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Pinus roxburghii constituents augment human lymphocytes mediated cytotoxicity towards cancer cells.

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Running title: Pine needles enhance anticancer potential of immune cells

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Abstract

Background: *Pinus roxburghii* has been used in the Himalayan region as folkloric remedy while nothing is yet known about its immunomodulatory potential.

Methods: The crude extracts of green and fallen pine needles were subjected to sequential fractionation to get partially purified fractions. Human peripheral blood lymphocytes (PBL) were used to analyze immunoenhancing potential of these fractions. Microcytotoxicity assay was employed to investigate improved cancer cells killing capabilities of PBL against various cancer cell lines. GC-MS was carried out to identify the major compounds in the bioactive fractions.

Results: The lymphocyte proliferation assay depicted the immunoenhancing potential of extracts and fractions of fallen and green needles of *P. roxburghii*. The ethyl acetate fractions of both fallen and green needles displayed highest mitogenic activity on human PBL. Both fractions heightened the expression of cell surface markers (CD3, CD8, and CD56) and significantly increased the production of cytokines (IL-2, IFN-γ and TNF-α). The enhanced intracellular granulysin (immunomarker for activated CTLs and NK cells) expression also confirmed immune stimulatory potential of these fractions on human lymphocytes. The ethyl acetate fractions of pine needles enhanced the cytotoxicity of PBL towards various cancer cells (HCT-116, HeLa, PC-3 and A549) as compared to untreated PBL. GC-MS analysis showed presence of major compounds like 3-α-mannobiose, octakis (trimethylsilyl) ether, methyloxime in ethyl acetate fraction of the green needles and cyclodecasiloxane, eicosamethyl in ethyl acetate fraction of the fallen needles.

Conclusion: The bioactive fractions of the fallen and green needles of *P. roxburghii* stimulate cancer cells killing potential of human lymphocytes.

Key words: Anticancer, immunomodulation, granulysin, GC-MS, Pine needles.

1. INTRODUCTION

Cancer is the primary health problem and one of the leading reasons of deaths worldwide. The link between the immune system and cancer has been widely appreciated. Dysfunction of host systemic immunity, explains tumor progression in spite of the presence of tumor-specific T-cells.[1] Previous studies demonstrated that the number of effector cells (T lymphocytes and NK cells) and levels of Th1 cytokines such as interleukin-2 (IL-2) and interferon-γ (IFN-γ) were found to be reduced in the peripheral blood from cancer patients.[2, 3] To restore normal signaling in T cells and NK cells that can be translated into improved anti-tumor functions, natural compounds can be utilized as immune modifying agents. *In-vitro* and *in-vivo* data strongly advocate that several phytochemicals have a significant immunomodulating potential in the maintenance of human health and treatment of various immunological disorder. [4-6]

Pinus roxburghii usually known as "chir pine" belongs to the family Pinaceae and is one of the ornamental trees with tremendous medicinal values. According to the Ayurvedic and Unani systems of medicines, all parts of the *P. roxburghii* have medicinal properties. The crude extract of the pine bark was shown to have high anti-inflammatory and analgesic activities.[7] The essential oil from *P. roxburghii* needles displayed anticancer potential and apoptosis induction on lung, pancreatic, colon, multiple myeloma, head and neck and leukemic cancer cells.[8] Our lab had recently demonstrated that the silver nanoparticles synthesized from the *P. roxburghii* needles showed significant anticancer potential towards A549 (lung cancer) and PC-3 (prostate cancer) cell lines.[9] In other studies, the methanolic extract of *P. densiflora* needles was shown to enhance the secretion of hematopoietic growth factors such as GM-CSF and IL-6 from Peyer's patch cells of C3H/He mice.[10]

Since the immunomodulatory potential of the *P. roxburghii* needles have not been explored much, therefore, we pursued our research work on the immune modifying activity of the fallen and green pine needles. In this context, the special attention needs to be given to the fallen pine needles, which are lying in abundance on forest floor. In India, more than one-million-ton pine needles are available annually as litter which are the major cause of the forest fires.[11] Therefore, the current study was carried out to explore the capability of the phytocomponents from pine needles to stimulate human peripheral blood lymphocytes (PBL) to guard against cancer cells. The anticancer potential of these pre-activated lymphocytes was analyzed against HCT-116 (colon), HeLa

(cervical), PC-3 (prostate) and A549 (lung) cancer cell lines. In this context, till date nothing has been reported on immune cells modulating properties of *P. roxburghii* fallen and green needles extracts and fractions. The identification of the major compounds present in bioactive fractions was carried out by GC-MS analysis.

2. EXPERIMENTAL

2.1 Materials and cell lines maintenance

Chloroform, ethyl acetate, n-butanol, petroleum ether, methanol, Folin-Ciocalteau reagent, sodium carbonate, aluminum chloride, potassium acetate were procured from Merck. All reagents were of analytical grade. Dimethyl sulphoxide (DMSO), phosphate buffer saline (PBS), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT), RPMI-1640 medium, and lymphocyte separation media (LSM-1077), concanavalin A (ConA) and gallic acid were purchased from Himedia, India. Cytokine ELISA kits were from procured from Biolegend. Human cancer cell lines, HCT-116 (colon cancer), PC-3 (prostate adenocarcinoma), HeLa (cervical cancer), and A549 (lung carcinoma) were acquired from the National Centre for Cell Sciences, Pune, India. All cell lines were cultured in DMEM containing 10% FBS and penicillin and streptomycin (100 units/ml and 100 mg/ml, respectively). Cells were kept at 5% CO_2 and 37 °C in a humidified atmosphere.

