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Immune modulation and apoptosis induction: Two sides of antitumoural activity of a standardised herbal formulation of *Withania somnifera*

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ABSTRACT

Deregulated apoptosis and suppressed tumour reactive immunity render tumour cells to grow amok in the host body. Traditionally used botanicals may offer potential anticancer chemo-immunotherapeutic leads. We report in this study a chemically standardised herbal formulation (WSF) of *Withania somnifera* possessing anticancer and Th1 immune up-regulatory activities. WSF produced cytotoxicity in a panel of human cancer cell lines *in vitro*. The molecular mechanism of cell cytotoxicity, IC_{50} 48 h ~20 μg/ml, was investigated in HL-60, where it induced apoptosis by activating both intrinsic and extrinsic signalling pathways. It induced early generation of reactive nitrogen and oxygen species (RNOS), thus producing oxidative stress mediated mitochondrial membrane potential (MMP) loss leading to the release of cytochrome c, the translocation of Bax to mitochondria and apoptosis-inducing factor to the nuclei. These events paralleled the activation of caspase-9, -3 and PARP cleavage. WSF also activated caspase-8 through enhanced expression of TNF-R1 and DR-4, suggesting also the involvement of extrinsic pathway of apoptosis. WSF at 150 mg/kg, i.p., inhibited >50% tumour growth in the mouse tumour models. In tumour-bearing mice, WSF inhibited the expression of pStat-3, with a selective stimulation of Th1 immunity as evidenced by enhanced secretion of IFN-γ and IL-2. In parallel, it enhanced the proliferation of $CD4^+/CD8^+$ and NK cells along with an increased expression of CD40/CD40L/CD80. In addition, WSF also enhanced T cell activation in camptothecin treated tumour-bearing mice. WSF being safe when given orally up to 1500 mg/kg to rats for 6 months may be found useful in the management of malignancy by targeting at multiple pathways.

1. Introduction

Cancer development largely depends on the ability of cancerous cells to exploit the normal physiological processes of the host for its progression and development. It arises from normal cells through the acquisition of several genetic alterations. There is a complex network of pro- and anti-apoptotic proteins machinery and cell–cell interaction which regulates

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the normal cellular proliferation and differentiation critical for survival. Cancer cells harbour mutations in this network, which endow them with the phenotypes associated with the malignant state.[1]

Another check point in cancer cell proliferation is tumour reactive immune system. The cell-mediated T-helper 1 (Th1) immune response is generally regarded as tumour inhibitory, and the expression of Th1 cytokines is associated with a favourable clinical outcome, whilst the expression of Th2 adversely affects the prognosis.[2] Cancer patients exhibit the enhanced expression of Th2 cytokines, whilst the expression of Th1 cytokines is decreased systemically as well as in the local tumour microenvironment thereby evading the Th1 immunosurveillance.[3,4] A fully functional immune response otherwise is critical to the recognition and elimination of tumour cells.

Despite employing various treatments like surgery, radiation and chemotherapy, cancer still remains one of the leading causes of mortality. One of the mechanisms by which chemotherapeutics destroy cancer cells is by targeting intrinsic and extrinsic signalling pathways of the cell by the activation or inhibition of certain pro- and anti-apoptotic cascades converging ultimately into cancer cells death.[5–7]

Treatments with chemotherapy have produced modest results, whilst immunotherapy has not proven to be encouraging. Cytotoxicity of chemotherapeutic drugs is not limited to the cancer cells only, but these drugs may also target the dividing lymphocytes required for the development of an effective immune response. However, new platforms for chemotherapy as tumour antigens releaser and immunotherapy as immunomodulatory conditioner have introduced a rational for the association of these therapies in a chemo-immunotherapy protocol in order to improve the results of the treatment.[8]

Plant based products provide enormous opportunity to stimulate components of immune system towards discovering more effective and safe antitumour agents. So the development of agents that might elicit multimodal action can be effective against the management of this complex disease. The use of herbal medicine is prevalent in developing world since long time, and is now rapidly growing in industrialised countries.[9,10] Herbs have been shown to possess a broad spectrum of activities and their standardization, safety and mechanistic details needed to be addressed.[11] Based on chemo-immunotherapeutic approach to address tumour at multiple levels, we prepared a herbal composition from *Withania somnifera* as a multi target therapeutic. *W. somnifera* DUNAL (Solanaceae) is a small subtropical under shrub and has been in use in the Indian traditional system of medicine Ayurveda for its medicinal properties.[12,13] We recently reported that the root extract of *W. somnifera* and its major constituent withanolide-A, elicit Th1 dominant cell-mediated immune up-regulation.[12] On the other hand, leaf extract of the plant has been found to have strong tumour-inhibitory activity by selectively killing the cancer cells.[14] In our early report we showed that withaferin-A predominantly found in the leaves of the plant, induced apoptosis in HL-60 cells is mediated through the activation of both intrinsic and extrinsic signalling cascade[13] and further that the alcoholic extract of leaves produced apoptosis (unpublished) through pathways operated solely by withaferin-A.[13] Because of inherent difficulties in the synthesis of the withanolides, we became interested in exploiting the immunostimulatory (Th1) and pro-apoptotic properties of the extracts to devise a formulation useful for cancer treatment. WSF showed strong tumour-inhibitory properties and Th1 immune stimulation without any toxicity in experimental animals. This formulation may also be found highly useful when used as adjunct with conventional anti-cancer therapeutics.

2. Materials and methods

2.1. Chemicals and reagents

The source of the chemicals and reagents used in the present study is the same as described earlier.[12,13]

2.2. Preparation and chemical characterisation of W. somnifera formulation (WSF)

WSF was prepared from the roots and leaves of an elite variety of *W. somnifera*.[15] Aqueous alcoholic (50% v/v) extracts of roots and leaves were prepared as described earlier.[12] Both the dried extracts were mixed in 1:1 ratio (w/w) to designate this mixture as *W. somnifera* formulation (WSF), which was characterised for chemo profiling as described earlier for root extract.[12]

2.3. Animal care and housing

BALB/c and Swiss albino mice were procured from the institute's animal house. The animals were housed in standard size polycarbonate cages fed with standard pellet diet (Gold Muhor, Lipton India Ltd.) and autoclaved water was given ad libitum. They were housed in controlled conditions of temperature (25 ± 2 °C), humidity (50–60%) and 12:12 h of light: dark cycle. The study and the number of animals used were approved by the Institutional Animal Ethics Committee.

2.4. Cell cultures and treatment

Various cell lines used in this study were procured from National Cancer Institute (NCI), Bethesda, United States of America (USA). Primary Human Gingival fibroblast (hGF) cell line developed from healthy gingiva[16] was a kind gift from Dr. Anil Balapure, Scientist, Central Drug Research Institute, Lucknow, India. The cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (0.3 mg/ml), pyruvic acid (0.11 mg/ml), and 0.37% $NaHCO_3$ at 37 °C in an atmosphere of 95% air and 5% CO_2 with 98% humidity. WSF was dissolved in 30% DMSO in water and was used for *in vitro* cell cultures so that the final concentration of DMSO does not exceed 0.5% v/v.

2.5. In vitro evaluation of cytotoxicity against a panel of human cancer cell lines

WSF was evaluated for its *in vitro* cytotoxicity against the indicated human cancer cell lines. A protocol of 48 h continuous

drug exposure and a sulphorhodamine B (SRB) protein binding assay was used to estimate cell growth.[17] Doxorubicin (Adriamycin) was used as positive control.

2.6. Assay of cell proliferation in HL-60 by MTT

HL-60 cells were plated in 96-well plates at a density of 2.5×10^4 cells/well/200 μl of RPMI medium containing 10% FBS. Cultures were treated with different concentrations of WSF and were incubated for 48 h. Cell proliferation using MTT was assayed as described earlier.[13] Control cultures were simultaneously treated with culture medium containing 0.5% DMSO v/v.

2.7. Flow cytometric analysis of apoptosis and necrosis

During early events of apoptosis, the plasma membrane phospholipid phosphatidylserine is translocated from the inner side of membrane leaflet to the outer side, which has a very high affinity for annexinV antibody. HL-60 cells treated with indicated concentrations of WSF for 12 h were collected, washed with PBS and stained with annexinV–FITC/PI.[13] Cells were immediately analysed on a BD-LSR flowcytometer (Becton Dickinson, USA) for apoptotic and necrotic populations.

2.8. Analysis of sub-G_0/G_1 population

HL-60 Cells (1×10^6/ml) treated with different concentrations of WSF for 12 h were washed with PBS and fixed in cold 70% ethanol overnight at 4 °C. Cells were washed, digested with DNase-free RNase (400 μg/ml) at 37 °C for 45 min. and stained with propidium iodide (5 μg/ml) before flow cytometric analysis.[13] The fluorescence intensity of sub-G_0/G_1 cell fraction represents the apoptotic cell population.

2.9. Flow cytometric analysis of reactive oxygen species (ROS)

Influence of WSF on the endogenous generation of reactive oxygen species was measured with ROS probe DCFH-DA.[13]

2.10. Flow cytometric analysis of intracellular nitric oxide using DAF-2-DA

Intracellular nitric oxide was measured by employing a low molecular weight fluorescent probe diaminofluoresceine 2-diacetate (DAF-2-DA), which is a membrane permeable and usually serves as a reporter of nitric oxide synthase activity.[18] Cells (10^6/ml) were pre-incubated for 30 min with DAF-2-DA (5 μM) then incubated together with different concentrations of WSF. Cells were collected, washed in PBS and analysed on flowcytometer in FL-1 channel for the evaluation of NO positive cell population.

