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Aqueous extract of *Pinus wallichiana* inhibits proliferation of cervical cancer cell line HeLa and represses the transcription of angiogenic factors HIF1α and VEGF

Swati Joshi[1], Sakshi Painuli[1], Nishant Rai[1], Ramesh C. Meena[2], Kshipra Misra[2] and Navin Kumar[1]*

[1]Department of Biotechnology, Graphic Era Deemed to be University, Dehradun, Uttarakhand, India
[2]Defence Institute of Physiology & Allied Sciences (Defence Research Development Organization), Timarpur, Delhi 110 054, India

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ABSTRACT

Aqueous extract of the needles of *Pinus wallichiana* was prepared through pressurized liquid extraction. Various assays were performed to analyze the phytochemical, anti-oxidant potential and anti-proliferative activities against cancer cell lines. Phytochemical analyses showed the presence of phenolics, flavonoids and antioxidant properties in the extract. HPTLC analysis revealed the presence of ascorbic acid and quercetin. XTT assay demonstrated anti-proliferative activity of the extract against human cervical cancer cell line, HeLa. The extract inhibited 58.02 % HeLa cells at a concentration of 31.2 µg mL^{-1}, which was significantly greater than the inhibition of non-cancerous VERO cells, i.e. 3.84 %. Extract induced repression of key angiogenic factors, HIF-1α and VEGF was analyzed through real-time PCR. The extract down-regulated the transcription of HIF-1α to 0.54 folds and VEGF to 0.31 folds. Results indicated anticancer potential of the needles of *P. wallichiana*.

Key word : *Antioxidant, HIF-1, HPTLC,VEGF, Pinus wallichiana*

Introduction

Uncontrolled production of intracellular free radicals may cause damage to proteins and DNA causing genomic instability, and ultimately resulting in cancer, diabetes, neurological, pulmonary and cardiovascular disorders (Lobo *et al*., 2010; Reuter *et al*., 1996). Cancer is associated with the highest mortality after cardiovascular disorders and is characterized by abnormal cell proliferation due to defective DNA replication, checkpoints and apoptotic pathways (Nojima 2004; Jemal *et al*., 2007).

Oncogenic tissues are characterized with the up-regulation of a transcription factor, hypoxia inducible factor-1 (HIF-1), which up-regulates the expression of angiogenic factors and vascular endothelial growth factor (VEGF) (Forsythe *et al*., 1996). HIF-1 is made up of HIF-1α and HIF1-β. The transcript level of HIF-1β remains steady but the transcription of HIF-1α keeps fluctuating and decides the concentration of HIF-1(Ke and Costa, 2006). Repression of HIF-1α increases survival of cancer patients as it inhibits the growth of tumor cells (Giaccia *et al*., 2003; Nagle and Zhou, 2006). Similarly, inhibition of VEGF expression increases survivability of cancer

patients (Hicklin and Ellis, 2005). Radiotherapy, surgery and chemotherapy are main anti-tumour and anticancer therapies but these possess several side effects and lesser survivable rate in patients, giving rise to need of new anticancer drugs of natural origin (Gandhiappan and Rengasamy, 2012; Baharum *et al*., 2014).

Pinus wallichiana (common name- Blue pine and A.B. Jackson) is a conifer, known for its superior wood quality, gum and resins. The plant is generally found in the temperate Himalayan region, ranging in altitude from 2000 to 3500 meter above sea level and is the native of India, Pakistan, Nepal, China, Afghanistan and Bhutan. A survey from Kaghan Valley, Pakistan has revealed its ethno-medicinal practice against cough, ulceration, itching, intestinal worm infections, and as diaphoretic agent and as CNS stimulant. Hussain and Ghani (2008) found that being good bio-sorbent, plant wood exhibited the property to remove arsenic from water sample (Saqib *et al*., 2013). High levels of phenolics, flavonoids and antioxidants have been reported in the bark and needles of the plant (Willfor *et al.*, 2009; Naeem *et al*., 2010; Maimoona *et al*., 2011). The oil fractions extracted from needles of *P. wallichiana* showed good radical scavenging and anti-proliferative activities (Dar *et al*., 2012).

In the present study, aqueous extract of the needles of *P. wallichiana* was analysed for phytochemical, antioxidant, anti-proliferative activities against cancer cell line, and repression of VEGF and HIF-1α transcription.

