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# A novel protein fraction from *Sesbania grandiflora* shows potential anticancer and chemopreventive efficacy, *in vitro* and *in vivo*

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## Abstract

We report mechanism-based evidence for the anticancer efficacy of a protein fraction, SF2 (Sesbania fraction 2) isolated from the flower of the medicinal plant, *Sesbania grandiflora* (*S. grandiflora*). The fraction was evaluated in two murine ascites tumour cell lines and human cancer cell lines of different origin for its anticancer effect. SF2 inhibited cell proliferation and induced apoptosis as demonstrated by DNA fragmentation and externalization of phosphatidyl serine in Daltons lymphoma ascites (DLA) and colon cancer cells (SW-480). Sensitivity to SF2 in these cells was associated with activation of caspases 3, 8 and 9, poly (ADP-ribose) polymerase cleavage and cytochrome C release which attests apoptosis induced cell death. Mechanistically, SF2 down-regulated phorbol myristate acetate (PMA) induced NF-κB, a transcription factor which controls the expression of genes encoding proteins involved in cell regulation and growth control. Additionally, SF2 also down-regulated anti-apoptotic factors such as Bcl-2, p-Akt and cyclooxygenase-2 induced by the tumour promoter PMA suggestive of a possible explanation for its anticancer effect. *In vivo* studies using ascites and solid tumour models strongly support *in vitro* findings as SF2 administration increased the life span and decreased the tumour volume in mice bearing tumour. *In vivo* toxicological evaluation revealed the pharmacological safety of SF2 and may serve as a potential anticancer drug candidate.

**Keywords:** *Sesbania grandiflora* • apoptosis • chemoprevention • anticancer

## Introduction

Ethnobotanical knowledge coupled with rationale-driven scientific research may help in identifying potent new drugs against cancer. Here we report the results of a complex protein isolated from *S. grandiflora*, a member of the family Papilionaceae. *S. grandiflora* is a small tree commonly known as 'sesbania' and 'agathi' and is an important source of dietary nutrients in Southeast Asian countries [1]. Some pharmacognostic studies have been reported on the bark of this plant [2]. In the Siddha system of Indian traditional medicine, different parts of this plant have been accredited for alleviating a spectrum of ailments including inflammation, leprosy, gout and rheumatism [3]. The flowers and leaves are enriched with vitamins and minerals and have been reportedly associated with anti-inflammatory, analgesic and antipyretic effects [4, 5]. Additionally, the leaves have demonstrated anxiolytic and anticonvulsive activity in experimental animals [6] and have proven effective against hepatitis [7] and in inhibiting HIV-1 protease activity [8], thus enabling significant protection against such maladies. Recent reports indicate that an aqueous suspension of *S. grandiflora* leaves exhibit protective effects against cigarette smoke-induced oxidative damage in rats [9], whereas alcoholic extracts of the leaves have been documented to provide significant protective effects against hepatotoxicity [10]. Additionally, the anti-urolithiatic and

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antioxidant properties of this plant has been well documented [11, 12]. The flowers of this plant are edible and are consumed as a popular traditional remedy for night blindness, bronchitis, nasal catarrh, headache and frontal sinus pain alleviation [13]. Recently, two proteins, namely SGF60 and SGF90 isolated from the flowers of this plant have been shown to inhibit α-glucosidase, and hence has been speculated as a potential drug against type 2 diabetes [14]. Even though Ayurvedic literatures [15] mention the anti-tumour effect of *S. grandiflora* fruit, there is no mechanism-based evidence in the literature on the anticancer therapeutic potential of *S. grandiflora*. In this study, we report some promising results obtained from a complex protein fraction of the flower against ascites and colon cancer cells characterizing apoptosis by morphological features such as caspase activation, cytochrome C release and poly (ADP-ribose) polymerase (PARP) degradation. We also assessed the potential molecular mechanism in support of its anticancer effect, and additionally present evidence for the first time in line with our *in vitro* observations, the anti-tumour effect of *S. grandiflora* in ascites and solid tumour models.

# Materials and methods

## Reagents and antibodies

Annexin V apoptosis detection kit and antibodies against cytochrome C, Bcl-2, p65, p50 and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-Akt, caspases 3, 8 and 9 and PARP were purchased from Cell Signaling Technology (Beverly, MA, USA) and the fluorimetric substrates for the caspases were obtained from Calbiochem (La Jolla, CA, USA). The rest of the chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA).

## Drug extraction

Fresh flowers of *S. grandiflora* were ground in phosphate buffered saline (PBS) in ice and the extract was filtered using cheese cloth and was further subjected to centrifugation at 2000 rpm for 10 min. at 4°C. The clear supernatant was subjected to protein precipitation using ammonium sulphate saturation and the three fractions (0–40% [SF1], 40–70% [SF2] and 70–100% [SF3]) obtained were centrifuged, dialysed, passed through Sephadex G-25 column and lyophilized as described earlier [16].

## *In vitro* studies

The murine ascites tumour cell lines – DLA and Ehrlich ascites (EAC), the normal murine cell line – murine embryonic fibroblast (MEF), the human lung cancer cell lines – A549 and H1299, the human cervical cancer cell lines – HeLa and ME-180 and the human colon cancer cell lines – SW-480, SW-620 and HT-55 were used for the *in vitro* studies. The cytotoxicity was measured by MTT assay [17]. Inhibition of DNA synthesis and DNA damage was assessed by [$^3$H]thymidine incorporation assay and DNA fragmentation assay, respectively [18]. Annexin V–FITC staining was done according to manufacturer's protocol [17]. The cleavage of caspases 3, 8 and 9 induced was detected by Western blot and assayed spectrofluorimetrically as described earlier [19]. Cleavage of PARP, the inhibition of Akt phosphorylation and down-regulation of Bcl-2 and COX-2 expression were detected by Western blot [19]. Mitochondria-free cytosol was isolated as described earlier [17] and immunoblotted against anti-cytochrome C. NF-κB down-regulation was studied by electrophoretic mobility shift assay (EMSA) [18].

## *In vivo* studies

### Ascites tumour model

Five randomized groups (eight per group) of inbred male *Swiss albino* mice of 9–11 weeks age were used for the study. While group I was used as negative control (PBS injection), group II was initially injected with the drug from second day onwards for 10 alternate days (100 mg/kg body weight in sterile saline, intraperitoneally [i.p.]) and thereafter twice a week for 6 months. These mice were later evaluated to study parameters related to chronic toxicity. Groups III, IV and V received $1 \times 10^6$ DLA cells in 0.1 ml sterile saline i.p. (day 1). Group III animals were designated as positive control. Mice belonging to group IV were treated with the drug from second day onwards for 10 alternate days (50 mg/kg body weight in sterile saline, i.p.) whereas, mice of group V received the same mode of drug treatment, but 7 days later after the tumour cell implantation.