2.2 Fractionation of pine needles and phytochemical analysis

2.2.1 Collection and authentication of plant materials

The green and fallen needles of *P. roxburghii* (common name: chir pine or long leaf Indian pine) were collected from Solan, Himachal Pradesh, India during the months of April and March. The plant sample was identified by the Department of Forest Products, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, (H.P.), India (Voucher specimens - UHF- Herbarium Field book no. 2916, Receipt No. 051)**.**

2.2.2 Fractionation of *P. roxburghii needles*

The green and fallen needles of *P. roxburghii* were shade dried at room temperature, powdered and stored in air tight bottle until further use. The phytochemicals from needles were extracted with methanol (1:10 ratio) using soxhlet apparatus and the solvents were evaporated *in vacuo* at 50 °C to obtain the dried extracts. The extracts were dissolved in 100 ml of triple distilled water

and sequentially partitioned by petroleum ether, chloroform, ethyl acetate and butanol. The fractions thus obtained from each solvent were dried and dissolved in DMSO at 100 mg/ml for further studies.[9]

2.2.3 Phytochemical analysis

The crude extract and fractions of green and fallen needles of *P. roxburghii* were investigated using the standard protocols for the presence of phytochemical constituents like alkaloids, proteins, amino acid, flavonoids, carbohydrates, tannins, saponins glycosides, steroids and terpenoids.[12]

2.3 Total phenolic content (TPC)

The crude extract and fractions were allowed to mix with 0.02 N Folin-Ciocalteau's reagents at a ratio of 1:5 for 5 minutes followed by addition of 2.0 ml of sodium carbonate (75 g/l).The absorbance was measured after 2 hr of incubation at room temperature at 760 nm using systronics double beam spectrophotometer-2205. Results were analyzed with reference to standard curve and were presented as mg gallic acid equivalents per gram of dry weight (mg GAE/g dry wt.). Each assay was carried out in triplicate.[13]

2.4 Total flavonoid content (TFC)

Each sample was mixed with methanol (10% w/v) and 0.5 ml of the mixtures were dissolved in 0.1 ml of aluminum chloride (10%), 2.8 ml of distilled water, 0.1 ml of potassium acetate (1 M) and 1.5 ml of methanol followed by 30 min incubation at room temperature. The reaction mixture absorbance was recorded using systronics double beam spectrophotometer-2205 at 415 nm. Data was articulated with reference to standard curve and was reported as mg quercetin equivalents per gram of dry weight (mg QE/g dry wt).[13]

2.5 Preparation and cultivation of Peripheral Blood Lymphocytes (PBL)

Blood samples were obtained from healthy human volunteers (age 20-40). Informed consent was taken from all the human donors and the work was done according to the guidelines given by Institute Ethics Committee, Shoolini University, Solan (approval No. SUBMS/IEC/16/12). The blood sample was mixed with the equal volume of PBS and then layered on LSM 1077. The blood samples were centrifuged at RT for 40 min at 400×*g*. The undisturbed lymphocytes layer was

cautiously taken out and washed with PBS twice and resuspended in complete RPMI-1640 media. The PBL cell number was counted by using trypan blue.[14]

2.6 MTT Assay

PBL were seeded in complete RPMI-1640 medium for 24 h in 96-well plate at 37 °C and 5% CO_2. Next day, the cells were treated with extract and fractions (25-200 µg) of *P. roxburghii* to incubate further for 48 h. Con A (10 µg/ml, a standard T cell activator) or DMSO treated cells were utilized as positive and negative controls, respectively. After 24 h and 48 h of treatment, MTT (5 mg/ml) was added and incubated for another 4 h to generate formazan. Followed by removal of the supernatant and addition of 100 µl DMSO to suspend purple formazan crystals for 30 min. The absorbance was observed at 595 nm on microplate reader. The assay was conducted thrice in triplicate and percentage proliferation was calculated.

$$\%\ Proliferation = \frac{OD_{sample} - OD_{control}}{OD_{control}}\ x\ 100$$

Where, $OD_{control}$ showed optical density of the untreated cells and OD_{sample} was the optical density of the cells treated with extracts and fractions.[14]

2.7 Cytokines Analysis

The MTT assay revealed that ethyl acetate fractions of both green and fallen needles of *P. roxburghii* exhibited highest immuno-stimulatory potential therefore, only these two fractions were used for further studies. The human PBL were treated with methanolic crude extracts and ethyl acetate fractions of green and fallen pine needles for 24 h. Untreated and Con A treated lymphocytes were used as negative and positive controls, respectively. The cytokine (IL-2, IFN-γ and TNF-α) production was analyzed in the culture supernatants by ELISA as per the manufacturer's guidelines (Elisa Max™Biolegend).