2.11. Measurement of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was measured by using a mitochondrial membrane sensor kit containing JC-1 as described by the manufacturer (BD Bioscience, CA). Briefly, cells after treatment were washed twice with PBS and centrifuged at 300*g* at 4 °C for 5 min. Each cell pellet was suspended in 1 ml of diluted BD Mito-Sensor reagent and was incubated at 37 °C, 5% CO_2 for 15 min. The cells were washed and suspended in 1 ml incubation buffer and were analysed by flow cytometry for FL-1 fluorescence versus FL-2 fluorescence.

2.12. Caspase assays

Cells (2×10^6/2 ml) were incubated with WSF for the indicated time periods. At the end of treatment, cells were washed in PBS and cell pellets lysed in cell lysis buffer. Activities of caspase-3, -8 and -9 in the cell lysates were determined fluorimetrically using BD Apoalert caspase fluorescent assay kits as per the instructions given by the manufacturer.

2.13. Preparation of total cell lysates for the expression of PARP, Bcl-2 and apical death receptor proteins

HL-60 cells (3×10^6/3 ml) were treated with WSF (100 μg/ml) for different time periods. Cells were harvested and resuspended in RIPA buffer to prepare total cell lysates for the evaluation of pro- and anti-apoptotic protein expression by Western blotting as described earlier.[13]

2.14. Preparation of cytosolic and nuclear extracts

HL-60 cells (5×10^6/5 ml) were treated with WSF for indicated time periods. Cytosolic and nuclear cell lysates were prepared as described earlier.[13]

2.15. Western blot analysis

The conditions for the western blot analysis were the same as reported earlier.[13]

2.16. In vivo antitumour studies

The studies for *in vivo* anticancer activity in mouse models were conducted as per the guidelines of the National Cancer Institute (NCI), USA.[19]

2.17. Ehrlich Ascites Carcinoma (EAC)

Ehrlich Ascites Carcinoma (EAC) cells maintained in the peritoneal cavity of Swiss albino mice were collected from an animal having 8–10 d old ascitic tumour by aspirating ascitic fluid. Mice of single sex weighing 18–23 g were selected for the experiment, and were injected with 1×10^7 EAC cells intraperitoneally (i.p.) in each animal on day 0. On day 1, the animals were randomized and divided into different groups. Treatment groups contained seven animals each and a control group contained 15 animals. WSF was prepared in 1% Gum acacia in normal saline and was given both orally and intraperitoneally from day 1 to day 9. Similarly, Control group received 1% Gum acacia in normal saline, whilst treatment with 5-fluorouracil (5FU), 22 mg/kg i.p., was given to a group of mice serving as positive control.

2.18. Tumour evaluation

All the animals were sacrificed on day 13. Peritoneal fluid of all animals was collected and its volume was determined in each animal. Total number of tumour cells present in the peritoneal fluid of each animal was counted and percent growth inhibition was calculated as follows:

$$\%\ \text{Tumour inhibition} = \frac{\text{Av. no. of cells in control grp.} - \text{Av. no. of cells in test grp.}}{\text{Av. no. of cells in control grp.}} \times 100$$

2.19. Ehrlich Ascites Tumour (EAT solid)

The procedure for the tumour development and experimentation in the case of EAT was the same as that of EAC. In the case of EAT, the intramuscular injection of EAC cells (1×10^7) was given in the right thigh of each animal on day 0 and the treatment of WSF started from day 1. The tumour weight was calculated on day 13 in the animals treated for 9 d whilst in the case of prolonged treatment of 21 d, tumour evaluation was performed on 23rd day. Etoposide (25 mg/kg) was used as a positive control. Tumour weight was calculated by the formula given below.[19]

$$\text{Tumour weight (mg)} = \frac{\text{Length (mm)} \times [\text{Width (mm)}]^2}{2}$$

The average tumour weight for each group was calculated, and the percent tumour growth inhibition in treated groups was calculated as follows:

$$\%\ \text{Tumour inhibition} = \frac{\text{Av. tumour wt. of control grp.} - \text{Av. tumour wt. of test grp.}}{\text{Av. tumour wt. of control grp.}} \times 100$$

2.20. Sarcoma-180 solid tumour model

Sarcoma-180 cells were maintained in the peritoneal cavity of BALB/c mice. Mice of single sex weighing 18–23 g were selected for the experiment and injected with sarcoma-180 cells (1×10^7) in the right thigh of each animal on day 0. Intraperitoneal treatment of WSF and the positive control 5FU (22 mg/kg b.wt. normal saline) started from day 1 after the randomization of the animals. Treatment groups contained seven animals each and a control group contained 15 animals and treatment was followed the same way as described in EAT above.

2.21. Flow cytometric analysis of cell surface markers

The expression of different cell surface markers was evaluated in blood samples by flow cytometry using the corresponding FITC/PE conjugated monoclonal antibody. Fluorochrome conjugated anti-$CD3^+$ and anti-$CD4^+/CD8^+$ antibodies were used for T cell proliferation and differentiation analysis, whilst anti-NK1.1 antibodies were used for NK cells. For the evaluation of costimulatory molecules, cells were stained with anti-CD40/CD40L and anti-CD80 antibodies. Briefly, blood samples from the tumour-bearing WSF treated and untreated mice were collected and incubated with different antibodies for 30 min. in dark. Cells were washed before acquisition, and analysis was performed by flow cytometry, using Cell Quest Pro software (BD Bioscience).

2.22. Isolation of peritoneal macrophages and estimation of nitric oxide production

Macrophages were isolated from naive BALB/c mice and cultured in 24-well plates using RPMI + FBS (10%) for 48 h.[12] Macrophages were incubated with LPS (1 μg/ml) along with different concentrations of WSF at 37 °C for 48 h. Supernatants were harvested for the estimation of nitrite content using Griess reagent.[20] The absorbance was measured at 550 nm, and the nitrite content was determined by using a standard calibration curve.

2.23. Isolation of splenocytes and estimation of cytokines

Splenocytes were isolated from naïve BALB/c mice and cultured as described earlier.[12] Cells were primed with Con A (0.5 μg/ml) and co-incubated with different concentrations of WSF for 48 h. Supernatants were harvested and assayed for the cytokine secretion using BD OptEIA sets as described previously.[12]

2.24. Evaluation of different haematological parameters

Blood samples were collected from WSF treated and untreated tumour-bearing mice on day 23 of the WSF treatment (21 d). Different haematological parameters analysed are as, white blood cell count (WBC), haemoglobin (HB), haematocrit (HCT), lymphocyte, monocyte, granulocytes, mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) using haematology analyser (Humacount GMBH Germany).

2.25. *Assay of Th1/Th2-like cytokines in tumour-bearing mice sera by ELISA*

Sera were collected from WSF treated and untreated tumour-bearing mice for cytokine estimation. Graded doses of WSF were given to tumour induced mice for 21 d and sera were collected on day 23 for the estimation of Th1/Th2 cytokine expression. Cytokines were estimated by ELISA using BD Opt-EIA sets as reported earlier.[12]

2.26. *Evaluation of signal transducer and activator of transcription-3 (Stat-3) expression in tumour tissue*

Mice were sacrificed 2 d after 21 d of WSF treatment. Tumour tissues were excised and homogenised in cold RIPA buffer. Homogenates were centrifuged at high speed to collect supernatants for evaluating the expression of Stat-3 by immunoblotting.

2.27. *Statistical analysis*

Data were analysed for statistical significance by Student's *t*-test and presented as mean ± SD of the number of experiments indicated. *p*-Values less than 0.05 were considered statistically significant.

3. Results

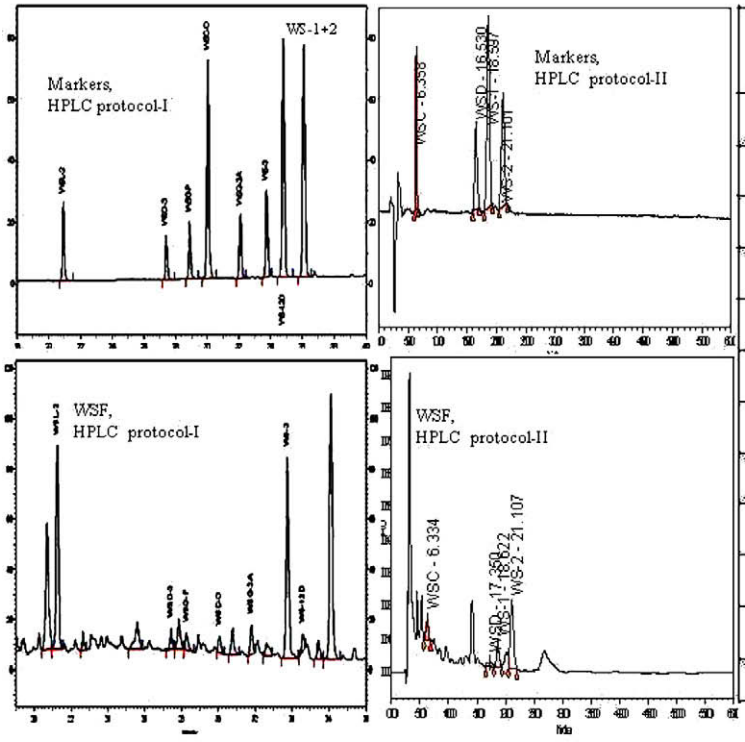
3.1. *Preparation and chemical standardization of WSF*

Keeping in view the immunostimulatory properties of root extract and anticancer activities of leaf extract, a novel formulation (WSF) consisting equal proportions of both root and leaf extracts was prepared. Chemo profiling data of WSF were performed based on eleven markers (Fig. 1) employing HPLC resolution.[12] The contents of withanolides present in the WSF are the result of average contents of the withanolides present in leaf and root extracts taken together as shown in Box 1 of Fig. 1.