### Solid tumour model

*Swiss albino* mice (male, eight per group) of 9–11 weeks were used for the solid tumour reduction study. Groups II, III and IV were injected with $1 \times 10^6$ DLA cells in 100 μl PBS subcutaneously on the lower hind flank. For group I (control) and III animals, SF2 (50 mg/kg body weight in 100 μl PBS) was injected subcutaneously on the following day and continued for 10 alternate days. A similar course of treatment was given to group IV, but after 7 days of cell injection and was continued for 10 alternate days. Group II animals were kept as the positive control (PBS injection). The solid tumours were measured from day 6 onwards and the volume was calculated as reported previously [20].

## Toxicological analysis

### Chronic toxicity

*Swiss albino* mice ($n$ = 8) were injected (i.p.) every alternate days for the first 10 days with SF2 (100 mg/kg body weight) followed by twice weekly regimen for 6 months. Equal number of control mice received only PBS injection. After 6 months, animals were killed by cervical dislocation and the liver tissue was collected. One portion of the liver was preserved in 10% buffered formalin. Sections were stained with haematoxylin and eosin [21]. Blood collected from the ocular vein was studied for the total and differential counts of white blood cell (WBC) and the haemoglobin content [22]. The activity of serum alkaline phosphatase (ALP) was determined by the method of King and Armstrong [23, 24] and the level of blood urea nitrogen (BUN) was evaluated according to standard protocol [25]. The activity of serum and liver glutamate oxaloacetic transaminase (GOT) and glutamate pyruvic transaminase (GPT) was determined by the Reitman and Frankel method [26].

**Acute toxicity**

To determine acute toxicity, doses of 0 (control), 100, 200, 500 and 700 mg/kg of SF2 was given to groups of six mice each. The mice were observed continuously for 1 hr for any gross behavioural changes and death, and intermittently for the next 6 and 24 hrs after dosing. The behaviour parameters observed were convulsion, hyperactivity, sedation, grooming, food and water intake, etc.

### Statistical analysis

Statistical significance was calculated using one-way ANOVA followed by Tukey *post hoc* analysis ($P < 0.0001$ for *in vitro* studies and $P < 0.001$ for *in vivo* studies).

## Results

### A complex protein fraction, SF2 isolated from *S. grandiflora* induces cytotoxicity, nuclear membrane damage and externalization of phosphatidyl serine in mouse ascites cells

Three protein fractions from *S. grandiflora* flower extract (SF1, SF2 and SF3) were prepared as described under the section 'Materials and methods'. The ascites tumour cell lines, DLA and EAC were exposed to each of the three fractions of *S. grandiflora* and the cell viability was determined by MTT assay after 72 hrs of treatment. Among the three fractions investigated, only SF2 showed growth inhibitory effect, whereas both SF1 and SF3 did not show any effect up to 200 μg/ml in both the cells studied (Fig. 1A). We observed a dose-dependent growth inhibition in both cell lines, when exposed to the SF2 fraction in the range, 1–100 μg /ml. As DLA turned out to be the most sensitive cell line (IC50–8.5 μg/ml), it was selected for further studies (Fig. 1A). In [$^3$H] thymidine incorporation assay, SF2 also inhibited DNA synthesis in DLA cells (Fig. 1B). However, SF2 was non-toxic up to 100 μg/ml towards the normal murine fibroblasts (Fig. 1C). Evidence supporting SF2-induced apoptosis was obtained by staining with annexin V which binds to the externalized phosphatidyl serine which is an early marker of apoptosis. As shown in Fig. 1D, in DLA cells, 22% and 51% of the cells were annexin positive after treatment with 5 and 10 μg/ml of SF2, respectively, for 16 hrs. Some cells also showed typical PI staining (yellowish red) suggesting the appearance of late apoptotic or necrotic cells (Fig. 1D).

### SF2 induces caspase activation, PARP cleavage, DNA fragmentation and cytochrome C release in DLA cells

SF2-induced activation of caspases 3, 8 and 9 in DLA cells was assessed both spectrofluorimetrically and by Western blot (Fig. 2A). Treatment with 10 μg/ml SF2 in DLA cells for 24 hrs produced 4.4-, 2.5- and 2.8-fold increases in enzymatic activity of caspases 3, 8 and 9, respectively, compared to untreated control (Fig. 2A).

DNA fragmentation representing the extent of DNA damage is a hallmark of apoptosis. Figure 2B clearly indicates that 5 and 10 μg/ml SF2 induce DNA fragmentation of DLA cells. Next, we examined cleavage of the DNA repairing protein, PARP, which is a substrate of caspase 3. When DLA cells were treated with 5 or 10 μg/ml SF2, the 116-kD form of PARP was cleaved into the 85-kD forms (Fig. 2C). We also observed a dose-dependent release of cytochrome C from the mitochondria to the cytoplasm, strongly indicating that the apoptosis induced by SF2 is through the mitochondrial pathway (Fig. 2D).

### SF2 reduces Ascites as well as solid tumour development in *Swiss albino* mice

As the *in vitro* studies showed promising results, we conducted an in *vivo* study of SF2 using ascites and solid tumour models as described in the section 'Materials and methods'. In the ascites tumour model, the average lifespan of the positive control mice (group III) was 25 days and developed a visible tumour after 14 days, whereas the animals that were treated with SF2 24 hrs after tumour transplantation (group IV) did not develop any visible tumours for the first 25 days. These animals had an increase in lifespan of 105% compared to the positive control. In the case of group V animals, which were treated with the drug 7 days after tumour transplantation, even though the growth of tumour was delayed, the increase in lifespan was 49% indicating that chemopreventive efficacy of SF2 is much higher than its anti-tumour effect (Fig. 3B). There was no tumour development in the negative control groups (group I) which were kept under observation and monitored for 6 months.

In the solid tumour model, to our surprise, the group III animals, which received the drug 1 day after tumour transplantation, did not develop any tumour during the observation period. However, group IV animals that received the drug, 7 days after tumour transplantation, developed tumours initially, but further growth of tumour was arrested, again indicating the chemopreventive efficacy of the drug (Fig. 3C). A substantial increase in the lifespan (54%) was also noted.

### SF2 does not produce any toxicity in the blood, liver or kidney of Swiss albino mice

To rule out the possibility of any adverse side effects of SF2 we conducted a detailed toxicity study, both chronic and acute. In the chronic toxicity study, we evaluated the haematotoxicity, nephrotoxicity and hepatotoxicity due to the SF2 fraction by analysing the level of the total and differential WBC count (Fig. 4A), serum ALP (Fig. 4B), level of BUN (Fig. 4C) and GOT and GPT profiles in