2.8 Co-culture microcytotoxicity assay

All the target (T) cancer cells (HCT-116, HeLa, PC-3 and A549) were cultured and seeded (1 x 10^4) overnight in 96-well plates at 37 °C and 5% of CO_2. The PBL (Effectors-E) were treated with methanolic crude extracts and ethyl acetate fractions of green and fallen needles (50 µg/ml) for 24

h. The activated PBL were washed twice with media and co-cultured with cancer cells at various E:T ratios (2.5:1, 5:1, 10:1 and 20:1) followed by 48 h of incubation in CO_2 incubator. After that, media and the suspended dead cells (150 µl) were removed and washed twice with media and MTT assay was carried out as given above.The percentage of cell death was analyzed by using following formula:

$$\% \, cell \, death = \frac{OD_{sample} - OD_{control}}{OD_{control}} \, x \, 100$$

Where, $OD_{control}$ displayed optical density for target cells alone and OD_{sample} was the optical density of the co-cultured target cells.

2.9 Granulysin expression analysis

PBL cells ($1x10^5$) were seeded in 12-well tissue culture plate and treated with methanolic extract and ethyl acetate fractions of green and fallen needles of *P. roxburghii* for 48 and 72 h in 5% CO_2 incubator. Con A was used as positive control. After incubation, cells were washed with PBS and fixed in 4% paraformaldehyde at RT for 20 mins. Then cells were washed twice with permeabilization/washing buffer (0.1% Triton X and 1% FBS in PBS). The primary antibody against granulysin (rabbit anti-granulysin, Santa Cruz) was added (1:100 dilution) and incubated for 30 mins on ice followed by incubation with anti-rabbit IgG allophycocyanin (1:400) for 30 mins. Granulysin expression was analyzed by Flow cytometer (BD FACS Canto II) at central facility of Post Graduate Institute of Medical Education & Research, Chandigarh.[15]

2.10 Immunophenotyping by Flow cytometry

The PBL were incubated with ethyl acetate fractions of green and fallen needles (50 µg/ml) for 48 h at 37 °C and thereafter, stained with FITC-tagged anti-CD8, perCP-Cy5-5-A-tagged anti-CD3 and PE-A-tagged anti-CD56 (BD Oncomark™ CD8/CD56/CD3) at 4 °C for 20 mins. Untreated and Con A treated immune cells were used as negative and positive controls, respectively. Following washing with PBS, cells were inspected by flow cytometry (BD FACS CANTO II).[16]

2.11 GC-MS analysis of the bioactive fractions

The ethyl acetate fractions of green and fallen needles of *P. roxburghii* samples were sonicated for 30 min and then passed through an ultra-membrane filter (pore size 0.45 µm) before injecting in the sample loop. Filtrate was used for GC-MS analysis (Agilent GC 7890A) with ion trap gas-chromatograph supplied with HP5 capillary column (30 m x 0.25 mm; coating dimension 0.25 µm). The MS settings encompassed an EI ion source at a temperature of 230 °C, a mass scan range of 40 - 600 amu and ionization energy of 70 eV. Analytical conditions: the maximum temperature was 300 °C; helium at 1 ml/min was used as carrier gas; injection 0.2 µl of n-hexane. Running conditions included:HS SPME injection technique and HS 2.5 mL syringe. The phyto-components were identified by comparing their retention times and liner retention indices with pure samples and by matching with in-built GC-MS software and mass literature.[17]

2.12 Statistical analysis

The data was demonstrated as mean ± SD (standard deviation). The statistical investigation was done by using one-way ANOVA and by Bonferroni's multi-comparison test. GraphPad Prism (version 6.0) was utilized and a probability (p) value < 0.05 was considered as significant.

3. RESULTS AND DISCUSSION

3.1 Phytochemical screening of extracts and fractions from needles

The methanolic crude extracts of *P. roxburghii* green and fallen needles were fractionated sequentially using various solvents to obtain partially purified fractions. The qualitative phytochemical analysis of both green and fallen needle extract and fractions showed the presence of flavonoids, alkaloids, carbohydrates, glycosides, tannins, phenolics and steroids. Both types of needles were found to have similar phyto-components while fatty acids and glycerol were negligible in the fallen needles (data not shown). Recently, alkaloids, tannins and terpenoids, saponins and glycosides have been detected in *P. roxburghii.*[18] Additionally**,** Naeem *et al*., have shown presence of flavonoids in the various fractions of *P. roxburghii* needles.[19]

The TPC analysis revealed highest amount of phenolics in the ethyl acetate fraction of the green leaves (121.58 ± 0.17 mgGAE/g dry wt.) trailed by the ethyl acetate fraction of the fallen needles (108.20 ± 0.03 mg GAE/g dry wt.) of *P. roxburghii* as compared to other fractions. The TFC

estimation showed maximum flavonoids in methanolic extracts of green and fallen leaves (346.11 ± 1.16 mg QE/g dry wt. and 352.14 ± 1.20 mg QE/g dry wt., respectively) (Table 1). Whereas, ethyl acetate fractions of green and fallen needles also displayed appreciable flavonoid content (253.31 ± 1.23 mg QE/g dry wt. and 286.24 ± 3.21 mg QE/g dry wt., respectively**)**. Previously, the ethyl acetate fraction of pine needles were reported to have a high amount of phenolic and flavonoid content.[20]