Methodology

Collection of Plant material

Needles of *Pinus wallichiana* were collected from Barkot region of Uttarkashi district of Uttarakhand India, situated at latitude 30° 48' 24.16'' N and longitude 78.12' 22.63''° E, elevation 4293ft. The species were identified and authenticated by the expert Dr. Rakesh Mohan Painuli, Department of Botany, H.N.B. Garhwal University, India. The specimen sample is stored in the herbarium vide specimen voucher number GUH20744.

Preparation of extract (Mamta *et al*., 2015)

Needles of the plant were dried and crushed. The extraction procedure was done in Accelerated Solvent extraction system equipped with a solvent controller unit (ASE350, DIONEX, and Corporation Sunnyvale, CA, USA). In the present study water was used for extraction. The plant extracts were lyophilized and stored at 4 °C till further use.

Phytochemical analyses

Analyses of Total Phenolic Content (TPC) (Singleton and Rossi 1965; Cheng *et al*., 2003)

Total Phenolic Content (TPC) was determined by Folin-Ciocalteu Reagent method following with slight modifications (Singleton and Rossi 1965; Cheng *et al*., 2003).

Plant extracts (150 µL) was mixed with an equal amount (150 µL) of 0.25N FCR (Folin-Ciocalteu reagent) and 2400 µL of nanopure water and kept for incubation. After incubation for 3 min in dark 300 µL of 1N Na_2CO_3 was added in it. It was mixed thoroughly and incubated for 2h. The absorbance was measured at 765 nm. Different dilutions of gallic acid (0.01 mg $mL^{-1,}$ 0.02 mg $mL^{-1,}$ 0.04 mg mL^{-1}, 0.08 mg mL^{-1} and 0.1 mg mL^{-1}) were used as a standard for drawing the calibration curve. The result was expressed as mg g^{-1} gallic acid equivalent (GAE) of extract (mg g^{-1} GAE).

Analyses of Total flavonoid Content (TFC) (Singleton and Rossi, 1965; Cheng *et al*., 2003)

One mL aliquot of the extract was mixed with 2 mL of distilled water followed by mixing 0.15 mL of $NaNO_2$ (5%) and incubated for 6 min. After incubation 0.15 mL of $AlCl_3$ (10 %) solution was added to it and mixed thoroughly and allowed to stand for next 15 min. After 15 min absorbance was taken at 510 nm. Different dilutions of rutin (0.01 mg mL^{-1}, 0.02 mg mL^{-1}, 0.04 mg mL^{-1}, 0.08 mg mL^{-1} and 0.1 mg mL^{-1}) were used as a standard for drawing the calibration curve. The result was expressed as mg g^{-1} rutin equivalent of extract (mg g^{-1} RE).

HPTLC Analysis (Menon *et al*. 2012)

Flavonoids (hesperidin and quercetin), phenolic (gallic acid) and vitamin C (ascorbic acid) were used as a standard for HPTLC analysis. Lyophilized extract (20 mg) was dissolved in 1 mL methanol. A standard mixture, consisting 200 µL each of gallic acid, ascorbic acid, hesperidin, and quercetin, was prepared from a stock solution of 1 mg mL^{-1} in methanol of each standard. The samples (10 µL) were spotted in the form of bands with CAMAG microliter syringe on a 0.2 mm thick pre-coated silica gel 60 F_{254} HPTLC plate using CAMAG

LINOMAT 4 Applicator. Bands of 6 mm width were applied using the automatic LC sampler 4 (ATS4, CAMAG, Muttenz, Switzerland) with a track distance of 8 mm. The spots were dried in a current of air. The mobile phase used was ethyl acetate: dichloromethane: glacial acetic acid: formic acid: methanol in the ratio of (5/5/0.5/0.5/1 v/v). Then the plate was placed inside the chamber and developed until the solvent front had travelled at a distance of 150 mm above the base of the plate. After development, plates were dried for 15 min, and then documented through Reprostar 3 documentation system using illumination at 254 nm and 366 nm. Detection and quantification were performed with CAMAG Scanner III at a wavelength of 254 nm. For quantification retention factor (Rf) and Area Under Curve (AUC) were analyzed with winCATS Planar Chromatography Manager software. Experiments were repeated at least three times and the best representative of the results are shown.

