mg/kg and 100 mg/kg body weight, respectively, whereas the expression level of p53 was upregulated after the drug treatment.

4. Discussion

Cancer chemoprevention has become an important area of cancer research. There is compelling clinical and epidemiological evidence that dietary constituents [18] are associated with reduced risk of cancer including precancerous lesions of the buccal mucosa, second primary tumors in patients treated for squamous cell carcinoma of the head and neck [19], and lung. Experimental studies have revealed that the process of carcinogenesis can be modulated by timely intervention including administration of chemopreventive agent [20] or intake of protective agents through regular food and drinks.

In the present study, the root extract of *P. senega* at doses of 200 μg and 400 μg caused cytotoxicity and cell death in a significant number of normal culture cells. However, lower doses of 50 μg and 100 μg were found to be relatively nontoxic to the lung cells in primary culture, while they significantly suppressed the proliferation of A549 cells in culture. Moreover, the nuclear morphology analyzed quantitatively by Hoechst 33258 suggested that an apoptotic cell death mechanism was also potentially involved in the direct cytotoxicity of the cancer cell line. On the other hand, *in vivo* studies demonstrated that benzo[a]pyrene is a major carcinogenic constituent in tobacco smoke [21] which is metabolically activated by the CYP system to reactive diolepoxides
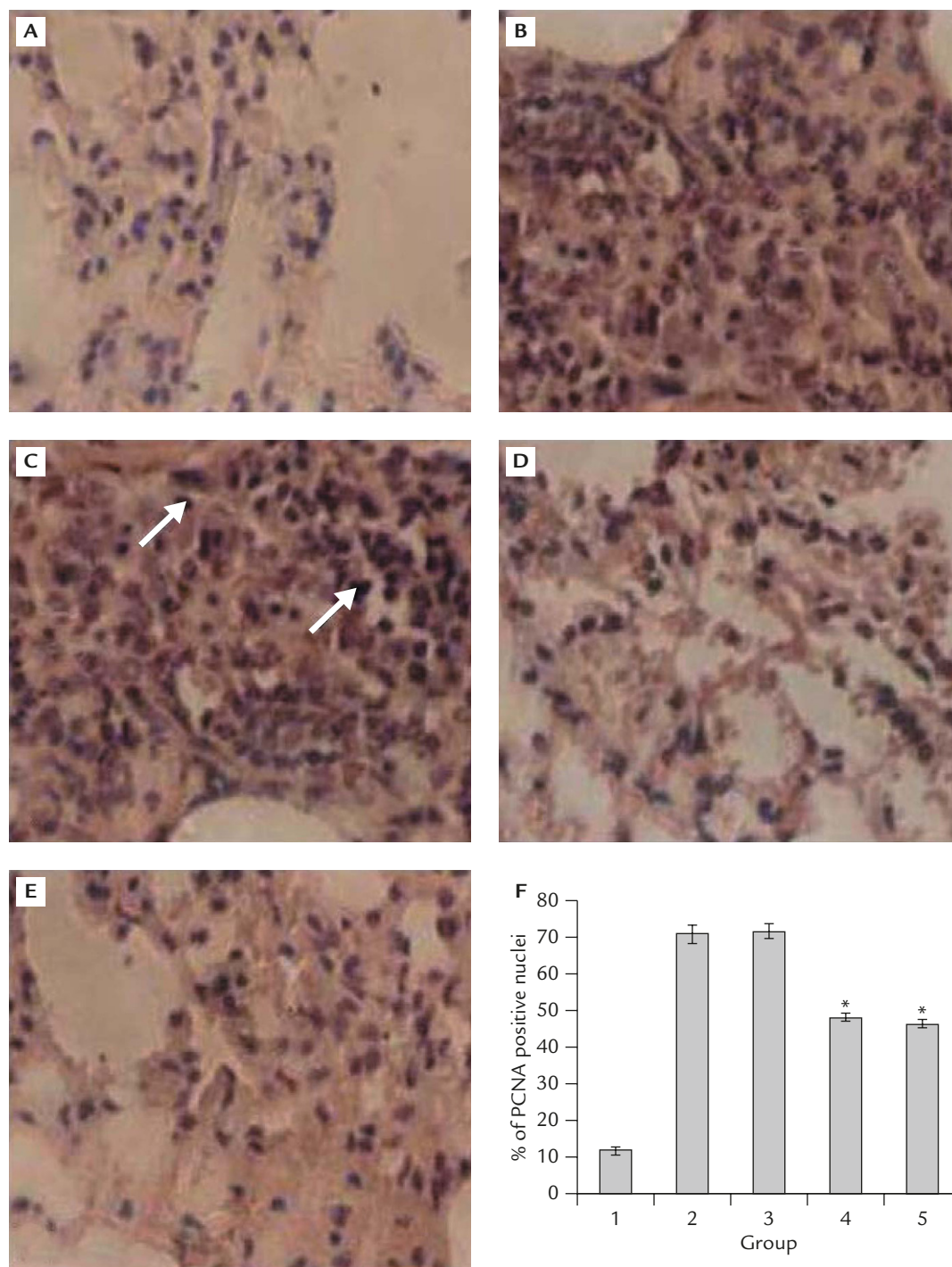
Figure 3 Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) in the lung of control and experimental groups of animals. (A–E) The lung sections of groups 1–5 experimental animals respectively. (F) Histogram representing the percentage of PCNA-positive cells in control and experimental groups. *$p<0.05$. Arrow represents PCNA-positive cells. (A) Group 1: Normal (olive oil); (B) Group 2: benzo[a]pyrene+olive oil; (C) Group 3: benzo[a] pyrene+olive oil+alcohol; (D) Group 4: benzo[a]pyrene+olive oil+50 mg/kg bw of root extract of *Polygala senega*; (E) Group 5: benzo[a]pyrene+olive oil+100 mg/kg bw of root extract of *P. senega*.