3.2 *P. roxburghii* needles possess immunomodulatory activity

The immunomodulatory potential of the extracts and fractions of the green and fallen pine needles was examined on human peripheral blood lymphocytes (PBL). The MTT assay showed an increased lymphocytes proliferation with the increase in the concentration of extracts and fractions (25-200 µg/ml) of the pine needles (Figure 1). The ethyl acetate fraction of both green and fallen needles exhibited highest proliferative activity with EC_{50} values of 36.64 ± 0.01 and 25.85 ± 5.84 µg/ml, respectively as compared to other fractions and methanolic extracts (Table 2). These results suggested that the phytoconstituents present in *P. roxburghii* needles exhibit stimulatory effect on human PBL. As shown above, the high amount of the TPC and TFC present in the ethyl acetate fractions could attribute to the highest proliferative effect of these fractions on human PBL. Polyphenols are a large group of compounds synthesized by plants having several beneficial effects on human health. Several researchers have reported the antitumor effect of polyphenols through enhancing the immune response and counteracting the immune escape.[21] To the finest of our knowledge, this is the first study depicting the immunoenhancing potential of the green and fallen needles of the *P. roxburghii* on human lymphocytes. The phenolic extract from *P. massoinana* Lamb bark have been shown to stimulate proliferation of mouse splenocytes in a dose-dependent manner.[22] Similarly, *P. radiata* bark extract have displayed immunomodulatory effects on Korean chickens causing enhanced proliferation of PBL, thymocytes and splenocytes.[23]

3.3 *P. roxburghii* needles boost the cytokine production by PBL

As shown above that ethyl acetate fraction of both green and fallen needles of *P. roxburghii* exhibited the highest immuno-stimulatory potential. Therefore, we utilized these two fractions crude methanolic extract (for comparison) for further studies. The secretion of pro-inflammatory cytokines IFN-γ, IL-2 and TNF-α was examined after 24 and 48 h of treatment of PBL with

extracts and fractions. As shown in (Figure 2), as compared to the untreated lymphocytes, the levels of IFN-γ was found to increase by 4-fold and 2-fold after 24 h of incubation with ethyl acetate fractions of fallen and green needles, respectively. After 48 h of treatment, a further increase in the IFN-γ secretion was observed. Similarly, the IL-2 levels were found be enhanced by □3-fold and 5-fold following incubation with both ethyl acetate fractions for 24 h and 48 h, respectively. Interestingly, the highest levels of TNF-α was observed by PBL upon the treatment with methanolic extract of fallen pine needles, whereas the ethyl acetate fractions of both green and fallen pine needles cause □3 and 2-fold increase in TNF-α levels after 48 h of incubation, respectively.

The enhanced secretion of these cytokines by ethyl acetate fractions of green and fallen pine needles suggest immuno-stimulation of human PBL. These cytokines are known to have a vital role in the immune cell expansion and activation. IL-2 and IFN-γ augment antitumor immune responses by enhancing the cancer cells killing capabilities of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells.[2, 3]

3.4 Immune cells profiling

Both CTL and NK cells have been implicated in tumor cells lysis(3). The FACS analysis showed significantly enhanced presentation of CD3, CD8, and CD56 markers on PBL after 48 h of incubation with ethyl acetate fractions and methanolic extracts of pine needles as compared to that of untreated lymphocytes (Figure 3). The results depicted a 3-fold increased expression of CD3 marker whereas □2 to 3-fold upregulation of CD8 and CD56 markers on PBL treated with *P. roxburghii* methanolic extracts and ethyl acetate fractions as compared to unactivated PBL. The upregulation of these markers on human PBL suggest immune cells expansion and activation via *P. roxburghii* phytoconstituents.

3.5 Granulysin expression analysis

Granulysin is a proinflammatory and cytolytic protein expressed after 3-5 days of (late expression) after stimulation of human peripheral blood immune cells. It is a multifaceted granular protein found in human NK cells and CTLs, with cytolytic potential towards different tumors and microorganisms.[24] Flow cytometric analysis showed up-regulation of intracellular granulysin expression after 48 h (~24- and 21-fold increase) and 72 h (8 and 12-fold increase) of incubation

with ethyl acetate fractions of green and fallen pine needles as compared to untreated PBL respectively (Figure 4a & 4b). These results strongly suggested that the pine needle fractions were capable of modulating expression of cytolytic protein like granulysin in human immune cells. Earlier studies have shown that enhanced granulysin expression can act as an immuno-marker depicting antitumor potency of human immune cells.[15]