Antioxidant assays

***DPPH Assay* (Thai Pong *et al*., 2006)**

Plant extract (150 µL) at the concentration of 1 mg mL^{-1} was added to DPPH solution (2850 µL) and allowed to stand for 2 h in the dark. After 2 h absorbance was taken at 515 nm. Trolox was used as a standard at different concentration. Different dilutions (0.01, 0.02, 0.04, 0.08 and 0.1 mg mL^{-1}) of the standard antioxidant, trolox were used to plot standard linear graph. Control (without extract and standard drug) was also included in the study. Results were shown as µg gm^{-1} TE (microgram gm^{-1} trolox equivalent of dry weight of extract).

ABTS Radical Scavenging activity (Thai Pong *et al*., 2006)

Plant extract (150 µL) was mixed with 2850 µL of ABTS solution and kept in dark for 2 h. The O.D. of the mixture was measured at 734 nm wavelength. Different dilutions (0.01, 0.02, 0.04, 0.08 and 0.1 mg mL^{-1}) of the standard antioxidant, trolox were used to plot standard linear graph. Results were shown as per cent ABTS scavenging activity and mg gm^{-1} TE of dry weight of extract.

***FRAP Assay* (Thai Pong *et al*., 2006)**

Total antioxidant activity is measured by Ferric reducing antioxidant power assay. Stock solution used in FRAP assay contained acetate buffer 300 mM (3.1 g $C_2H_3NaO_2.3H_2O$ and 16 mL $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2-4-6 tripyridyl-s-triazine) solution in 40 mM HCL solution, and 20 mM $FeCl_3.6H_2O$ solution. FRAP working solution was freshly prepared by mixing 25 mL of 30 Mm acetate buffer, 2.5 mL of 10Mm TPTZ and 2.5 mL of 20 Mm $FeCl_3.6H_2O$ solution which was warmed at 37 °C before use. Plant extract (150 µL) was allowed to react with 2850 µL of the FRAP solution for 30 min in the dark. Absorbance was taken at 593 nm. Results were further expressed as µg g^{-1} TE of the plant extract.

Total Reducing Power (Jayanthi and Lalitha, 2011)

One mL of each extract (1 mg mL^{-1}) was added to phosphate buffer (2.5 mL, pH 6.6) and potassium ferricyanide (2.5 mL), and kept for incubation at 50 °C for 20 min. Afterward, 2.5 mL trichloroacetic acid (10%) was added to the solution and centrifuged at 3000 rpm for 10 min. The supernatant was taken and mixed with 2.5 mL distilled water. To this solution 0.5 mL ferric chloride was added and O.D. was taken at 700 nm. Ascorbic acid, at different concentrations (0.025, 0.05, 0.10, 0.15, 0.20, and 0.25) was used as standard to plot the standard graph. Results were expressed as µg g^{-1} AE of plant extract (ascorbic acid equivalent of microgram gm^{-1} of extract).

***In vitro* anti-proliferative assay**

Anticancer property of the aqueous extract of the needles of *P. wallichiana* was evaluated by XTT (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide salt) method (Zheng *et al*. 2005). The 100 µL of cell cultures (1×10^5 cells $well^{-1}$) in DMEM medium, were seeded in each well of 96-well microtiter plate. Fresh DMEM containing extract in decreasing log_2 concentration (1000 µg mL^{-1} to 31.2 µg mL^{-1}) were dispensed in each well and plate was incubated for 48 h at 37 °C with 5% of CO_2. Hydrogen peroxide, in decreasing log_2 concentration (from 34 ng mL^{-1} to 1.06 ng mL^{-1}), was used as positive control in the study. Afterward, plates were incubated for 72 h at 37 °C with 5% of CO_2. After 48 h incubation, the cells were washed with PBS, and 40µL of XTT and 2 µL of 50 µM menadione reagent were added to each well. The plate was further incubated for another 2 h and absorbance was measured at 490 nm in microtiter plate reader (BioRad). Following is the formulae for the calculation of % inhibition.

% Cell Inhibition = 100 — % Cell Viability

Real time PCR analysis for modulation activities of HIF 1α and VEGF genes

The modulation of the HIF1α and VEGF mRNA expression by the plant extracts were investigated through Real time PCR (RT-PCR) (Lu *et al*., 2009). 18s rRNA gene was used as internal control. Doxorubicin, a standard anticancer drug, was used as positive control in the present study.