that are capable of interacting with DNA or proteins to form adducts. In the lung, CYP1A1 and CYP1B1 are important in the biotransformation of benzo[a] pyrene. In the present investigation, downregulation of CYP1A1 was observed after treatment of the root extract of *P. senega*, suggesting that it inhibited DNA adduct formation.

Results of the current study imply that root extract of *P. senega* interfere with the process of programmed cell death through modulation of caspase-3
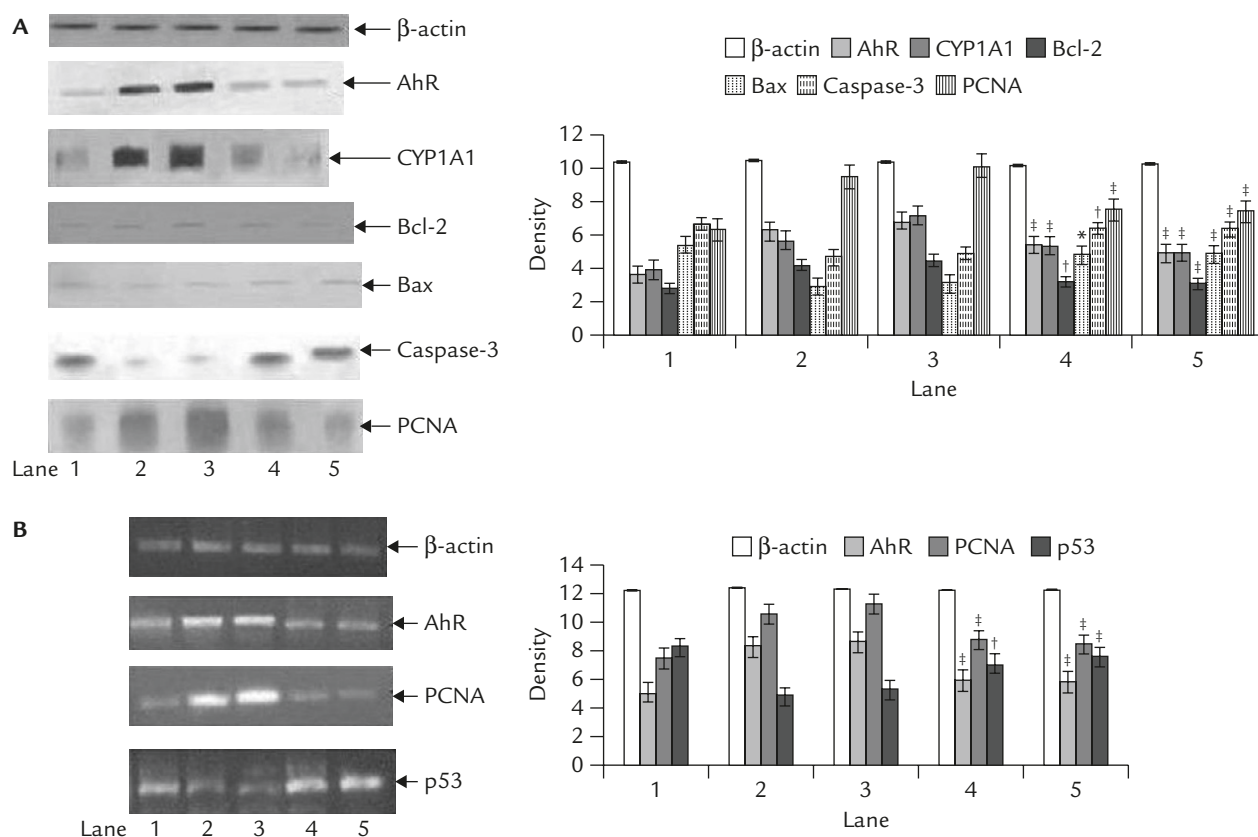
Figure 4 (A) Western blot analysis shows the effect of root extract of *Polygala senega* on the expression of β-actin, Aryl hydrocarbon receptor (AhR), cytochrome P450 (CYP1A1), Bcl-2, Bax, Caspase-3, proliferating cell nuclear antigen (PCNA) during mouse lung carcinogenesis induced by benzo[a]pyrene. mRNA expression of AhR, p53, PCNA with internal control β-actin. $*p<0.05$; $\dagger p<0.01$; $\ddagger p<0.01$. Lane 1: normal; lane 2: benzo[a]pyrene+olive oil; lane 3: benzo[a]pyrene+olive oil+alcohol; lane 4: benzo[a]pyrene+olive oil+50 mg/kg bw of root extract of *P. senega*; lane 5: benzo[a]pyrene+olive oil+100 mg/kg bw of root extract of *P. senega*. Histogram represents the band densities expressed as mean±SE of the three independent experiments.