3.6 Pine needles stimulate the anticancer activity of PBL

To assess the anticancer potential of the ethyl acetate fractions of the green and fallen pine needles, the activated human lymphocytes (effector cells) were co-cultured with target cancer cells. The co-incubation of immune cells and target cells imitate *in vivo* conditions where lymphocytes induce the cell death in cancer cells. For this purpose, cancer cell lines of different origins (HCT-116, HeLa, PC-3 and A549) were used to analyze the broad-spectrum action of activated immune cells. The co-culture assay showed that with the increase in effector:target (E:T) ratios (2.5:1 to 20:1), enhanced target cancer cells killing was detected (Figure 5). In case of HCT-116, maximum killing (40 %) of cancer cells was observed at 20:1 E:T ratio as compared to only 6 % target cells death via untreated PBL. In both A549 and HeLa cells maximum 36 % and in PC-3 cells 29 % cell death was observed. Our data clearly showed that ethyl acetate fractions (50 µg/ml) of both green and fallen needles were capable of enhancing the anticancer potential of effector cells and displayed similar target cell killing capabilities. Moreover, the activated lymphocytes were capable of killing different types of cancer cells likewise irrespective of their origin. As cancer patients encounter suppressive immune environment, therefore herbal immunotherapy is an alternative and effective strategy.[21] These results clearly demonstrated that phytoconstituents present in both green and fallen needles can act as promising immune-enhancers for the treatment of cancers of several origins. Earlier, *P. densiflora* needles have shown antitumor effects to rats incubated with mammary carcinogen, 7, 12-dimethylbenz[a]anthracene or to the mice injected with sarcoma-180 cells.[25]

3.7 GC-MS analysis

The ethyl acetate fractions of green and fallen needles of *P. roxburghii* were examined by gas chromatography combined with mass spectrometry (supplementary Table 1 and 2, Figure 1). The GC-MS investigation leads to the identification of 28 and 32 compounds present in green and

fallen needle fractions, respectively. The various compounds present in the bioactive fractions of green and fallen needles are listed in order of their elution in supplementary tables 1 and 2. The dominant compound revealed in ethyl acetate fraction of green needles was 3-α-mannobiose, octakis (trimethylsilyl) ether, methyloxime (66.93%) **(1)** and ethyl acetate fraction of the fallen needles was found to be dominated by cyclodecasiloxane, eicosamethyl (25.95%) **(2)**, 3,9-β-14,15-diepoxypregn-16-en-20-one,3,11-β-,18-triacetoxy (15.45%) **(3)** and γ-sitosterol (10.43%) **(4).**

Till date, nothing has been reported on the biological activities of compounds 1, 2 and 3, however these compounds have been found in various plants with anti-neurotoxicity, anti-onchocerca activity and antioxidant activity, respectively.[26, 27] Interestingly, compound 4 (sitosterol) have been shown to boost antitumor immunity and the regression of breast tumor tissues in mouse model.[28, 29]

4 Conclusion

Green and fallen pine needles phytoconstituents showed a promising immuno-stimulatory effect on human PBL. The ethyl acetate fractions of both fallen and green needles of Himalayan *P. roxburghii* displayed highest PBL proliferative capabilities as compared to other fractions. The immune cells profiling, cytokine secretion and co-culture cytotoxicity assay confirmed the immunoenhancing potential of these fractions. The co-incubation assay depicted anticancer response of activated immune cells towards HCT-116, HeLa, PC-3 and A549 cancer cells. Moreover, enhanced granulysin expression in human lymphocytes after treatment with these fractions also showed anti-cancer potential of these immune cells. Based on these results, ethyl acetate fractions of both green and fallen needles of *P. roxburghii* can act as novel cancer immuno-therapeutic agents for a wide range of cancers.

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Conflict of interest

None

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Tables

Table 1: Analysis of TPC and TFC of different fractions of green and fallen needles of *P. roxburghii.* Results are expressed here as mean ± SD; Data was significant as $p < 0.05$. [E = Extract; F = Fraction]

P.roxburghii	**Fractions**	**TPCmgGAE/g dry wt.**	**TFC mg QE/g dry wt.**
Green needles	Methanol (E)	92.12±0.22	346.11±1.16
	Petroleum-ether (F)	12.11±0.15	27.02±1.02
	Chloroform (F)	14.18±0.44	42.24±1.45
	Ethyl acetate (F)	121.58±0.17	253.31±1.23
	Butanol (F)	78.04±0.11	211.03±1.13
	Aqueous (F)	64.11±0.07	156.22±1.41
Fallen needles	Methanol (E)	84.20±0.11	352.14±1.20
	Petroleum-ether (F)	16.28±0.12	31.21±1.46
	Chloroform (F)	19.15±0.27	17.43±1.04
	Ethyl acetate (F)	108.20±0.03	286.24±3.21
	Butanol (F)	82.17±0.16	198.42±1.11
	Aqueous (F)	78.22±0.15	137.01±1.42

Table 2: EC_{50} (µg/ml) values of crude extracts and fractions of green and brown needles of *P. roxburghi* on human PBL after 24 hr of incubation. (F= fraction)

	Methanolic extract	**Petroleum ether F**	**Chloroform F**	**Ethyl acetate F**	**Butanol F**	**Aqueous F**
Green Needles	210.6±0.035	284.3±0.044	394.2±0.058	36.64±0.017	135.2±0.032	Interrupted
Fallen Needles	103.2±0.022	172.6±0.041	Interrupted	25.85±5.84	60.76±0.12	238.6±0.22