Drug treatment and RNA isolation

HeLa Cells were sub-cultured in DMEM media in 90 mm petri-dishes till confluency reaches to 80-90%. Then, cells were exposed to the doxorubicin (5μg mL^{-1}) and plant extract (500.0 μg mL^{-1}) and no drug (untreated control), separately for 24 h before the isolation of RNA. Morphological changes were visualized microscopically (for signs of deterioration like granularity around the nucleus, detachment of the cells, and cytoplasmic vacuolation) with the help of inverted light microscope (LEADZ Trinocular microscope) at 10X (Figure 1).

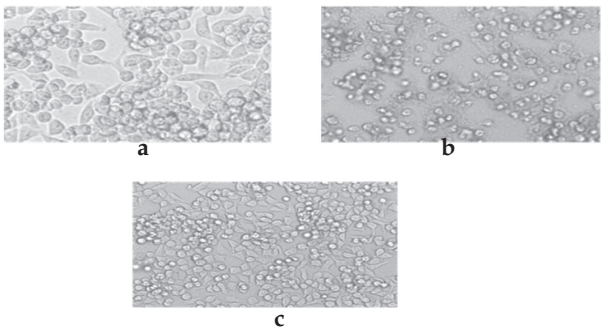

Fig. 1. Effect of the extracts on the morphology of HeLa cell line after 24 h exposure in adherent culture. Cells were photographed at 10X through inverted light microscope (LEADZ trinocular); a) Without any treatment; b) Treated with positive control, Doxorubicin; c) Treated with PWA: *Pinus wallichiana* extract.

After 24 h incubation, RNA was extracted from each sample with the help of RNA sure mini kit ® mini kit (Nucleo-pore™, Catalogue No- NP-84105) following manufacturer's instructions. RNA was quantified by spectrophotometer (Thermo Scientific, NanoDrop-2000C) and purity was ascertained by $A_{260/280}$ ratios of different RNA samples. Intactness of RNA samples was determined by the visualizing 28s rRNA and 18s rRNA bands through agarose gel electrophoresis (Figure 2).

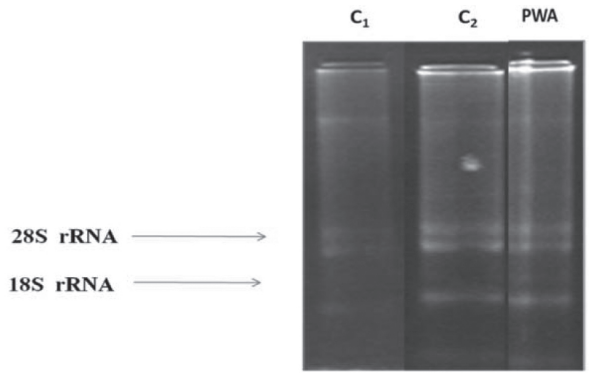

Fig. 2. Agarose gel electrophoresis of total RNA isolated. C_1: from untreated sample; C_2: from doxorubicin treated; PWA: *Pinus wallichiana* extract.

cDNA were synthesized using High-Capacity cDNA Reverse-Transcription Kit of Applied Biosystems (Catalog no. 43-688-14), following manufacturer's instruction.

PCR amplification

To detect the expression of HIF-1α and VEGF mRNA, quantitative RT-PCR was performed using cDNA products as a template in applied biosystems 7500 Fast Real-Time PCR System, using SYBR® Green PCR Master Mix (Catalog number: 4309155) of the applied biosystems. 18s rRNA was used as a control and each reaction was repeated three times. Gene specific primers were used for amplification (Table 1).

Amplification was carried out with an initial incubation at 95º C for 10 minutes, followed by 95 ºC for 15s and 60 ºC for 1 minute, for a total of 40 cycles, and 60 ºC for 10 minutes. Data analysis was carried out using 7500 Sequence Detection Software v2.0.6 and the $2^{-\Delta\Delta Ct}$ method based upon Ct (threshold cycle) values (Livak and Schmittgen, 2001).

Results

Aqueous extract of *P. wallichiana* needles (PWA) was prepared by modern method, pressurized liquid extraction using accelerated solvent extractor at high pressure (1500 psi) and room temperature, with an aim to prepare a polyphenol rich extract.