3.2. *Pro-apoptotic and anticancer activities of WSF*

3.2.1. *In vitro* cytotoxicity against a panel of human cancer cell lines

WSF was evaluated for its *in vitro* cytotoxicity against various human cancer cell lines of colon (HT-29, HCT-15, SW620, 502713 and Colo-205), lung (A-549 and HOP-62), liver (Hep-G2) central nervous system (SK-N-SH), neuroblastoma (IMR-32) and prostate (DU-145 and PC-3) origin (Table 1). WSF exposure was given to cells for 48 h and a sulphorhodamine B (SRB) protein binding dye was used to estimate cell



Chemical composition of the formulation, % dry weight of the extract

Markers used for chemoprofiling	g/100g	S.D.
Withanolide A (WS – 1)	0.689	0.006
Withanone (WS – 2)	0.815	0.006
Withaferin-A (WS – 3)	0.608	0.005
Withastramonolide (WSC)	0.333	0.005
27-Hydroxy withanone (WSCO)	0.032	0.003
Withanolide-D (WSD)	0.041	0.003
Withanoside-IV (WSG-3)	0.267	0.004
Withanoside –VI (WSG-3A)	0.075	0.003
Physagulin-D (WSGP)	0.098	0.004
12-Deoxywithastramonolide (WS-12D)	0.011	0.001
Unidentified Withanolide (WSL-2)	1.212	0.003

Box-1

Fig. 1 – Finger printing of WSF formulation employing HPLC profile of isolated chemical markers. According to protocol-I, HPLC of WSF was performed using acetonitrile: water gradient over a period of 60 min: acetonitrile 10%, 0.01–5 min; 10–60%, 5–30 min; 60–98%, 30–40 min; 98%, 40–45 min; 98–10%, 45–55 min; 10%, 55–60 min. Since WS1, WS2, WSC and WSD were not resolved on HPLC under these conditions, the markers and WSF were separately resolved on HPLC by protocol-II, which involved isocratic resolution employing methanol:H2O (60:40). (Box 1) Box showing the quantification of eleven withanaloids/glycowithanaloids from HPLC chemoprofile of WSF. Names of chemical markers represented by alphabets are also shown. Data are mean ± SD (*n* = 3).

Table 1 – In vitro evaluation of WSF-induced cell growth inhibition (%) in a battery of human cancer cell lines employing protein binding SRB dye assay.

WSF (μg/ml)	Colon					Liver	Lung		Neuroblastoma	CNS	Prostate	
	HCT-15	SW-620	502713	COLO-205	HT-29	Hep-G2	HOP-62	A-549	IMR-32	SKN-SH	DU-145	PC-3
1	0	36	0	6	8	61	6	7	29	37	3	5
3	34	68	0	9	9	77	10	10	63	54	11	21
10	81	80	0	22	34	91	35	38	69	87	38	44
30	97	92	69	36	56	91	56	77	90	94	80	87
100	100	92	88	96	90	100	77	85	99	95	99	99
Adria 2 μM	68	79	69	79	92	87	83	88	92	99	95	89

WSF-induced cytotoxicity in various human cancer cell lines was evaluated as described in Section 2. Cells were treated with indicated concentration of WSF and adriamycin (Adria) for 48 h. The results are expressed as the percent of cell growth inhibition determined relative to that of untreated control cells. Adria dissolved in DMSO was added to cultures (DMSO 0.5% v/v) as positive control. Data are mean value of 8 wells and representative of one of three similar experiments.

growth. The results are expressed as the percent of cell growth inhibition determined relative to that of untreated control cells. It was observed that WSF produced dose-dependent inhibition of cell growth in all cancer cell lines used in the study. WSF at 30 μg/ml inhibited cell growth by more than 50%.

3.2.2. *WSF induces selective cancer cell growth inhibition compared to normal cells*

The extent of WSF-induced cytotoxicity was investigated in normal human gingival fibroblast and human leukaemia 'HL-60' cell lines. The cells were treated with the indicated concentrations of WSF for 48 h and growth inhibition was assayed using MTT. Compared to IC_{50} value of 20 μg/ml in HL-60 (Fig. 2A), it required ⩾600 μg/ml of WSF to produce a comparable effect on hGF cells (Fig. 2B) suggesting that WSF is safe for normal non-cancerous cells.

3.3. *Mechanism of WSF-induced cancer cell cytotoxicity in HL-60*

3.3.1. *WSF induces apoptosis in HL-60 cells*

To determine if WSF-induced cytotoxicity is due to apoptosis or necrosis, human leukaemia HL-60 cells were incubated with different concentrations of WSF for 12 h, and the percentage of cells undergoing apoptosis/necrosis was determined by staining with annexinV–FITC and PI (Fig. 2C). WSF at 30 and 100 μg/ml produced about 37% and 54% of combined apoptotic and post-apoptotic cells. The increase in annexinV/PI positive cell population suggests that WSF is a potent inducer of apoptosis and triggers events leading to apoptotic cell death.

3.3.2. *WSF increases hypo-diploid sub-G_0/G_1 DNA fraction in HL-60 cells*

Another end-point of apoptosis is assayed by the extent of increase in hypo-diploid DNA fraction. For this purpose, HL-60 cells were treated with WSF for 24 h. The cells exhibited concentration dependent increase in hypo-diploid sub-G_0/G_1 DNA fraction (<2*n* DNA) (Fig. 2D). The sub-G_0/G_1 fraction was <7% in untreated control cells which increased to ~34% and 67% in cells treated with 30 μg/ml and 100 μg/ml, respectively.

3.3.3. *WSF induces early generation of ROS in HL-60*

WSF was found to produce significant endogenous ROS in HL-60 cells after 12 h of the treatment. For this purpose, cells were stained with DCFH-DA, a selective probe for ROS measurement. WSF increased the cell population of DCF-derived fluorescence by 34% and 55% when cells were treated with 30 and 100 μg/ml of WSF, respectively (Fig. 3A). This increase in ROS was time-dependent and that the population of DCF-positive cells arrived at ~70% after 24 h of treatment (Fig. 3B). Studies were further extended to measure if WSF also induced NO.

3.3.4. *WSF causes robust increase in nitric oxide (NO) in parallel to ROS*

The extent of NO generation in cells was analysed by flow cytometry using NO probe DAF-2-DA (FL-1). It was observed

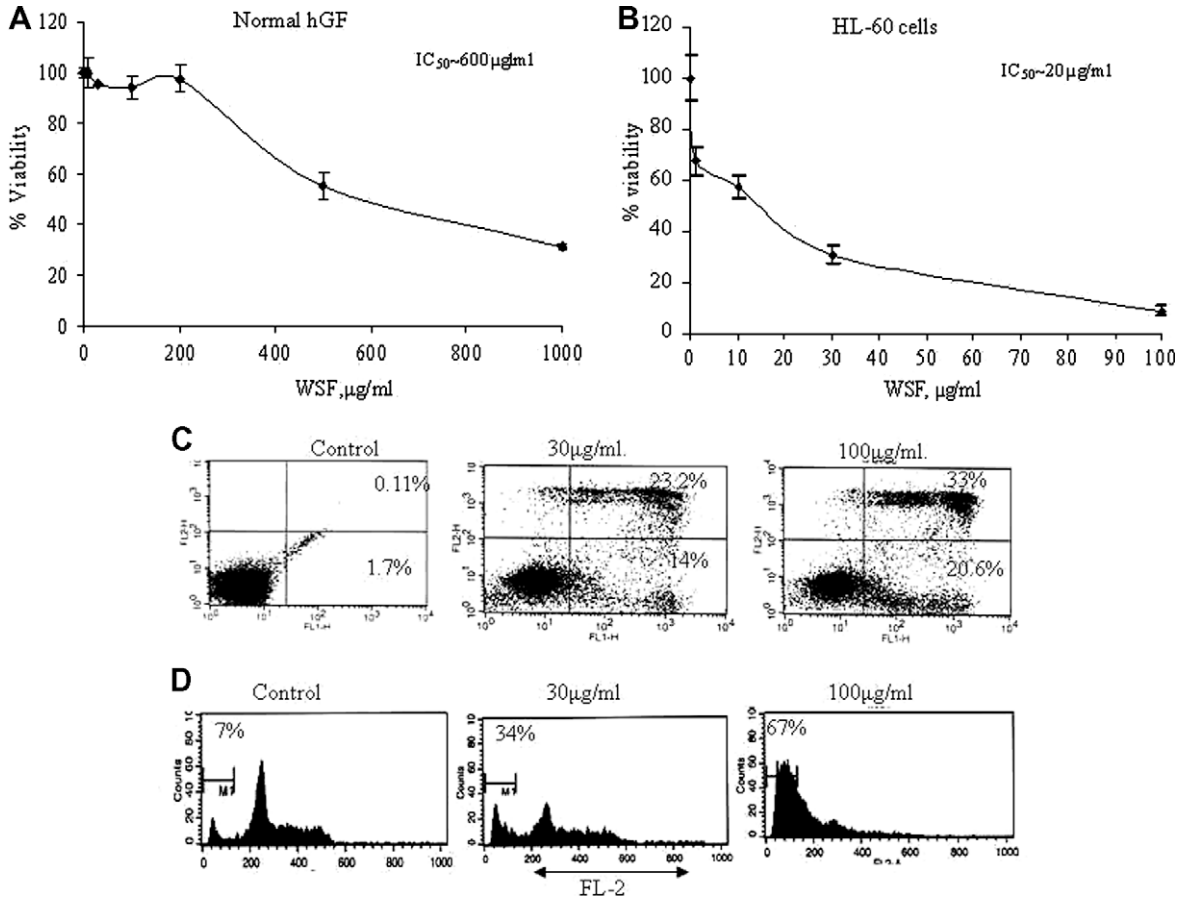


Fig. 2 – Influence of WSF on cell growth inhibition. (A) Normal cell line (hGF) was grown in 96-well culture plate and treated with indicated concentrations (10–1000 µg/ml) of WSF for 48 h, thereafter cultures were incubated with MTT for 2 h at 37 °C for colour formation. Data are mean ± SD (n = 8 wells) and representative one of three similar experiments. (B) HL-60 cells were cultured in 96-well plates and treated with various concentration of WSF for 48 h. Cell viability was measured by MTT assay as described. (C) Flow cytometric analysis of WSF-induced apoptosis in HL-60 cells using annexinV–FITC/PI. Cells were incubated with indicated concentrations of WSF for 12 h and stained with annexinV–FITC/PI to analyse apoptotic and necrotic cell populations. Data are representative of one of three similar experiments. (D) DNA cell cycle analysis in WSF treated HL-60 cells. Cells were exposed to different concentrations of WSF for 24 h. Cells were stained with PI to determine DNA fluorescence and cell cycle phase distribution. Fraction of cells for hypo-diploid (sub-G_0/G_1, <2n DNA) population analysed from FL-2-A versus cell counts (%) is shown. Data are representative of one of the three similar experiments.