Figure Legends

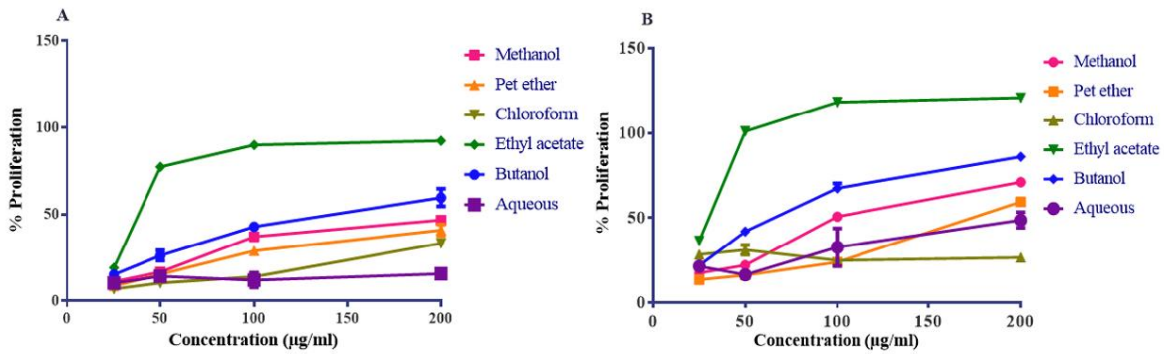

Figure 1: MTT assay showing mitogenic effect of green (A) and fallen (B) needles crude extracts and fractions at various concentrations. Methanol refers to crude extract and Pet ether, chloroform, ethyl aceatate, butanol and aqueous are solvents used to partially purify fractions of the pine needles. Results are expressed here as mean ± SD; Data was significant as $p < 0.05$

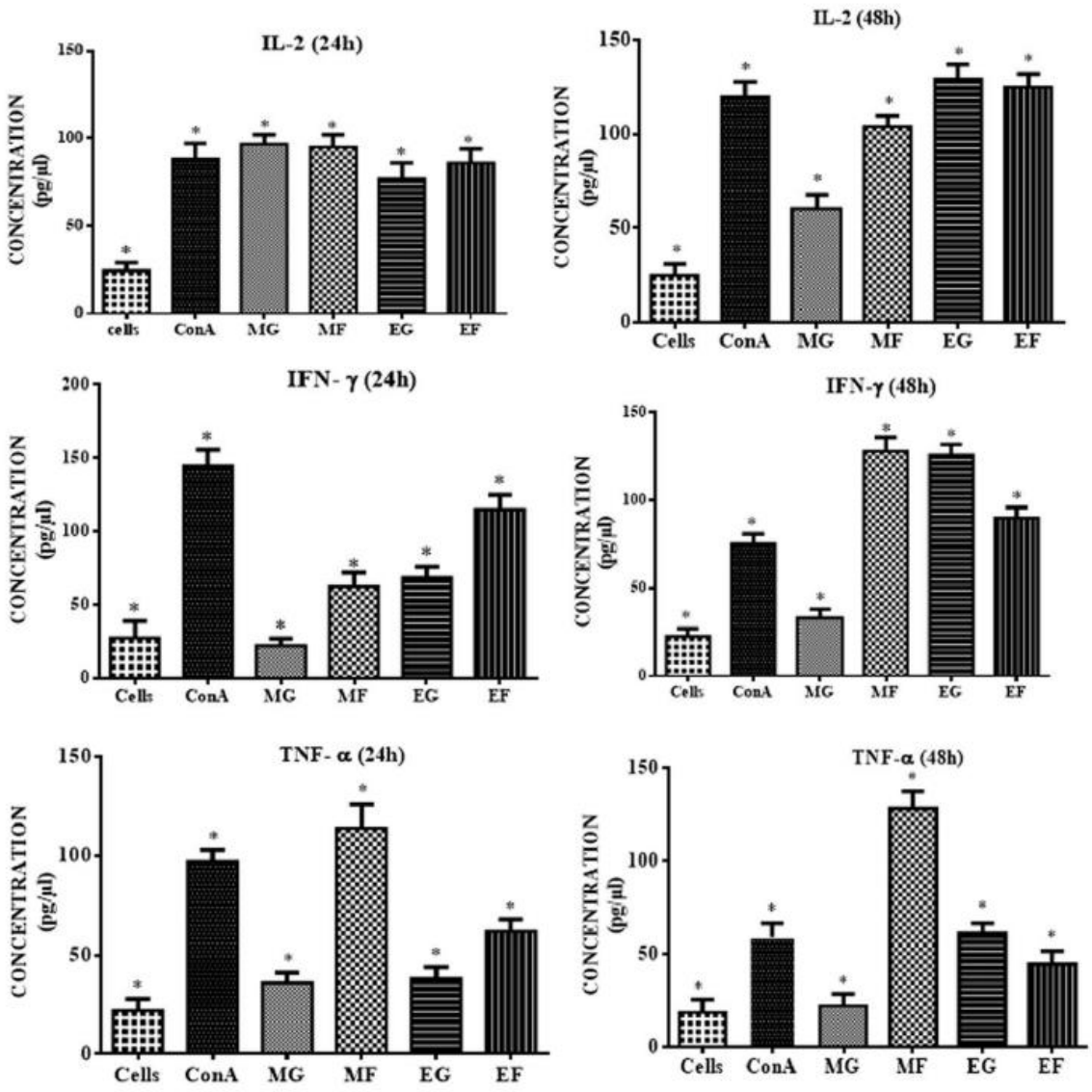

Figure 2: IFN-γ, IL-2, and TNF-α production by PBL after 24 and 48 hr of treatment with extracts and fractions (50 µg/ml) of *P. roxburghii* needles. **MG and MF**: Methanolic extracts of green and fallen needles, **EG and EF**: ethyl acetate fractions of green and fallen needles, respectively. Con A was used as a positive control. Values were expressed as mean ± SD of three independent experiments.* indicates significance of treated groups versus control (*$p \leq 0.05$).