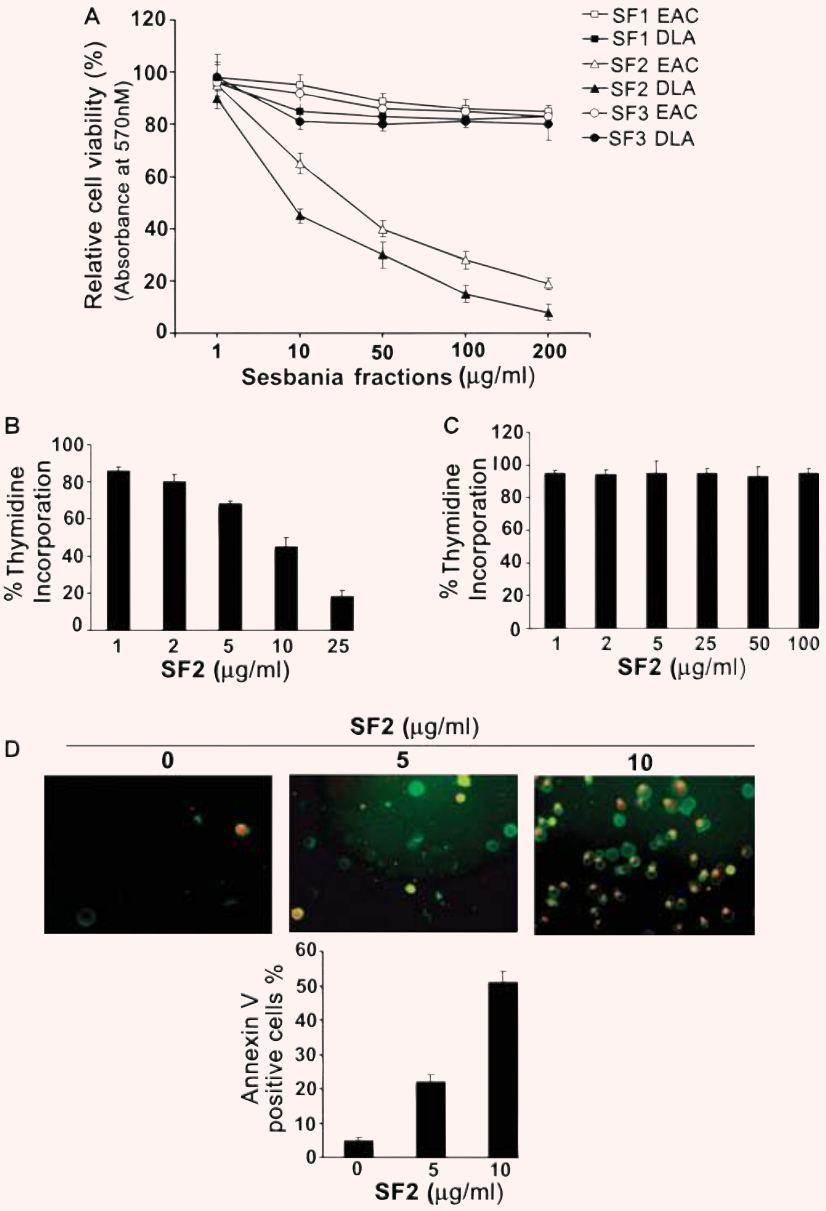


**Fig. 1** SF2 induces cytotoxicity to ascites cells and brings about membrane flip-flop in DLA cell lines. (**A**) Murine ascites cell lines, DLA and EAC ($5 \times 10^3$) were plated in 96-well plates, treated with indicated concentrations of Sesbania protein fractions – SF1, SF2 and SF3 for 72 hrs, MTT was added and viability was determined. (**B**) DLA cells were seeded as in MTT assay, treated with SF2 for 24 hrs, [$^3$H] thymidine was added during last 6 hrs and thymidine incorporation was measured. For both (**A**) and (**B**) the results are expressed as the mean percentage of control of quadruplicate determinations from three independent experiments. (**C**) MEF cells were seeded as mentioned in Fig. 1A, treated with indicated concentrations of SF2 for 72 hrs, MTT was added and viability was determined as described. (**D**) Cells were treated with SF2 for 16 hrs, washed with PBS and stained with annexin V–FITC/propidium iodide mixture, and photographed (20×). Annexin V positive cells were counted and expressed graphically.

serum and hepatic tissues (Fig. 4D). Results do not reveal any significant changes in any of the parameters studied proving that the drug is non-toxic and pharmacologically safe, *in vivo*. The histopathology data also supported these results (data not shown).

In the acute toxicity study, when the mice were observed for behavioural changes after i.p. administration of a single dose of SF2, the groups treated with 100 and 200 mg/kg did not exhibit any abnormal behavioural responses. However, mice which received 500 and 700 mg/kg of SF2 showed toxic symptoms. These include hyperactivity, water intake and sedation. These symptoms appeared almost within 1 hr after the administration of SF2. All the animals at the dose level of 700 mg/kg and 60% of the animals at the dose level of 500 mg/kg died within 2 days. The LD 50 value for a single i.p. dose was found to be 416 mg/kg.

## SF2 induces cytotoxicity to human cancer cells and induces apoptosis in the colon cancer cell line, SW-480

The promising data obtained using the murine ascites cell lines both in the *in vitro* and *in vivo* conditions encouraged us to extend