that NO levels increased significantly in cells exposed to increasing concentrations of WSF (Fig. 3C). WSF enhanced the NO positive population of cells by 55–90% at the concentrations of 30 and 100 µg/ml, respectively. WSF caused robust generation of NO with time so that cells were more than 90% DAF positive after 12 h (Fig. 3D). WSF produced overwhelming early increase in NO over ROS, though the generation of both ROS and NO proceeded simultaneously in WSF treated HL-60 cells.

3.3.5. *Effect of WSF on mitochondrial membrane potential*

RNOS are known to disrupt the mitochondrial functions and arrest cell growth consequent to persistently high NO generation.[21] As RNOS generation is related to mitochondrial dysfunctions, the effect of WSF on MMP loss ($\Delta\psi_m$) in HL-60 cells was examined. Cells were treated with indicated concentrations of WSF for 12 h and MMP was measured by flow cytometry using specific fluorescent Mito-Sensor JC-1 dye (Fig. 3E). WSF treatment caused concentration dependent loss in MMP evidenced by increase in green fluorescence intensity (FL-1) due to the monomeric JC-1 dye with simultaneous decrease in red fluorescence. The MMP loss was found to be 35% and 63% in cells treated with 30 and 100 µg/ml of WSF, respectively, when only a 15% of MMP loss was observed in untreated control cells.

3.3.6. *WSF is a potent activator of caspases*

After validating cell death by apoptosis measured by several end-points, it is important to determine the involvement of major caspases in apoptotic death by WSF, for instance the activation of caspase-9 and -8 suggests the engagement of both intrinsic and extrinsic pathways of apoptosis which culminates in the activation of caspase-3. The activation of caspase-3, -8 and -9 in HL-60 cells treated with WSF (100 µg/ml)

was measured for indicated time periods that were examined. WSF produced time-dependent activation of caspase-3, -8 and -9 by two to three folds through 12 h whilst prolonged treatment through 24 h the activity reached almost saturation (Fig. 4A–C). The increase in caspase-3 activity exhibited strong correlation with time-related cleavage of PARP in WSF treated HL-60 cells analysed by Western blotting. PARP, an enzyme involved in DNA repair, is a preferential substrate for caspase-3. WSF treatment caused the cleavage of PARP, 116 kDa into 89 kDa in less than 6 h treatment (Fig. 4D).

3.3.7. WSF gears up apoptotic machinery by altering the steady state level of pro- and anti-apoptotic proteins

Reactive oxygen and nitrogen species (RNOS) generation is known to contribute to mitochondrial damage because of oxidative stress. As a result, Bax from cytosol is translocated and integrated into the outer mitochondrial membrane to form pores to allow the release of cytochrome *c* into the cytosol as a prerequisite for mitochondrial mediated pathway of apoptosis.[22] To address the possibility that the WSF-induced apoptosis is related to contributions from the mitochondrial pathway as evidenced by caspase-9 activation, time-dependent influence of WSF on the release of cytochrome *c*, Smac/DIABLO and translocation of Bax into the mitochondria by Western blot analysis of proteins of WSF treated HL-60 cells was evaluated (Fig. 5A). WSF induced time-dependent progressive release of cytochrome *c* and Smac/DIABLO from mitochondria to the cytosol with simultaneous translocation of the Bax from cytosol to mitochondria (Fig. 5A). It may be mentioned that cytochrome *c* and Smac/DIABLO are pro-

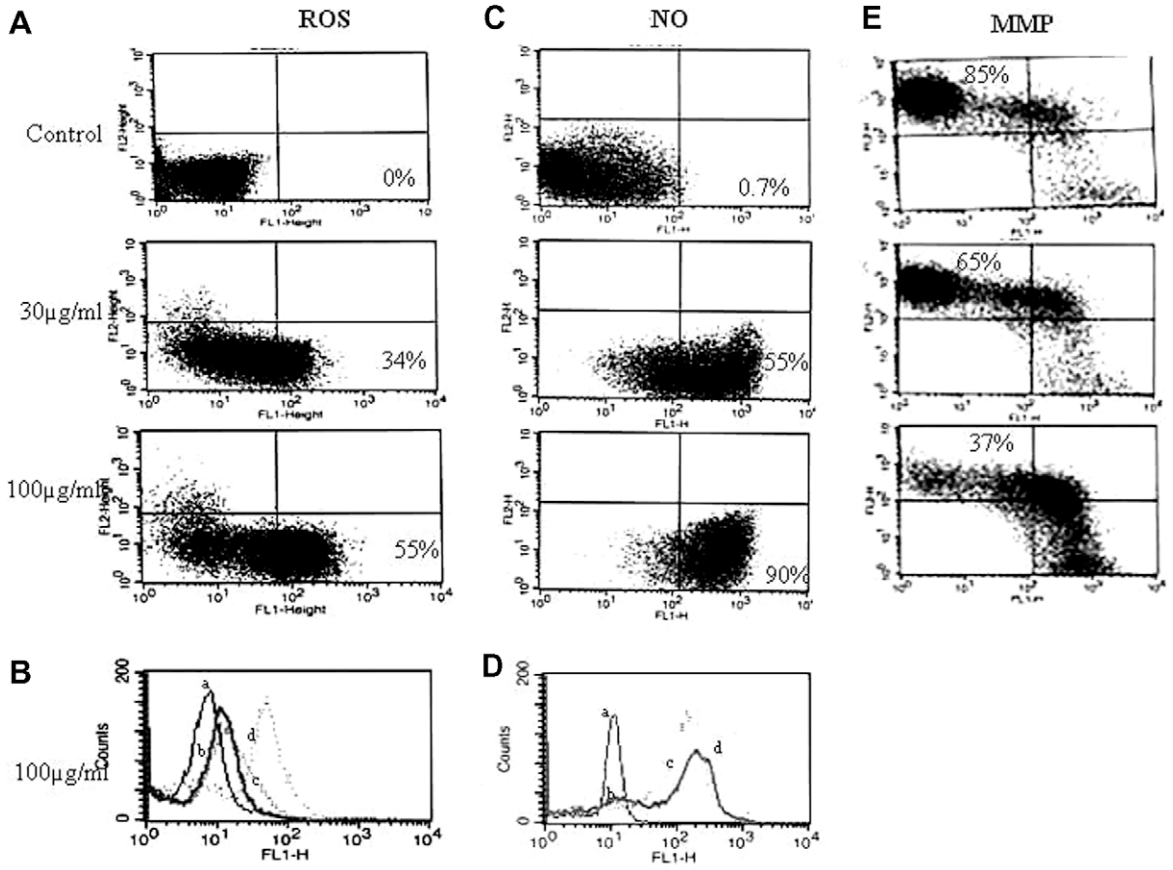


Fig. 3 – (A and B) WSF mediated early generation of ROS in HL-60 cells. Cells (1×10^6/ml) were treated with indicated concentrations of WSF for12 h, followed by incubation with DCFH-DA (5 μM) for 30 min. Cells were analysed for DCF-fluorescence on flowcytometer in the FL-1 (DCF-fluorescence) channel. Data are representative of one of two similar experiments. (B) HL-60 cells were treated with WSF (100 μg/ml) for various time periods and the time-dependent increase in ROS generation is depicted in the histogram, a: control; b: 6 h; c: 12 h; d: 24 h. (C and D) WSF induces early intracellular nitric oxide generation in HL-60 cells. Cells (1×10^6/ml) were exposed to various concentrations of WSF for 12 h, and NO probe DAF-2-DA (5 μM) was added 30 min before WSF treatment. The cells were analysed by flow cytometry for DAF positive NO producing cell fluorescence intensity in FL-1 channel. Other conditions were same as described in Section 2. Data are representative of one of two similar experiments. (D) HL-60 cells were treated with WSF (100 μg/ml) for various time periods and the time-dependent increase in NO generation is depicted in the histogram: a, control; b, 6 h; c, 12 h; d, 24 h. (E) WSF induces mitochondrial membrane potential ($\Delta\psi_m$) loss in HL-60 cells. FACScan analysis of a typical dot plot of $\Delta\psi_m$ loss in HL-60 cells treated with indicated concentrations of WSF. A decrease in FL-2 fluorescence and a concurrent increase in FL-1 fluorescence were indicative of mitochondrial depolarisation. Data are representatives of one of two similar experiments.

apoptotic proteins which are released from the mitochondria during the intrinsic pathway of apoptosis. On the other hand, the expression of Bcl-2, an anti-apoptotic protein that inhibits the translocation of Bax and the release of cytochrome *c*, remained unchanged during the course of WSF treatment. However, the results indicated that WSF potentially disarranged the ratio of Bax:Bcl-2, a factor responsible for the cells to undergo apoptosis (Fig. 5A).