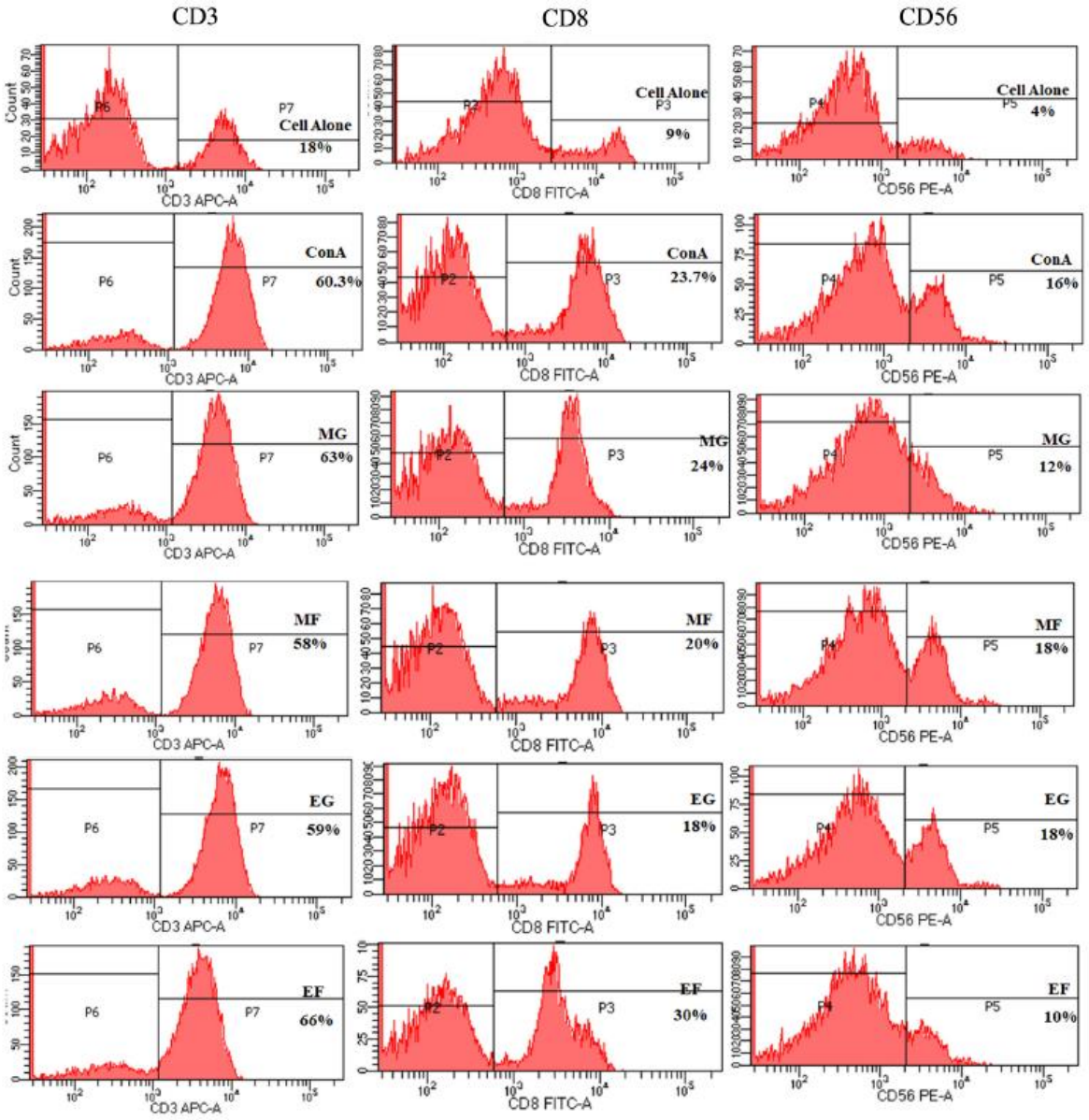

Figure 3: Representative FACS data revealing percentage of the cells having CD3, CD8, and CD56 markers on PBL after 48hr of treatment with methanolic extract and ethyl acetate fractions of green and fallen needles at 50 µg/ml. Untreated PBL (cells alone) and Con A were taken as negative and positive controls, respectively.