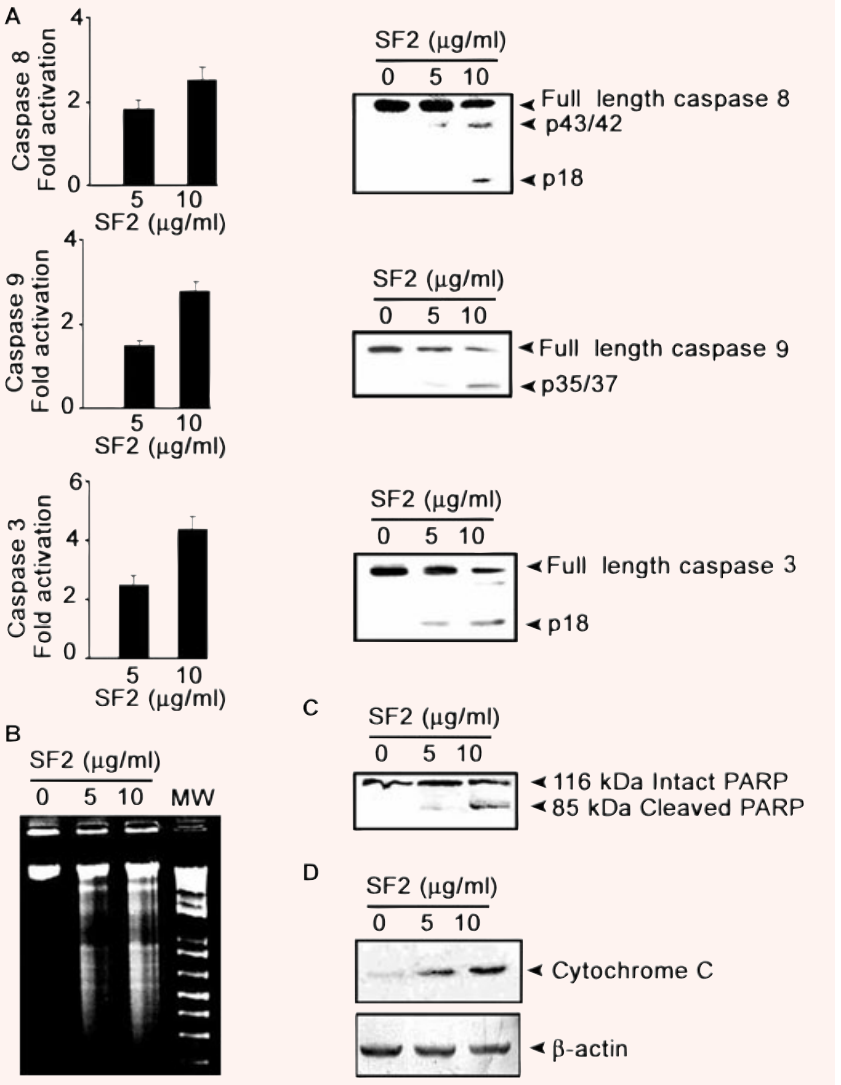


**Fig. 2** SF2 induces activation of caspases, DNA fragmentation, PARP cleavage and cytochrome-C release in DLA cells. (**A**) Cells treated for 24 hrs with indicated concentrations of SF2 were lysed, centrifuged and the supernatant was used for assaying the caspases 8, 9 and 3 activities, respectively, using appropriate fluorogenic substrates. Cell lysates were blotted against specific antibodies for the respective caspases. (**B**) The cells were treated with indicated concentrations of SF2 for 24 hrs; DNA was extracted and subjected to electrophoresis. (**C**) Cells treated for 24 hrs with indicated concentrations of SF2, were lysed and blotted against anti-PARP antibody. (**D**) Cells were treated with SF2 for 24 hrs, and mitochondria-free cytosol was prepared and blotted against anti-cytochrome-C and β-actin antibodies.

the study in human cancer cell lines. We screened human cancer cell lines of various origins. Even though all the investigated cells showed cytotoxicity on exposure to the SF2 fraction, in the range from 1 to 25 μg/ml, colon cancer cells were more sensitive (Fig. 5A). Among the colon cancer cell lines studied, SW-480 was the most sensitive (Fig. 5A) and the IC50 was found to be 10 μg/ml. SF2 also inhibited DNA synthesis in all the three colon cancer cell lines with maximum inhibition in SW-480 cells (Fig. 5B). Hence we focused on this cell line for further investigations.

Spectrofluorimetric analysis using fluorogenic substrates specific for caspases showed that SF2 activates caspases 3, 8 and 9 in a dose-dependent manner in SW-480 cells (Fig. 5C). Additional evidence supporting SF2-induced apoptosis was obtained by annexin V–propidium iodide staining in which 16% and 37% of the cells were annexin V positive after treatment with 5 and 10 μg/ml of SF2, respectively, for 16 hrs. Some cells also showed typical PI staining, especially with 10 μg/ml of SF2 treatment (Fig. 5D). Our results support the notion that SF2 treatment initiates signalling pathways leading to apoptosis.

## SF2 down-regulates the activation of anti-apoptotic factors, NF-κB, Bcl-2, Akt and COX-2 induced by PMA

We also explored whether SF2 can down-regulate various survival signals which are the key regulators of apoptosis. We observed a dose-dependent inhibition of NF-κB by SF2, in SW-480 cells

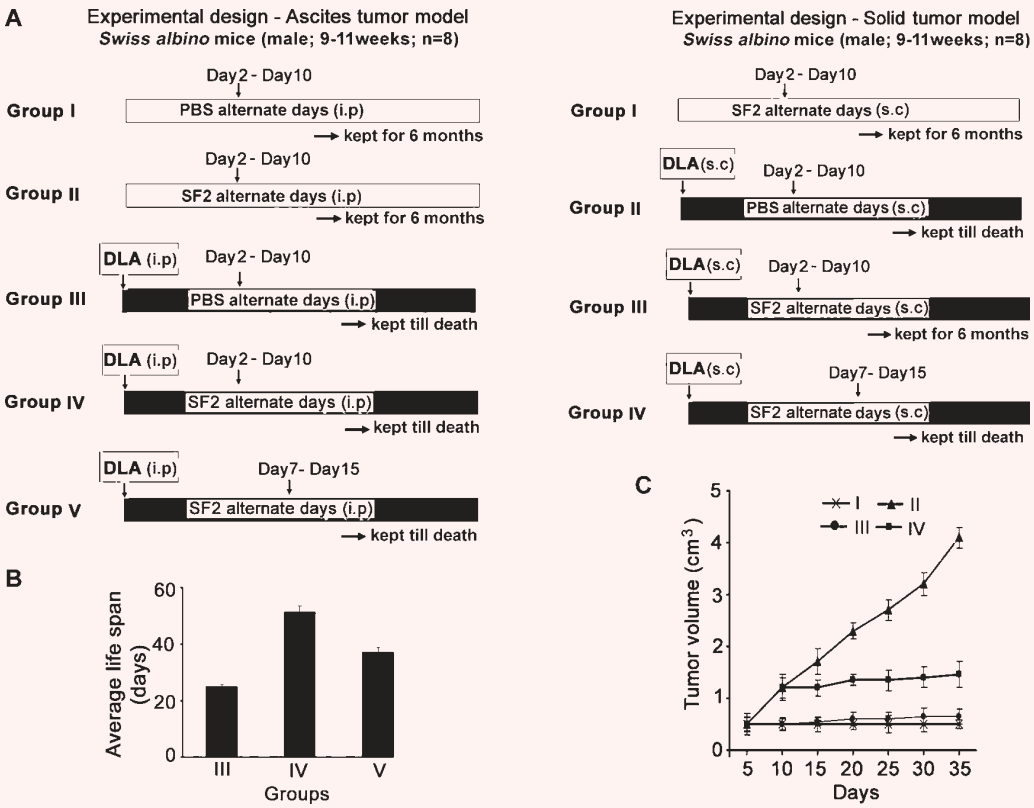


**Fig. 3** SF2 inhibits the development of ascites and solid tumours in *Swiss Albino* mice. (**A**) Schematic representation of different experimental groups and treatment schedule for Ascites and solid tumour model. (**B**) The average survival time of each group of mice in Ascites tumour model. The values are mean of eight mice per group ± S.D. Groups IV and V were compared with group III ($P < 0.001$). (**C**) Mean of tumour volume in mice treated with Sesbania fraction 2 subcutaneously. The solid tumour size was measured from day 6 onwards and volume was calculated. The values are mean of 8 mice per group ± S.D. Groups III and IV were compared with group II ($P < 0.001$).

treated with PMA, a well-known inducer of NF-κB (Fig. 6A). Antibodies against both p50 and p65 subunits of NF-κB shifted the active NF-κB complex producing super shifts whereas incubation with excess unlabelled oligonucleotide containing the NF-κB binding site completely removed the active complex confirming the specificity of the bands (Fig. 6B). Since SF2 induced the release of cytochrome C from the mitochondria, the involvement of mitochondrial pathway is evident in SF2-induced apoptosis. Hence we investigated the role of SF2 in regulating Bcl-2, the mitochondrial membrane bound anti-apoptotic factor. We observed a strong and dose-dependent down-regulation of Bcl-2 by SF2 (Fig. 6C). SF2 also down-regulated the expression of COX-2 which also provides survival advantages to cancer cells (Fig. 6D). Phosphorylation of Akt, another survival signal that in many cases is regulated by NF-κB [27, 28], was also inhibited by SF2 (Fig. 6E).