3.3.8. *Influence of WSF on the translocation of AIF from mitochondria to nucleus*

Effect of WSF was also evaluated on the release of apoptosis-inducing factor (AIF) and its translocation to nuclear fraction. Increased RNOS generation originating from electron transport chain (ETC) is also known to be associated with AIF release from mitochondria.[23] Time-dependent increase in the levels of nuclear AIF indicated that the protein has translocated from mitochondrial inter membrane space to nuclei of the WSF treated cells (Fig. 5B).

3.3.9. *WSF also activates the extrinsic apoptotic signalling pathway*

Extrinsic pro-apoptotic signalling occurs from binding of death ligands (e.g. TNF-α, Fas ligand and TRAIL) to their corresponding death receptors (TNF-R1, Fas, DR-4/5) there by recruiting the death domain to initiate apoptosis through the activation of caspase-8. The influence of WSF was observed in HL-60 cells. WSF time-dependently increased the expression of death receptor DR-4 and TNF-R1 levels indicating the activation of extrinsic apoptotic pathway (Fig. 5C). This time-related enhanced over expression of TNF-R1 and DR-4 in WSF treated cells exhibit strong correlation with the increase in caspase-8 activity suggesting the involvement of extrinsic signalling cascade in the induction of apoptosis in addition to intrinsic pathway as discussed above.

3.4. *WSF-induced inhibition of tumour growth in experimental mice*

WSF was compared with its parent extracts, similarly prepared from roots and leaves of *W. somnifera*, for its efficacy in the inhibition of tumour growth in solid tumour models of mouse (Table 2). WSF when administered intraperitoneally at doses of 150 mg/kg b.wt. daily for 9 d produced a significant tumour growth inhibition of 52% in sarcoma 180% and 76% in EAC mice tumour models, whilst at 100 mg/kg it produced 41% inhibition in EAT mouse model. The tumour growth inhibition by root extract at similar doses, however, was negligible poor. On the other hand, the leaf extract showed comparable tumour inhibition at the dose of 100 mg/kg whilst a higher dose (150 mg/kg) turned out to be highly toxic as all animals died during the course of treatment. Interestingly, WSF at

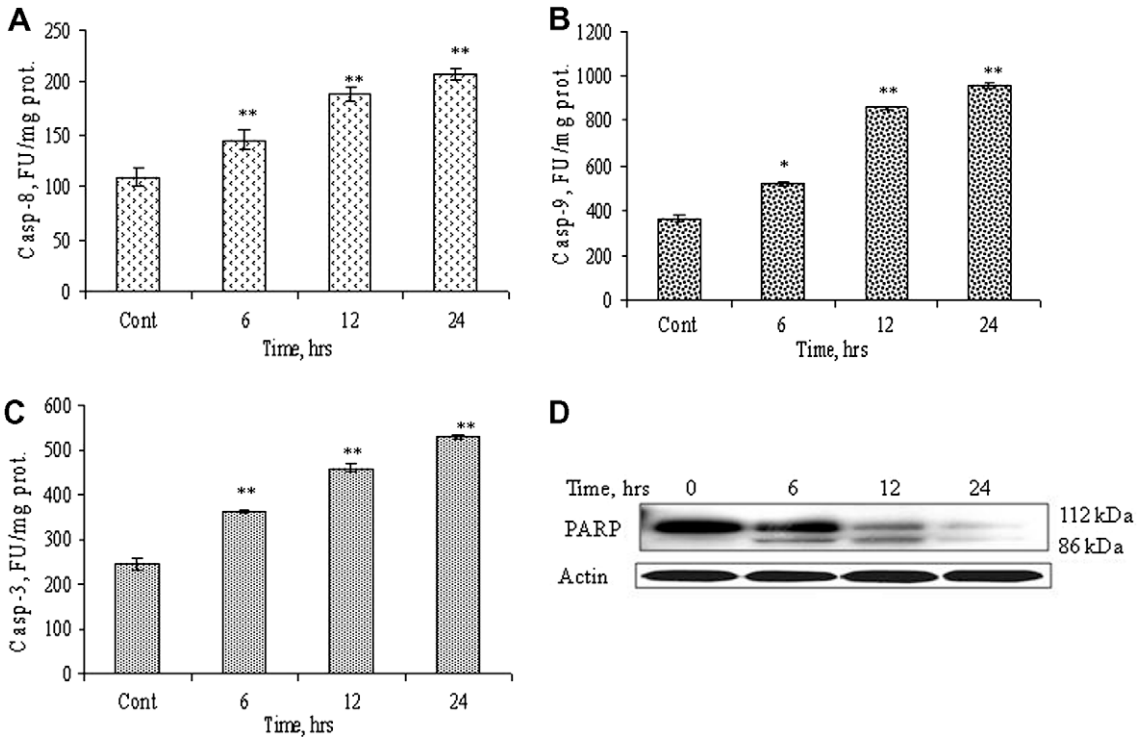


Fig. 4 – (A–C) WSF induced differential activation of various caspases in HL-60 cells. The cells in culture were exposed to 100 μg/ml of WSF for indicated time periods for the estimation of caspase-8 (A), caspase-9 (B) and caspase-3 (C) activities. The caspase activities were determined fluorimetrically in the cell lysate of HL-60 cells using BD ApoAlert caspase assay kits as per instructions of the manufacturer. Data are mean ± SD from three similar experiments. *p*-Values: *<0.05, **<0.01 compared to untreated control. (C) WSF caused cleavage of PARP. Western blot analysis of PARP cleavage was performed in total cell lysates of HL-60 cells for indicated time periods. Data are representative of one of two similar experiments.

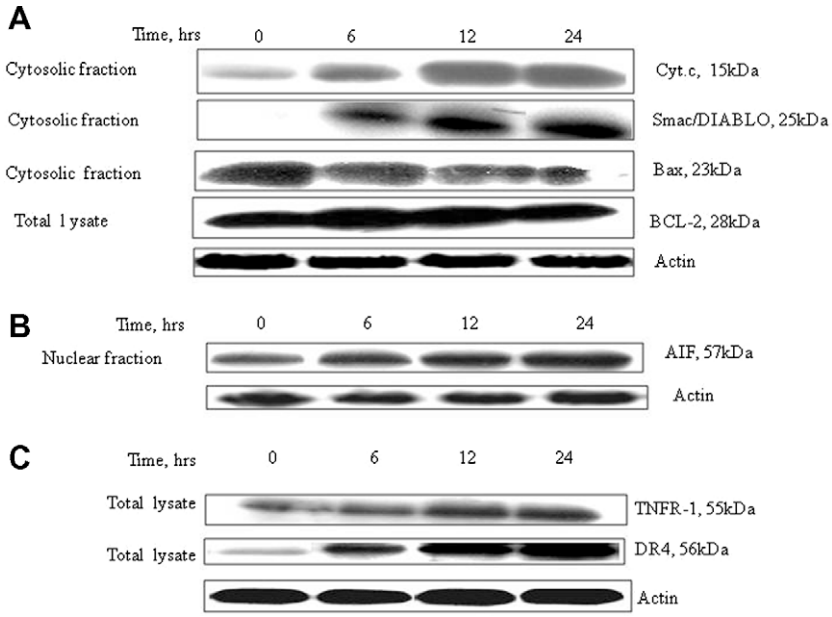


Fig. 5 – Influence of WSF on the expression of critical proteins involved in the initiation of apoptosis. (A) HL-60 cells (2×10^6/2 ml) were treated with 100 μg/ml of WSF for indicated time periods. Equal amount of protein was loaded for SDS–PAGE, and specific antibodies were used for detection of cytochrome c, Smac/DIABLO, Bax and Bcl-2. Data are representative of one of two similar experiments. (B) Immunoblot analysis of AIF in nuclear fraction of WSF treated HL-60 cells. Cells (5×10^6/5 ml) were treated with 100 μg/ml of WSF for indicated time periods. Nuclear fractions were prepared and protein (50 μg) was resolved on 10% SDS–PAGE gel for Western blot analysis. Data are representative of one of two similar experiments. (C) Western blot analysis of TNF-R1 and DR-4. HL-60 cells (3×10^6/3 ml) were treated with 100 μg/ml of WSF for indicated time periods. Whole cell lysate was prepared as described in Section 2. Protein samples were resolved on 10% SDS–PAGE and blotted with specific antibodies. Data are representative of one of two similar experiments.

Table 2 – Comparative analysis of anti- tumour effect of WSF with root and leaf extracts on various mouse tumour models.

Extracts (50% alcoholic)	Mouse tumour models	Dose – mg/kg (i.p.)	Tumour wt. (mg) ± SE	Tumour growth inhibition (%)
Ehrlich Ascitic Tumour (solid)				
		Control	2555.74 ± 45	
Leaf extract		100	1303.52 ± 32*	49
Root Extract		100	2044.25 ± 54*	20
WSF		100	1507.14 ± 29*	41
5FU		22	971.37 ± 17*	62
Sarcoma-180 (solid)				
		Control	2184.28 ± 55	
Leaf extract		150	Toxic	Intolerable
Root Extract		150	1703.57 ± 43*	22
WSF		150	1048.57 ± 52*	52
5FU		22	1070.16 ± 29*	51
Ehrlich Ascitic Carcinoma (suspension)				
			(Cells/ml) × 10^7	
		Control	336.42 ± 53	
WSF		150	80.7 ± 23*	76
WSF		350 (oral)	131.42 ± 24*	61
5FU		22	17.8 ± 3*	95

Efficacy of WSF was compared to that of its parent 50% alcoholic extracts of leaves and roots for the *in vivo* antitumour activity in different mouse models. One day after the injection of cancer cells (i.p. or i.m.), plant extracts and WSF were administrated intraperitoneally, and wherever indicated by oral route, to the animals for 9 d and tumour evaluation was done on day 13. Treated mice received different doses of WSF and extracts whilst control groups received vehicle only. 5FU treatment served as positive control. Comparative analysis for the efficacy in tumour growth inhibition by WSF to that of individual parent extracts was observed. Other conditions were the same as described in Section 2. Data are mean ± SE ($n = 7$).