expression. The expression of p53 protein is connected with cell DNA damage. Derangements of p53 protein expression are the most often observed lesions in the invasive types of lung cancer [22] and seem to play a vital role in the multidegree pathway of cancerous lesion development [23].

Bcl-2 protein has antiapoptotic qualities. Bcl-2 overexpression in atypical adenomatous hyperplasia cells is significant and reaches about 70% [24]. The antiapoptotic activity of Bcl-2 correlates with its intracellular ratio to Bax. It was reported that p53 induces apoptosis by either increasing transcriptional activity of proapoptotic genes such as Bax or suppressing the activity of the antiapoptotic gene of the Bcl-2 family [25]. RT-PCR and Western blot findings revealed that root extract of *P. senega* suppressed AhR mRNA and protein expression levels compared with those of the control. This finding demonstrates the antiapoptotic activity of root extract of *P. senega* associated with the downregulation of AhR expression.

The extract of *P. senega* did not significantly induce toxicity or cause any mortality in the treated normal mice. In animals bearing lung hyperplasia, the significant tumor progression may be due to the uncontrolled proliferation of lung cells. On drug treatment, regression of tumors might have been due direct inhibitory action of the drug on tumor growth. PCNA is a 36 kDa auxiliary protein to DNA polymerase-S, which is found in various concentrations within the cell throughout the cell cycle and in greatest quantities during the S-phase [26]. Decreased expression of PCNA upon treatment of drug revealed the antiproliferative effect of root extract of *P. senega*. This might help prevent cancer cells from proliferation. The principal active constituents of *P. senega* are saponin glycosides polygalic acid and senegin, which make up 5% and 4% of the dried root, respectively [27]. Earlier studies have revealed *P. senega* to have immune system stimulating activity, but not much evidence exists for its anticancer effects [28,29]. Findings of the present study

suggest that they also have considerable anticancer potentials against lung cancer.

The results obtained in the present study demonstrated that root extract of *P. senega* has apoptotic potential on A549 cell *in vitro* and benzo[a]pyrene-induced lung carcinogenesis *in vivo* with minor cytotoxic effects in primary lung cells.

Acknowledgments

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