Taken together, the above noted novel mechanistic observations strongly attest the potential anticancer efficacy of SF2, in both *in vitro* and *in vivo* model systems.

## Discussion

In this study we have documented findings pertinent to the prospect for therapeutic applications of *S. grandiflora*. An active fraction (SF2) derived from the flower of *S. grandiflora* contains a pharmacologically safe pro-apoptotic compound. Despite existing anecdotal information on anticancer properties associated with the fruit of this plant in the Indian system of medicine (Ayurveda), no information exemplifying anti-tumour efficacy of flowers of this plant exists in the literature. Our studies revealed that flowers of *S. grandiflora* are associated with more potent anticancer efficacy compared to the leaves and fruit (data not shown). We elucidated the mechanistic pathway through which an active fraction of the flower, SF2, induced apoptosis in murine, as well as in human cancer cell lines, and confirmed its anticancer effect in mice bearing ascites and solid tumours.

Although the mechanism by which SF2 achieves these effects remains unknown, several studies document that caspase activation

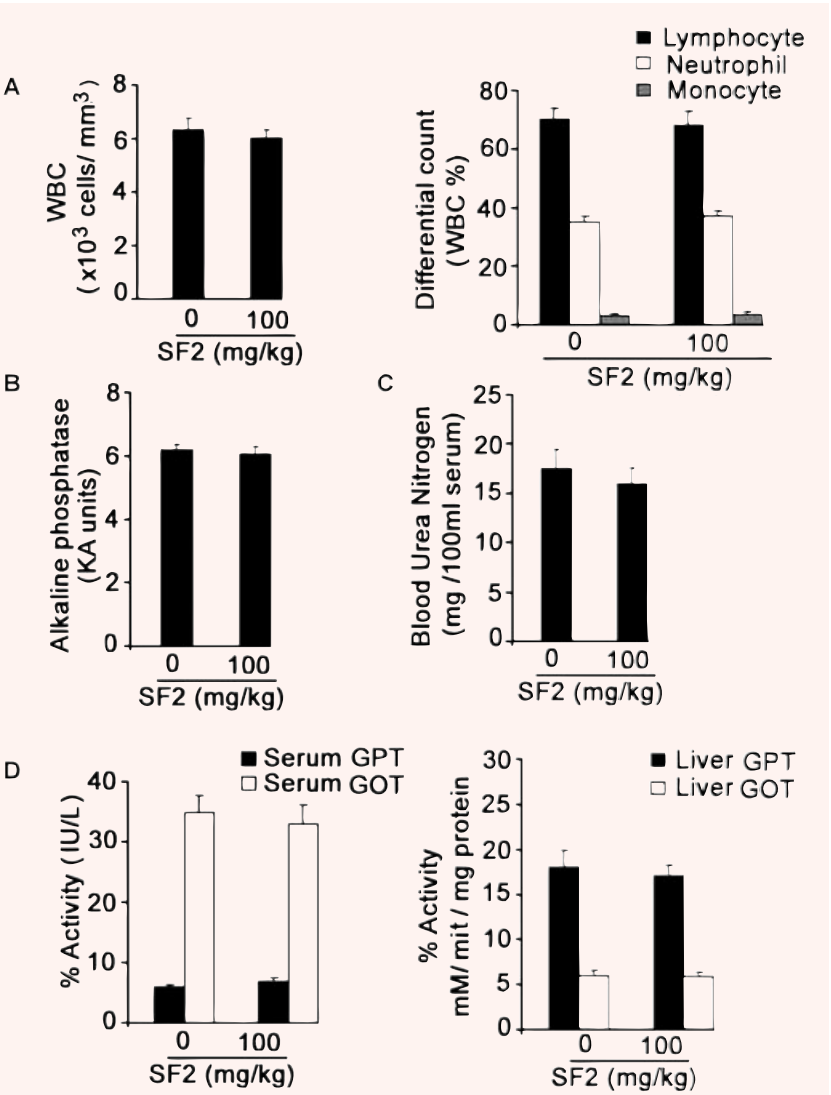


**Fig. 4** SF2 does not induce toxicity *in vivo*. *Swiss albino* mice were injected with and without SF2 and the blood and liver tissues were collected. (**A**) Blood taken from both groups were studied for differential counts of white blood cell (WBC). (**B**) The serum was separated from blood and activity of serum alkaline phosphatase was determined. (**C**) Blood collected from *Swiss albino* mice treated with or without SF2, were assayed for blood urea nitrogen. (**D**) Blood and liver collected from *Swiss albino* mice treated with or without SF2 were assayed for GOT and GPT levels in serum and liver using standard protocols.

and subsequent cleavage of functionally essential key enzymes perform a central role in the biological processing of apoptosis [29]. In concordance with the currently acceptable dogma for apoptosis, SF2 induced a dose-dependent activation of the caspases 3, 8 and 9 (both spectrofluorimetrically and by Western blot) and cleaved the full length PARP into the apoptotic fragments. Furthermore, a dose-dependent release of cytochrome C from the mitochondria to the cytoplasm strongly indicates that the apoptosis induced by SF2 is through the mitochondrial pathway. However, it remains to be tested whether SF2-mediated apoptosis also operates through caspase-independent apoptogenic proteins such as apoptosis inducing factor and endonuclease G [30, 31].

As biological therapy for treatment of cancer, SF2 treatment given to mice 24 hrs after the implantation of ascites and solid tumours exhibited reduction of tumour development and increased lifespan of mice strongly affirming efficient suppression of *in vivo* tumourigenesis by SF2. Furthermore, the tumour static effect seen in animals treated 7 days after tumour implantation illustrates the efficacy of SF2 in preventing further development of the tumour.

Almost all currently available chemotherapy schemes for the treatment of cancer are associated with considerable toxicities that fail to transcend into optimal clinical benefits for patients. The major problem associated with chemotherapy is the reduction in the count of haematological parameters such as lymphocytes, neutrophils and monocytes. To rule out this possibility, we conducted a toxicity study using the SF2 fraction. SF2 did not produce any significant difference in haematological parameters indicating that it does not cause any toxicity or immunosuppression in animals. Following the onset of hepatocellular damage, ALP, GPT and