* p-Values < 0.05.

comparative doses was not toxic in either suspension or solid tumour model.

WSF was also tested orally in Ehrlich Ascites Carcinoma (suspension) mouse model. It exhibited significant tumour cell growth inhibition of 62% when administrated daily for 9 d at the oral dose of 350 mg/kg b.wt. Animals treated with WSF appeared healthy and active, and no mortality occurred during the treatment period.

3.5. *Immune stimulatory properties of WSF*

3.5.1. *Influence of WSF on Th1 and Th2 cytokine expression in vitro*

Splenocytes isolated from BALB/c mice were incubated with Con A (0.5 μg/ml) and indicated concentrations of WSF for 48 h. Cytokine estimation was performed in the supernatants of culture using ELISA. WSF was found to stimulate the secretion of Th1-cytokine IFN-γ (Fig. 6A). The optimum secretion of IFN-γ was found at 0.1 and 1 μg/ml of WSF. However secretion of IL-4, a Th2 cytokine observed a negligible decline with the treatment of WSF and by large its level was comparable to the untreated cultures (Fig. 6B). This suggested that WSF activity follows the immune stimulation similar to the one observed for root extract[12] and that WSF skews immune system to Th1 immunity.

3.5.2. *WSF induces nitric oxide generation in peritoneal macrophages in vitro*

Similarly the effect of WSF was investigated *in vitro* on nitric oxide secretion by peritoneal macrophages obtained from normal mice. The macrophages were primed with LPS (1 μg/ml) and incubated with different concentrations of WSF. WSF treatments (1 μg/ml) in macrophages showed threefold increase in the production of nitric oxide over LPS control (Fig. 6C).

3.6. *Tumour growth inhibition with concomitant immune activation by WSF*

3.6.1. *WSF treatments inhibit tumour growth in mice*

Oral doses of 100, 200 and 400 mg/kg b.wt. were administrated to the mice on the day 1 of the induction of Ehrlich Ascites Tumour (EAT). One set of mice groups were treated with WSF for 9 d and tumour inhibition was evaluated on day 13 whilst another set of mice received similar treatment for 21 d and tumour evaluation was done on day 23. Etoposide (25 mg/kg) was used as positive control whilst untreated control groups

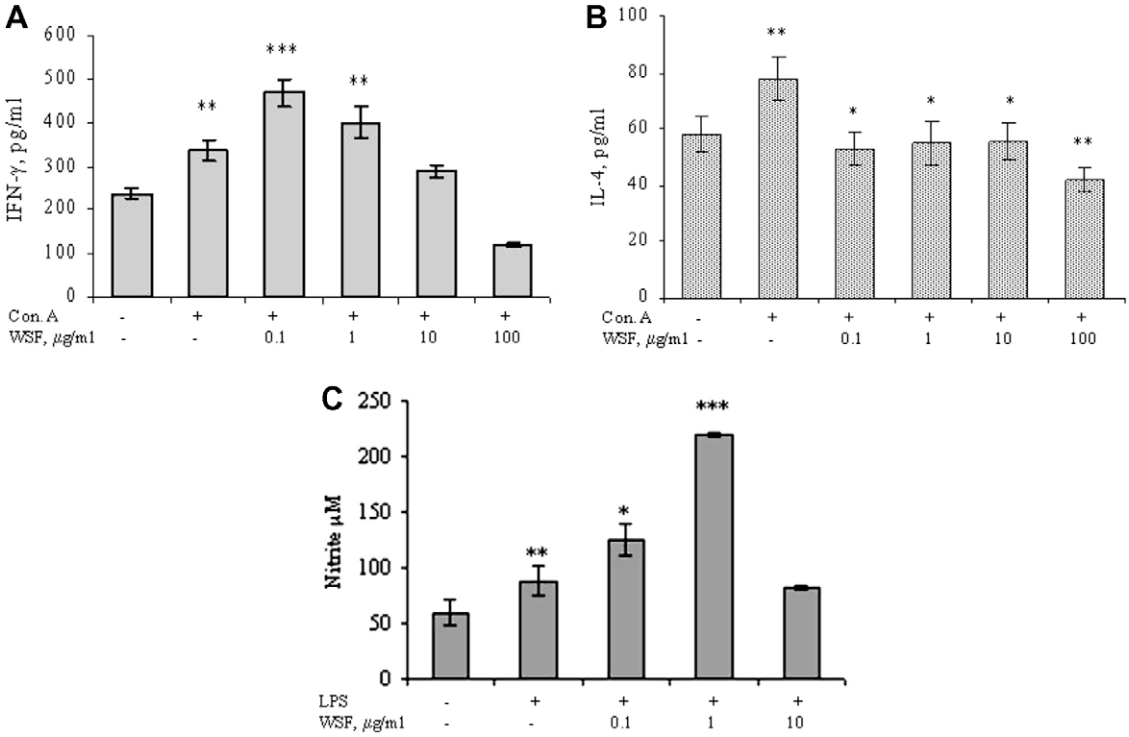


Fig. 6 – (A and B) Influence of WSF on cytokines expression in mouse splenocytes *in vitro*. Splenic lymphocytes isolated from BALB/c mice were grown in 24-well cell culture plates (2×10^6/well) in RPMI + FBS (10%). Cells were co-incubated with 0.5 μg/ml Con A and different concentrations of WSF (0.1–100 μg/ml) for 48 h. The cytokines IFN-γ (A) and IL-4 (B) were assayed by ELISA. The results are presented as mean ± SD ($n = 4$). *p*-Values: *<0.05; **<0.01; *<0.001 (control versus Con A and Con A versus Con A + WSF). (C) Influence of WSF on nitric oxide production in mouse peritoneal macrophages *in vitro*. Macrophages (3×10^6 cells/well) cultured in 24-well culture plates were stimulated with LPS (1 μg/ml) and subsequently treated with WSF (0.1–10 μg/ml) for 48 h. The supernatants were used for nitrite assay as described in Section 2. The results are presented as mean ± SD ($n = 4$). *p*-Values: *<0.05; **<0.01; ***<0.001 (control versus LPS and LPS versus LPS + WSF).**