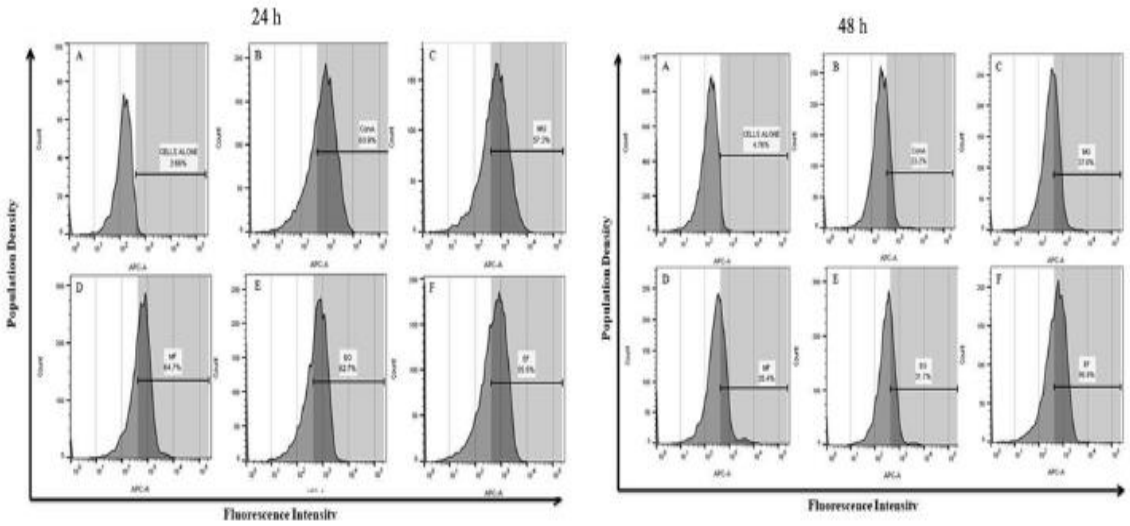

Figure 4: Intracellular granulysin expression of human PBL treated with crude extract and fraction of *P. roxburghii* leaves (50 µg/ml) after 24 and 48 hr. **A**: Untreated cells, **B**: Con A, **C and D**: methanolic extracts of green and fallen needles, **E and F**: ethyl acetate fractions of green and fallen needles, respectively.

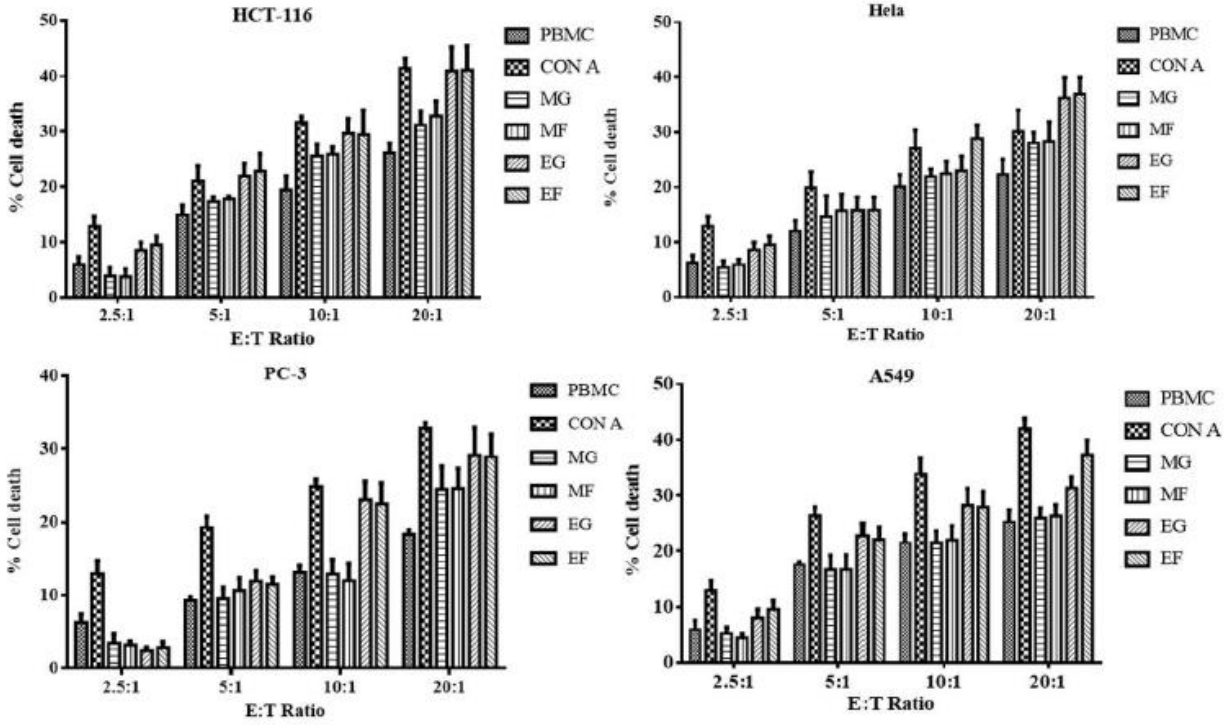

Figure 5: Anti-cancer effect of activated PBL on various cancer cell lines: HCT-116, HeLa, PC-3 and A549 cancer cells were co-incubated with preactivated PBL at different E:T ratios (n=3) for 48 hr. PBL were pre-treated with methanolic crude extract (MG and MF) and ethyl acetate fractions (EG and EF) of both green and fallen needles at 50 µg/ml, respectively. Results are expressed here as mean ± SD; Data was significant as $p < 0.05$.