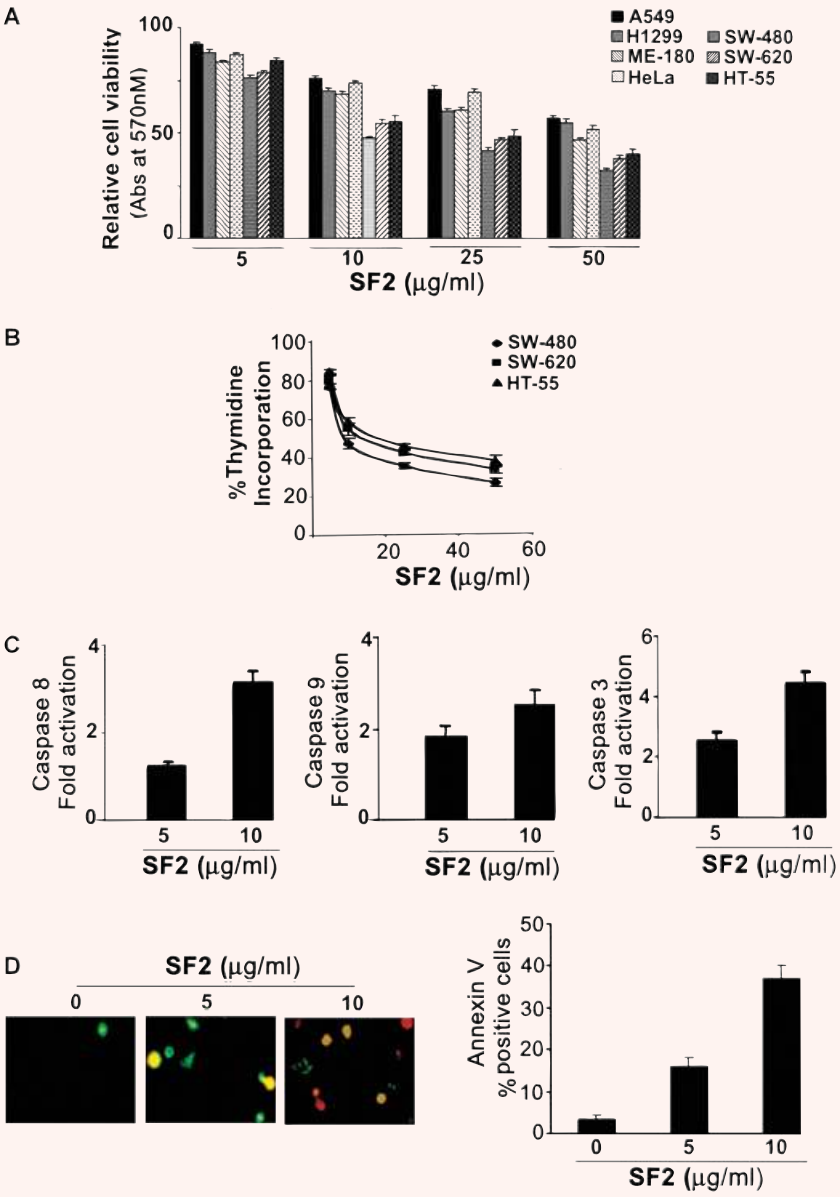


**Fig. 5** SF2 induces cytotoxicity to human cancer cells, inhibits DNA synthesis and induces caspase activation and membrane flip-flop in the colon cancer cell line, SW 480. (**A**) Cells of various origin ($5 \times 10^3$/well) were plated in 96-well plates, treated with indicated concentrations of SF2 for 72 hrs, MTT was added and viability was determined (**B**) SW-480 cells were seeded as in MTT assay, treated with SF2 for 24 hrs, [$^3$H] thymidine was added during last 6 hrs and thymidine incorporation was measured. For both (**A**) and (**B**) the results are expressed as the mean percentage of control of quadruplicate determinations from three independent experiments. (**C**) Cells treated for 24 hrs with indicated concentrations of SF2, were lysed, centrifuged and the supernatant was used for assaying caspases 8, 9 and 3 activities, respectively, with appropriate fluorogenic substrates. (**D**) Cells were treated with SF2 for 16 hrs, washed with PBS and stained with annexin–FITC/ propidium iodide mixture, and photographed (20×).

GOT is released from the damaged cells, elevating their levels in the serum. Further, to rule out any adverse hepatotoxicity owing to SF2, ALP profiles in serum and GOT and GPT profiles in serum and hepatic tissue were analysed. The results of serum and liver enzyme assays of the animals treated with SF2 were all in the normal range suggesting that the drug is non-toxic and pharmacologically safe *in vivo*. The level of BUN also indicated that the drug itself is not producing any severe toxicological manifestations in the kidney. Additionally, an acute toxicity study was also conducted using SF2 administered during a period not exceeding 24 hrs, in line with any pharmaceutical intended for human use, including preliminary identification of toxicity to target organs and obtain clues to the selection of starting doses for phase 1 human studies. SF2, up to 200 mg/kg, failed to exhibit any signs of cumulative adverse response in experimental animals as concluded from gross measures such as loss of body weight, ruffling of fur and change in behaviour and food intake indicating that the protein fraction is pharmacologically safe for *in vivo* administration up to this concentration.

For a mechanistic understanding of our novel findings, we further investigated whether SF2 can down-regulate the survival signals induced by external stimuli. We observed that SF2 is a potent

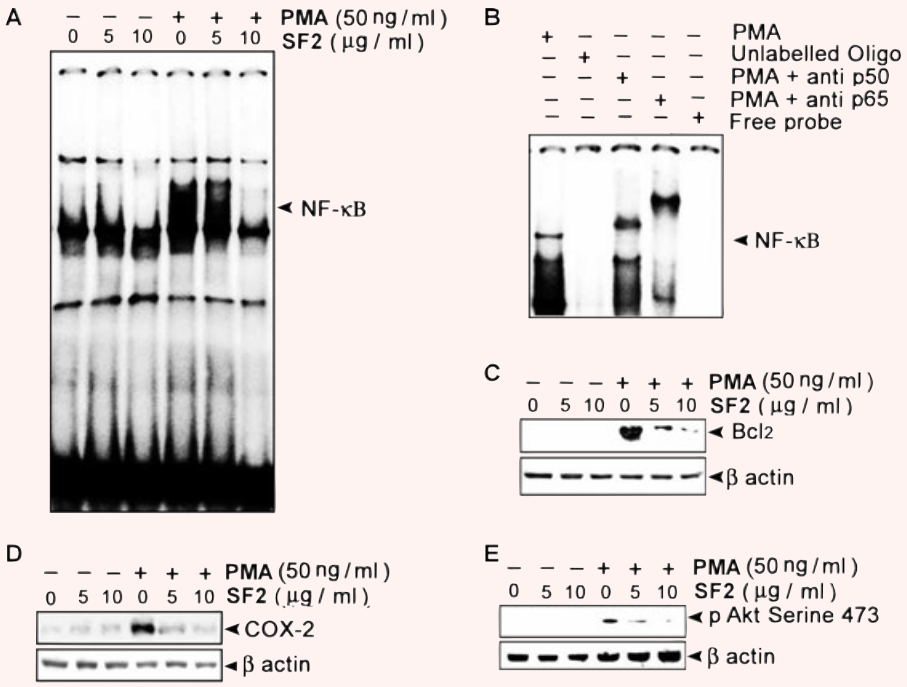


**Fig. 6** SF2 down-regulates the activation of NF-κB, phosphorylation of Akt and up-regulation of Bcl-2 and COX-2 induced by PMA. (**A**) SW-480 cells were treated with or without PMA for 1 hr and then with SF2 for 2 hrs as indicated, nuclear extracts were prepared, and EMSA was done. (**B**) The nuclear extracts from PMA-stimulated cells were incubated with either p65 or p50 antibody or unlabeled oligo. The *arrowhead* indicates the positions of the active DNA binding complex of NF-κB. (**C**) SW-480 cells were treated with or without PMA for 6 hrs and then with SF2 for 24 hrs, as indicated and the whole cell lysate was resolved on 15% gel and blotted against Bcl-2 antibody. (**D**) SW-480 cells were treated with or without PMA for 1 hr and then with SF2 for 24 hrs and the whole cell lysate was resolved on 8% gel and blotted against COX-2 antibody. (**E**) SW-480 cells were treated with or without PMA for 1 hr and then with SF2 for 2 hrs and the whole cell lysate was resolved on 10% gel and blotted against phospho-Akt serine 473.