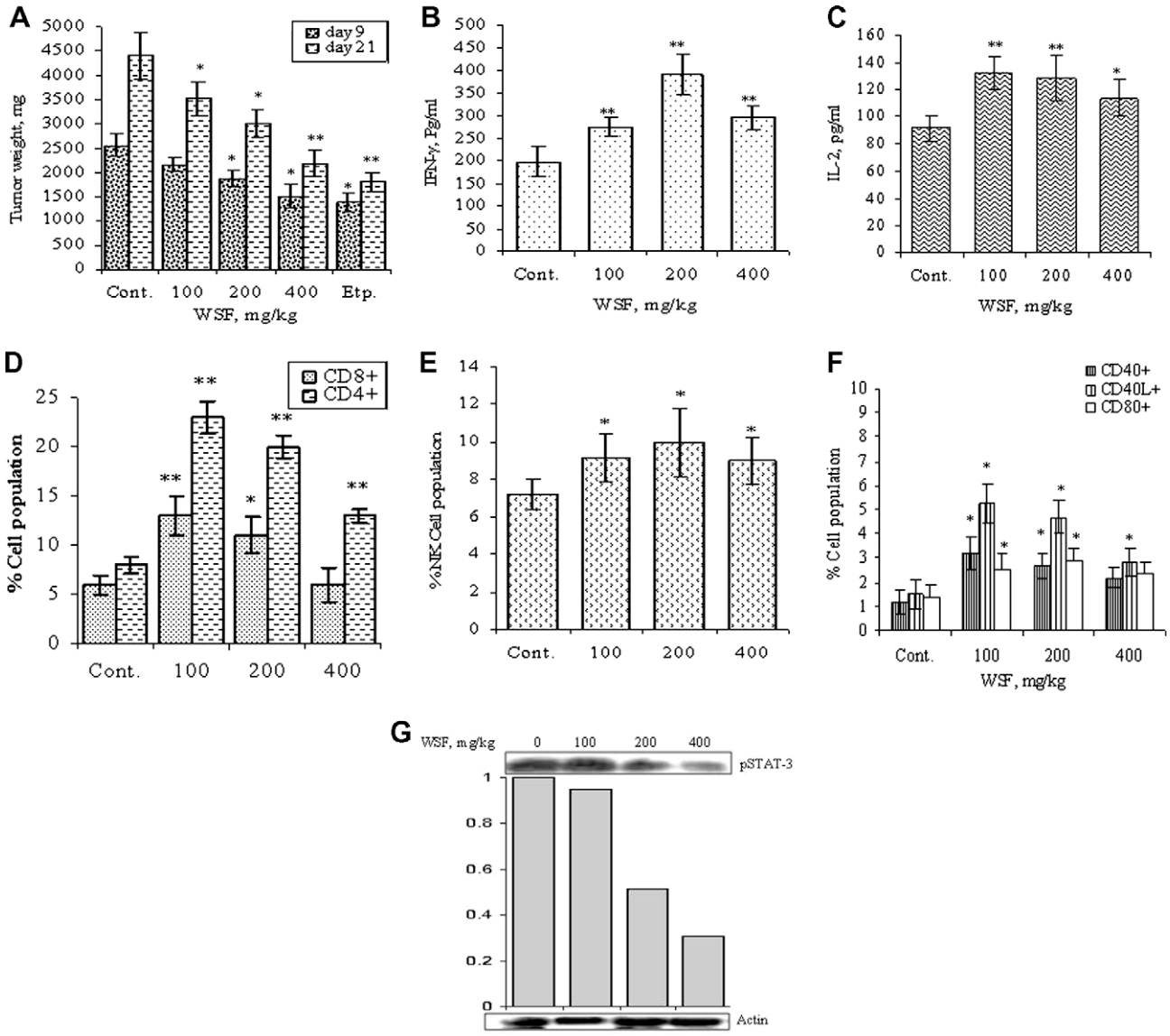


Fig. 7 – (A) Effect of WSF on tumour growth inhibition during the development of EAT in mice. Tumour was induced in Swiss mice as described in Section 2. WSF was given orally for 9 d and 21 d, 1 d after the injection of Ehrlich ascites cells and tumour sizes were evaluated on day 13 and 23. Etoposide (Etp.) was used as a positive control. Effect of WSF and Etp. on the inhibition of tumour growth is shown. Data are mean ± SE ($n = 6$). p-Values: *<0.05; **<0.01. (B and C) Cytokines expression in the sera of tumour-bearing mice. Serum samples were collected from tumour-bearing mice for cytokine analysis after treatment with different oral doses of WSF on day 23. Cytokines assay (B) IFN-γ and (C) IL-2 were performed by ELISA. The results are presented as mean ± SD ($n = 6$). p-Values: *<0.05; **<0.01. (D) FACS analysis of $CD4^+/CD8^+$ T cells in WSF treated mice. Blood samples (100 μl) taken from each mice on day 23 were evaluated for the expression of different cell surface markers. Briefly, samples were incubated with rat anti-mouse CD4–FITC and CD8–PE monoclonal antibodies for 30 min. Cells were washed and analysed on FL-1 versus FL-2 channels of flowcytometer corresponding to emission spectra of FITC and PE fluorochromes, respectively. Dot plot of percent gated population is shown in histograms. Other conditions were the same as described in Section 2. Data are mean ± SD ($n = 6$). p-Values: *<0.05; **<0.01. (E) FACS analysis of NK cells. Blood samples from WSF treated and untreated mice were stained with a FITC labelled anti-mouse NK1.1 antibody and subjected to flow cytometric analysis in the same way as described above. Data are mean ± SD ($n = 5$). p-Values: *<0.05. (F) A typical bivariate flow cytometric analysis of blood samples for the expression of CD40/CD40L and CD80. The percent gated population corresponding to each phenotype marker was carried out by staining blood samples with FITC conjugated anti-CD40, anti-$CD80^+$ and PE labelled anti-$CD40L^+$ antibodies. Dot plot of percent gated population is shown in histograms. Data are mean ± SD ($n = 6$). p-Values: *<0.05. (G) Stat-3 analysis by immunoblotting. Tumour tissues were excised from mice and homogenised in ice cold RIPA buffer by electric homogenizer. Homogenate was centrifuged and equal amount of protein samples were resolved on 10% SDS–PAGE and blotted with specific antibodies. Data are representative of one of two similar experiments.

received vehicle only. Studies showed that tumour growth was inhibited dose dependently in the mice bearing solid EAT when evaluated on different time periods (Fig. 7A). Tumour growth inhibition with the oral doses of 100, 200 and 400 mg/kg in WSF treated animals was found to be 8%, 20% and 37% in the set of animals treated for 9 d whilst prolonged treatment in another set of animals treated for 21 d showed 21%, 31% and 51% of tumour growth inhibition compared to untreated controls. Tumour growth inhibition by etoposide used as positive control was found 35% and 59% after 9 and 21 d treatment, respectively.

3.6.2. *WSF stimulates Th1 cytokine expression in tumour-bearing mice*

The effect of WSF was observed on the Th1 cytokine production in the sera of tumour-bearing mice on day 23. WSF dose dependently enhanced the secretion of IFN-γ and IL-2 in blood sera (Fig. 7B and C). In case the treatment of various doses of WSF the expression of tumour reactive cytokines (IFN-γ and IL-2) was significantly enhanced, whilst the level of Th2 cytokine, IL-4 was below the level of detection (not shown). These observations showed that WSF not only impaired the tumour size but also helped in the activation of Th1 immune system, whose down-regulation otherwise worsens the severity of the disease.

3.6.3. *WSF stimulates proliferation and differentiation of lymphocyte in tumour-bearing mice*

Blood samples of mice treated with WSF for 21 d were taken on day 23 for the evaluation of helper ($CD4^+$) and cytotoxic ($CD8^+$) T cell subsets. As these cells are major component of cell-mediated immunity and their count generally went down with the progression of the tumour. It was found that treatments with WSF showed optimum stimulation in the proliferation of $CD4^+$ (two–threefold) and $CD8^+$ cells (~twofold) at the doses of 100 and 200 mg/kg compared to untreated control, whilst at higher dose of 400 mg/kg the $CD8^+$ leveled to untreated control whilst $CD4^+$ still remains elevated by 50% (Fig. 7D).

3.6.4. *WSF enhances the natural killer cells in tumour-bearing mice*

Natural killer cells are vital part of tumour reactive immune system. NK cells directly suppress tumour growth and activate the rest of the immune system. Tumour-bearing mice treated with different doses of WSF (100, 200 and 400 mg/kg) registered an increase in NK cell population by 20–40% over untreated mice group (Fig. 7E).

3.6.5. *Influence of WSF on the expression of co-stimulatory molecules in tumour-bearing mice*

Blood samples from WSF treated and untreated tumour-bearing mice were stained with FITC labelled CD40 and CD80, and PE labelled CD40L and analysed by flow cytometry. CD40 is expressed on antigen presenting cells (APCs) and CD40L on activated T cells and their interaction plays a critical role in immune stimulation against tumours by activating cytotoxic T cells by CD4 cells.[24] It was observed that WSF enhanced the expression of CD40 and predominantly CD40L by threefold (Fig. 7F), indicating that WSF increases the activation of co-stimulatory molecules that are important in potentiation of adaptive immunity against tumours. CD80 another essential secondary signal molecule present on APCs for T cell activation and its interactions with CD28 enhance the immune response to tumours. WSF treated mice showed significant increase (1.5-fold) in the expression of CD80 positive cell population compared to that of control cells, and the increased expression was consistent with the given doses (Fig. 7F).

3.6.6. *WSF inhibits Stat-3 expression in tumour tissue*

Stat-3 is an important molecule that mediates cross talk between tumours and immune system. Constitutive expression of Stat-3 causes tumour progression with tumour induced immunosuppression by down-regulating Th1 immune response. Expression of Stat-3 was observed in tumour tissues of WSF treated (21 d) and untreated mice. WSF was found to suppress the expression of pStat-3 in a dose-dependent manner so that it was four fold lower than the constitutively expressed control level (Fig. 7G).

3.6.7. *WSF enhances the T cell activation in camptothecin treated tumour-bearing mice*

Splenocytes isolated from WSF treated and untreated tumour-bearing mice were analysed for T cell $CD3^+$ cell activation. WSF at a dose of 100 mg/kg significantly enhanced the $CD3^+$ population in the WSF treated tumour-bearing mice. WSF in combination with camptothecin (0.5 mg/kg) was able to enhance the $CD3^+$ cells by 40% over the camptothecin treated group that remained almost equal to control (Fig. 8A and B).

3.6.8. *Effect of WSF on haematological parameters*

The haematological parameters of WSF treated and untreated (21 d) tumour-bearing mice were evaluated (Table 3). Haemoglobin level that generally goes down during the progression of tumour was found to improve in the mice treated with various doses of WSF. The MCV and MCH which are related to the condition of the RBCs, and the HCT values were improved in WSF treated mice compared to that of untreated control. The number of important immune cells like WBCs in WSF treated mice was found significantly increased indicating immune stimulatory potential of WSF. Also a significant augmentation of lymphocytes, monocytes and granulocytes was found in the mice treated with indicated doses of WSF.

3.7. *Safety profile of WSF during acute and chronic studies*

During acute toxicity studies in mice, LD_{50} values were >2000 mg/kg and >1000 mg/kg by oral and intraperitoneal administration of WSF, respectively. No mortality occurred during or after the treatment of WSF up to 28 d of observation period. The animals in the oral route administration group showed better general appearance as compared to the control group. At the end of experiment, the animals' vital organs, i.e. liver, spleen kidney, lungs, heart, stomach and intestine, were observed for gross pathology. All the organs were found normal without any atypical appearance (data not shown).

The chronic toxicity of over a period of 6 months was performed in Wistar rats. Rats were daily given graded doses of WSF (500, 1000 and 1500 mg/kg b.wt.) orally for 6 months

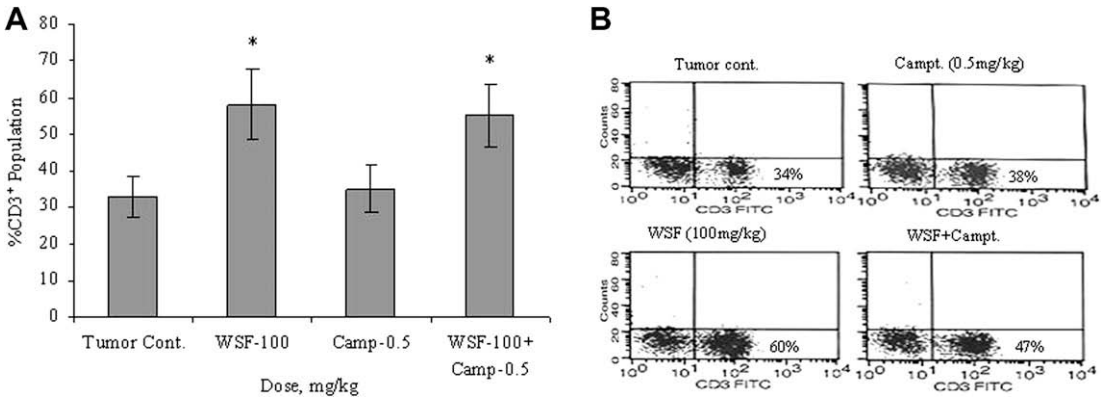


Fig. 8 – Flow cytometric analysis of CD3⁺ T cell proliferation of WSF treated tumour-bearing mice. (A) Splenocytes were isolated from mice treated with WSF alone and in combination with camptothecin. Cells were stained with FITC conjugated anti-CD3⁺ antibodies for the evaluation of the expression of T lymphocytes. Dot plot analysis of percent gated population is represented in histograms. Data are mean ± SD ($n = 5$). p-values: *<0.05. (B) Data are representative of one of the typical dot plots representing CD3⁺ T cell analysis by flow cytometry.

Table 3 – Haematological parameters of WSF treated tumour-bearing mice.

WSF (mg/kg)	WBC	Lymphocytes × 10^3/mm^3	Monocytes × 10^3/mm^3	Granulocytes × 10^3/mm^3	HCT	HB	MCV	MCH
Control	18 ± 3.9	6.1 ± 1.0	8.9 ± 0.8	7.6 ± 0.7	25.5 ± 3	8.2 ± 0.6	37 ± 2.5	11.9 ± 0.9
100	43.2 ± 3.5**	8.2 ± 1.5*	19.9 ± 2**	15.1 ± 1.8*	30.2 ± 2*	9.2 ± 1.3	39 ± 1.6	12.4 ± 0.6
200	59.8 ± 1**	10.9 ± 0.5*	30.1 ± 1.4**	18.9 ± 1.2*	34.5 ± 4*	11 ± 1.6*	40 ± 0.8*	12.8 ± 0.3
400	49.9 ± 3**	8.3 ± 0.6*	24.3 ± 0.5**	17.2 ± 0.8*	36.5 ± 7*	11 ± 1.2*	40 ± 0.9*	12.4 ± 0.6

Blood samples were collected from the WSF treated (100, 200 and 400 mg/kg) and untreated tumour-bearing mice on 23rd day, 2 d after the last dose. Samples were immediately taken for haematological analysis of various parameters like white blood cell count (WBC), haemoglobin (HB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), lymphocyte, monocyte and granulocytes by using automatic haematology analyser. Data are mean ± SE ($n = 5$).

* $p < 0.05$.

** $p < 0.01$.

and were constantly observed for feed and water intake, weight loss. Rats were also observed for haematological, biochemical and genotoxicity assays. The data were comparable to untreated control and nothing abnormal detected in WSF treated groups (data not shown).

4. Discussion

The objective of the present study was to prepare and study the chemo-immunotherapeutic activity of the herbal formulation targeting cancer cell proliferation and the immune system around the tumour microenvironment. WSF is a blend of extracts bearing cancer cell cytotoxicity along with tumour reactive immune up-regulation. The results of the present study demonstrate that WSF exhibited selective cytotoxicity against a panel of human cancer cell lines *in vitro* compared to normal cells and effectively inhibited tumour growth in mouse tumour models. Besides its antitumour effect, WSF also stimulated the cell-mediated Th1 immune response in tumour-bearing mice. HL-60 cells were used to elucidate the mechanism of cell death induced by WSF. The results demonstrate that the exposure of HL-60 to WSF enabled apoptotic cell death as evidenced by increased annexinV positive cell population and increase in sub-G_0/G_1 hypo-diploid DNA fraction. As enhanced production of RNOS has been shown to result in mitochondrial oxidative stress,[25,26] interestingly RNOS generation was overwhelmed during the early events of WSF exposure suggesting thereby an early pro-oxidative environment leading to apoptotic cell death. WSF in HL-60 cells has exquisitely enabled to induce disruption of mitochondrial function, with concurrent loss of mitochondrial membrane potential ($\Delta\psi_m$). Translocation of Bax may abet in the oxidative burst leading to the release of Cyt-c from mitochondrial inner membrane to cytosol where it binds to Apaf-1 to activate caspase cascade.[27,28] WSF induced early translocation of Bax to mitochondria consequent to the disruption of mitochondrial membrane function as was observed with withaferin-A.[13] The anti-apoptotic protein Bcl-2 is reported to block the release of cytochrome c and MPT opening by preventing ROS production.[29] No change was observed in the expression of Bcl-2 in WSF treated cells, but overall Bcl-2/Bax ratio was impaired. WSF treatment induced the release of Cyt-c and Smac/DIABLO in the cytosol suggesting the activation of intrinsic pathway of apoptosis. AIF, another pro-apoptotic

protein is translocated from mitochondria to nucleus during oxidative stress[23,30] and it was found that WSF treated cells showed timely accumulation of AIF in the nucleus. Apoptotic cell death also involve extrinsic signalling cascade emanating through the activation of apical death receptors leading to caspase activation.[31] In this study, we found that WSF treated cells over expressed the TNF-R1 and DR-4 with attendant caspase-8 activation showing the involvement of extrinsic pathway in the cell death. Phytomolecules, which confer apoptosis in cancer cells can be promising anticancer therapeutic candidates, this was validated from our *in vivo* studies where oral as well as intraperitoneal treatment of WSF effectively inhibited tumour growth and simultaneously neutralised the toxicity of leaf extract.

The next aim behind the preparation of WSF was to have a herbal product exhibiting its immunostimulatory properties besides its anticancer activity as discussed above. Cellular immune response is weakened in cancer patients or tumour-bearing animals,[32] with gradual shift from Th1 to Th2 cell phenotype leading to immune suppressive environment and tumour reactive immune dysfunction.[33] It follows that immunologic intervention with agents that selectively activate type-1 responses, whether directly or indirectly, by decreasing Th2 responses may be effective in activating NK cells, cytotoxic T lymphocytes, and tumouricidal macrophages, thus increasing the ability to kill tumour cells. The interesting outcome of WSF treatment was the significant up-regulation of Th1 cytokines in Con.A sensitised splenocytes *in vitro* eliciting a significant production of IFN-γ whilst the level of IL-4, however, remained unaffected. IFN-γ activates macrophages to generate large amount of NO consequent to enhanced inducibility of nitric oxide synthase (iNOS).[34] These macrophages in turn play an important role in immune surveillance against tumours during their development by presenting tumour antigen to cytotoxic T cells and releasing tumouricidal substances like cytokines and nitric oxide. Treatment of WSF when observed on nitric oxide production in macrophages showed augmented release of nitric oxide in LPS stimulated peritoneal macrophages suggesting its role in macrophage activation.

On the other hand, experiments in tumour-bearing mice were conducted to determine the efficiency of WSF in the inhibition of tumour growth vis-à-vis its effect on immune responses. Various oral and intraperitoneal doses of WSF given to mice bearing EAC EAT and Sarcoma-180 showed significant dose related inhibition in tumour growth. As WSF induced tumour growth inhibition in mice, its impact on different immunological parameters was evaluated in EAT bearing mice. Constitutive expression of Stat-3 controls various immunoevasive substances in tumour cells and its intrinsic signalling in haemopoietic cells hinder their performance in tumour immunity including dysfunction of NK cells, granulocytes and dendritic cells which become tolerogenic.[35] Oral treatment of WSF inhibited the expression of pStat-3 when evaluated in tumour tissues of mice showing it targets upstream of tumour promoting signal molecules. WSF treated animals showed significant enhancement in the production of Th1 cytokine (IFN-γ and IL-2) as compared to untreated group showing the promotion of tumour reactive microenvironment by WSF. These cytokines activate tumour antigen-specific cytotoxic T cells and MHC-unrestricted NK cells.[36] WSF treatment in tumour-bearing mice was able to enhance the $CD4^+$ and $CD8^+$ T cell proliferation, whose proliferation and differentiation play a vital role in tumour inhibition. Another important type of antitumour immune cells is natural killer cells that are important players of immune response characterised by having strong cytolytic activity against tumour cells and produce immune stimulating cytokines.[37] Treatment of WSF in tumour-bearing mice showed a significant enhancement of NK cell population showing immune response modifying the properties of WSF.

One of the major obstacles in generating an antitumour immune response is that the most tumours do not express costimulatory molecules like CD40, CD80 (APCs) and CD40L (T cells), whose activation is important in preventing tumour reactive immune dysfunction.[38,39] The present studies demonstrated that WSF administration produced remarkable activation of CD40/CD40L and CD80 co-stimulatory molecules having important role in the activation of cytotoxic lymphocytes and secretion of IFN-γ and nitric oxide. Further to study the adjunct therapeutic efficacy of WSF in terms of its immunostimulating conditioning properties, it was given to mice in combination with conventional anticancer drug. Studies revealed that WSF stimulated the total T cell ($CD3^+$) proliferation by almost 40% indicating again its useful adjunct immunostimulatory properties. Haematological analysis of treated mice showed that WSF treatment can be a better supplement during cancer therapy. Finally antitumour efficacy of WSF was compared to that of its parent individual extracts of roots and leaves. The results showed that WSF is not only more effective than root and leaf extracts in terms of tumour inhibition but also is extremely safe with LD50 > 2000 mg/kg.

In conclusion, the results of this study demonstrate that WSF of defined chemical signature with its immunostimulatory activities is a valuable addition to the therapeutic armoury of anticancer agents. WSF, being of least toxicological consequences is able to inhibit tumour growth in animals and simultaneously activate immune system favoring Th1 immunity. It enhanced macrophage activation, by augmenting the production of NO and activation of costimulatory molecules needed for successful management of malignancy though further in-depth studies are needed in this direction. From the present study, it may thus be concluded that WSF is a potent immuno-chemotherapeutic, which may find usefulness in the management of cancer either alone or as an adjunct to conventional radio or chemotherapy.

Conflict of interest statement

None declared.

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