inhibitor of NF-κB, a transcription factor which is currently envisaged to be therapeutically beneficial in cancer treatment armentorium. In recent years, naturally occurring compounds have gained considerable attention because of their alleged therapeutically beneficial effects attributable to multimodal actions including inactivation of NF-κB and other survival signalling cascades and fewer associated toxicities. Nuclear translocation of NF-κB and its binding to DNA at specific κB-sites, rapidly induce a variety of genes including COX-2 [32]. Furthermore, NF-κB induction up-regulates genes that antagonizes pro-apoptotic signals, including Akt, thereby suppressing cell death pathways [33]. It was also very interesting to note that inhibition of NF-κB by SF2 was accompanied by rapid down-regulation of the anti-apoptotic protein moieties – COX-2 and Akt. Akt/PKB has been shown to regulate IKK activity in both direct and indirect manners [34]. It is known that Akt may act through the NF-κB pathway and that the COX-2 gene is regulated at the promoter level by NF-κB [35]. It has also been reported that inhibition of COX-2 leads to inhibition of Akt, which in turn promotes apoptosis [36]. Recent studies indicate that constitutive COX-2 expression *via* constitutive NF-κB expression may be the principal mechanism for gastric carcinogenesis and tumourigenesis [37]. Epidemiological, animal and human data indicate that inhibitors of cyclooxygenase are chemopreventive for colon cancer in which COX-2 is overexpressed in 80–85% of cases [38]. In our study, we observed a significant down-regulation of COX-2 by SF2 in colon cancer cells, which strongly indicates a possible role for SF2 as a putative chemopreventive agent against colon carcinogenesis.

Several types of cancer cells constitutively express NF-κB, which contribute to their resistance to apoptosis induced by chemotherapeutic drugs [39]. Extensive studies reported in literature indicate that the anti-apoptotic protein Bcl-2 inhibits the release of apoptogenic cytochrome C from mitochondria into the cytosol [40, 41], suggesting that Bcl-2 may participate in stabilizing mitochondria [42]. Aberrant overexpression of Bcl-2 rescues precancerous cell clones from apoptotic elimination. Existing data suggest a potential role for Bcl-2 in altering NF-κB activity [43, 44]. It has been suggested that signalling pathways that lead to NF-κB down-regulation are mediated by one of the Bcl-2 homology domains that are conserved among members of the Bcl-2 gene family [45].

Several studies including ours have shown that antioxidants and vitamins significantly down-regulate anti-apoptotic factors contributing to anticancer effect [46, 47]. *S. grandiflora* is a rich source of amino acids, essential minerals, and vitamins such as vitamin A, vitamin C, thiamine, riboflavin and nicotinic acid, and recent studies demonstrate the hypolipidemic as well as antioxidant efficacy of *Sesbania* [12, 48–50]. Taken together, these observations indicate additional mechanisms by which Sesbania provides protection to DNA from oxidative damage induced by various carcinogens leading to the 'initiation' of cancer.

As further corollary to our study, we also separated the SF2 fraction by column chromatography and cytotoxicity of the fractions obtained were assessed by MTT assay. Unfortunately, none of the components induced significant cytotoxicity (data not shown) which indicates that various components in the SF2 fraction may afford a synergistic cytotoxic effect. Further studies are underway to isolate active compounds and to explore molecular pathways associated with chemoprotection and chemoprevention. Additionally, multiple signal transduction pathways regulating the apoptotic program induced by SF2 are also being actively pursued so as to make the formulation an interesting candidate which can be used in the treatment of colon cancer and other malignancies.

In conclusion, the results of this study demonstrate that a potent protein fraction isolated from flower of the medicinal plant *S. grandiflora* without any undesired toxicity potentiates apoptosis, reduces tumour cell viability and interferes in abrogating proliferative signals which are otherwise conducive for tumour growth.

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## References

1. **Ferantinos L**. Human consumption of Sesbania grandiflora. In: Macklin B, Evans DO, editors. Perennial Sesbania species in agroforestry systems. Hawaii: NFTA; 1990. p. 105.
2. **Chaudhari RH**. Comparative pharmacologistic studies on the stem bark of Sesbania sesban (L) and S.grandiflora (L). *Bull Bot. Surv Ind.* 1996; 8: 216–20.
3. **Joshy SG**. Leguminoceae. Text book of medicinal plants. New Delhi: Oxford and TBH Publishing Co. Pvt. Ltd.; 2000. p. 130.
4. **Thamboli S**. Antiinflammatory activity of *S. grandiflora*. *Indian Drugs*. 1996; 33: 504–6.
5. **Thamboli SA**. Analgesic and antipyretic activity of *S. grandiflora*. *Indian Drugs*. 2000; 37: 95–8.
6. **Kasture VS, Deshmukh VK, Chopde CT**. Anxiolytic and anticonvulsive activity of Sesbania grandiflora leaves in experimental animals. *Phytother Res.* 2002; 16: 455–60.
7. **Vijayakumar G**. Preliminary studies on the effect of indigenous plant *S. grandiflora* in carbon tetrachloride induced hepatitis in calves. *Indian J. Veterenery Med.* 1997; 17: 17–21.
8. **Tewtrakal SS, Rattanasuwan P**. HIV-1 protease inhibitory effects of some selected plants in Caesalpiniaceae and Papilionaceae families. *J. Sci. Technol.* 2003; 25: 509–14.
9. **Ramesh T, Begum V**. Protective effect of Sesbania grandiflora against cigarette smoke-induced oxidative damage in rats. *J Med Food.* 2008 11: 369–75.
10. **Pari LUA**. Protective effect of Sesbania grandiflora against erythromycin estolate-induced hepatotoxicity. *Therapie.* 2003; 5: 439–43.
11. **Doddola SPH, Koganti B, Prasad KV**. Evaluation of Sesbania grandiflora for antiurolithiatic and antioxidant properties. *Nat Med.* 2008; 3: 300–7.
12. **Ramesh TMR, Hazeena Begum V**. Effect of *Sesbania grandiflora* on lung antioxidant defense system in cigarette smoke exposed rats. *Int J Biol Chem.* 2007; 1: 141–8.
13. **Sivarajan VV, Indira B**. Ayurvedic drug sources. New Delhi: Oxford & IBH Publishing Co., New Delhi, India; 1995.
14. **Boonmee A, Reynolds CD, Sangvanich P**. Alpha-glucosidase inhibitor proteins from Sesbania grandiflora flowers. *Planta Med.* 2007; 73: 1197–201.
15. **Kirithikar KR, Basu BD**. Indian medicinal plants. Vol. 1. New Delhi: International Book Distributors; 1967. pp. 735–6.
16. **D'Silva I, Heath MC**. Purification and characterization of two novel hypersensitive response-inducing specific elicitors produced by the cowpea rust fungus. *J Biol Chem.* 1997; 272: 3924–7.
17. **Anto RJ, Mukhopadhyay A, Denning K, *et al.*** Curcumin (diferuloylmethane) induces apoptosis through activation of caspase-8, BID cleavage and cytochrome c release: its suppression by ectopic expression of Bcl-2 and Bcl-xl. *Carcinogenesis.* 2002; 23: 143–50.
18. **Anto RJ, Maliekal TT, Karunagaran D**. L-929 cells harboring ectopically expressed RelA resist curcumin-induced apoptosis. *J Biol Chem.* 2000; 275: 15601–4.
19. **Anto RJ, Venkatraman M, Karunagaran D**. Inhibition of NF-κB sensitizes A431 cells to epidermal growth factor-induced apoptosis, whereas its activation by ectopic expression of RelA confers resistance. *J Biol Chem.* 2003; 278: 25490–8.
20. **Ruby AJ, Kuttan G, Babu KD, *et al.*** Antitumour and antioxidant activity of natural curcuminoids. *Cancer Lett.* 1995; 94: 79–83.
21. **Prasad SB, Giri A**. Antitumor effect of cisplatin against murine ascites Dalton's lymphoma. *Indian J Exp Biol.* 1994; 32: 155–62.
22. **Mukherjee KL**. Medical laboratory technology. A procedure manual for routine diagnostic tests. Vol. 2. New Delhi: Tata McGraw Hill Publishers; 1988.
23. **Moss DW, Baron DN, Walker PG, *et al.*** The King-Armstrong method. *J Clin Pathol.* 1972; 25: 368–9.
24. **Narayanan S**. Alkaline phosphatase as tumor marker. *Ann Clin Lab Sci.* 1983; 13: 133–6.
25. **Wootton I**. Micro analysis in medical biochemistry. 4th ed. A and J Churchill London Ltd.; 1964.
26. **Reitman S, Frankel S.** A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am J Clin Pathol.* 1957; 28: 56–63.

27. **Pianetti S, Arsura M, Romieu-Mourez R, *et al.*** Her-2/neu overexpression induces NF-κB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IkappaB-alpha that can be inhibited by the tumor suppressor PTEN. *Oncogene.* 2001; 20: 1287–99.
28. **Ozes ON, Mayo LD, Gustin JA, *et al.*** NF-κB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature.* 1999; 401: 82–5.
29. **Miller DK**. The role of the Caspase family of cysteine proteases in apoptosis. *Semin Immunol.* 1997; 9: 35–49.
30. **Du J, Suzuki H, Nagase F, *et al.*** Methylglyoxal induces apoptosis in Jurkat leukemia T cells by activating c-Jun N-terminal kinase. *J Cell Biochem.* 2000; 77: 333–44.
31. **Li H, Zhu H, Xu CJ, *et al.*** Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell.* 1998; 94: 491–501.
32. **Santoro MG, Rossi A, Amici C**. NF-κB and virus infection: who controls whom. *EMBO J.* 2003; 22: 2552–60.
33. **Meng F, Liu L, Chin PC, *et al.*** Akt is a downstream target of NF-κB. *J Biol Chem.* 2002; 277: 29674–80.
34. **Kane LP, Shapiro VS, Stokoe D, *et al.*** Induction of NF-κB by the Akt/PKB kinase. *Curr Biol.* 1999; 9: 601–4.
35. **St-Germain ME, Gagnon V, Parent S, *et al.*** Regulation of COX-2 protein expression by Akt in endometrial cancer cells is mediated through NF-κB /I-κB pathway. *Mol Cancer.* 2004; 3: 1–11.
36. **Fan XM, Jiang XH, Gu Q, *et al.*** Inhibition of Akt/PKB by a COX-2 inhibitor induces apoptosis in gastric cancer cells. *Digestion.* 2006; 73: 75–83.
37. **Joo Weon Lim, Hyeyoung Kim, Kyung Hwan Kim**. Expressions of Ku70 and Ku80 mediated by NF-κB and cyclooxygenase-2 are related to cell proliferation in human gastric cancer cells *J Biol Chem.* 2002; 277: 46093–100.
38. **Williams CS, Mann M, DuBois RN**. The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene.* 1999; 18: 7908–16.
39. **Cusack JC, Liu R, Baldwin AS**. NF-κB and chemoresistance: potentiation of cancer drugs via inhibition of NF-κB. *Drug Resist Updat.* 1999; 2: 271–3.
40. **Kluck RM, Bossy-Wetzel E, Green DR, *et al.*** The release of cytochrome C from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science.* 1997; 275: 1132–6.
41. **Yang JLX, Bhalla K**. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science.* 1997; 275: 1129–32.
42. **Green DR, Reed JC**. Mitochondria and apoptosis. *Science.* 1998; 281: 1309–12.
43. **Tamatani M, Che YH, Matsuzaki H, *et al.*** Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NF-κB activation in primary hippocampal neurons. *J. Biol. Chem.* 1999; 274: 8531–8.
44. **Zong WX, Edelstein LC, Chen C, *et al.*** The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-κB that blocks TNF-alpha induced apoptosis. *Genes Dev.* 1999; 13: 382–7.
45. **Stefan G, Patrick AB, Klaus S-O**. Bcl-2 Down-regulates the activity of transcription factor NF-κB induced upon apoptosis. *J Cell Biol.* 1996; 134: 13–23.
46. **Bava SV, Puliappadamba VT, Deepti A, *et al.*** Sensitization of taxol-induced apoptosis by curcumin involves down-regulation of NF-κB and the serine/threonine kinase Akt and is independent of tubulin polymerization. *J Biol Chem.* 2005; 280: 6301–8.
47. **Kenichi Satake ET, Akiko I, Yasumasa K, *et al.*** Anti-tumor effect of vitamin A and D on head and neck squamous cell carcinoma. *Auris Nasus Larynx.* 2003; 30; 403–12.
48. The wealth of India: raw materials. New Delhi: Council of Scientific Industrial Research; 1972: 9. pp. 295–8.
49. **Ramesh T, Hazeena Begum V**. Hypolipidemic effect of *Sesbania grandiflora* on cigarette smoke exposed rats. *Pharmacologyonline.* 2006; 3: 309–23.
50. **Ramesh T, Mahesh R, Hazeena Begum V**. Effect of *Sesbania grandiflora* on membrane-bound ATPases in cigarette smoke exposed rats. *J Pharmacol Toxicol.* 2007; 2: 559–66.