As shown in Table 2, PWA contained higher amount of phenolics and flavonoids when compared to the aqueous extract prepared in a previous study (Maimoona *et al*., 2011). HPTLC analysis reported the presence of ascorbic acid (5.85 mg g^{-1})

Table 1. Primers used in the study

Name of Primer	Sequence
HIF1α forward	5′-GAGATGTTAGCTCCCTATATCCCA-3′
HIF1α reverse	5′-TAGGTTCTTGTATTTGAGTCTGCTG-3′
VEGF forward	5′-TACTGCCATCCAATCGAGAC-3′
VEGF reverse	*5′-GCATGGTTGATGTTGGACT-3′*
18srRNA forward	5′-TCGGAACTGAGGCCT-3′
18srRNA reverse	5-CTTTCGCTCTGGTCCGTCTT-3′

Table 2. Phytochemical and antioxidant property of *P. wallichiana* needle extract

Total phenolic content	45.00 ± 1.00 (mg gm^{-1} GAE ± SD)
Total flavonoid content	86.66 ± 2.081 (mg gm^{-1} RE ± SD)
DPPH scavenging activity	50.76 ± 0.002 (µg gm^{-1} TE ± SD)
ABTS scavenging activity	125 ± 0.142 (µg gm^{-1} TE ± SD)
Ferric reducing antioxidant power assay	469.48 ± 0.083 (µg gm^{-1} TE ± SD)
Total Reducing Power assay	17.15 ± 0.001 ((µg gm^{-1} AE)

Notes: GAE: Gallic acid equivalent; RE: Rutin equivalent; TE: trolox equivalent; AE: Ascorbic acid equivalent.

Table 3. Quantification of ascorbic acid, quercetin, gallic acid, and hesperidin in *P. wallichiana* needle extract

Standards	R*f*	Linear regression equation via area	Correlation coefficient (R^2)	Concentration of standards in PWA (mg gm^{-1})
Ascorbic Acid	0.31	Y = -1569.00 + 5.797 X	0.994	5.85
Quercetin	0.87	Y = - 457.20 + 4.571 X	0.990	3.50

and quercetin (3.50 mg gm^{-1}) in PWA (Table 3 and Figure 3).

Quercetin has been reported in the methanolic extract of needles of *P. wallichiana* (Naeem *et al*., 2010).

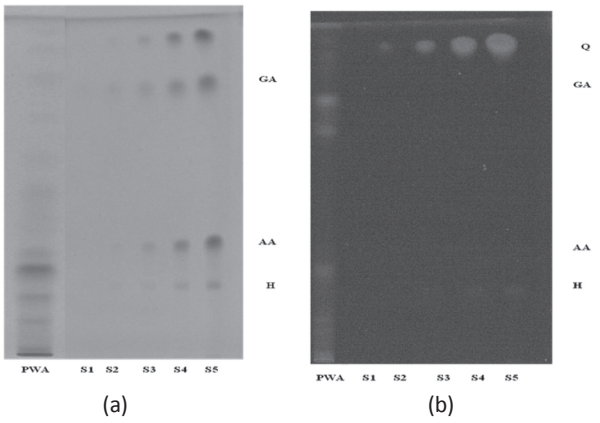

Fig. 3. HPTLC Chromatogram a) HPTLC Chromatogram at 254 nm: b) HPTLC Chromatogram at 366 nm Q: Quercetin, GA: Gallic Acid, AA: Ascorbic acid, H: Hesperidin, S1, S2, S3, S4, S5: Different dilutions of standards (S1: 200 ng, S2: 400 ng, S3: 800 ng, S4: 2000 ng, S5: 4000 ng).

Analysis of anti-cell proliferative activity revealed that the extract inhibited both cell lines in dose dependent manner, which was significantly higher against HeLa cells when compared with Vero cells (Figure 4a). For decreasing $\log_2$ range of PWA concentration (1000 gmL^{-1} – 31.2 μgmL^{-1}), the inhibitions ranged from 67.29 ± 1.41% to 58.02 ± 4.82% for HeLa cells, and 49.20 ± 2.38% to 3.84 ± 2.57% for Vero cells. Even at the lowest concentration used in this study (31.2 μgmL^{-1}), the extract showed greater inhibition of HeLa cells (58.02 %) than non-cancerous Vero cells (3.84%).

Many plant extracts have been studied to repress the expression of two most important transcription factors, HIF1α and VEGF which supports the tumour progression by angiogenesis (Nagle *et al*. 2006; Semenza 2010; Painuli *et al*., 2018). Therefore, we aimed to investigate the effect of PWA upon the transcription of HIF-1α and VEGF in HeLa cells through real-time PCR (Lu *et al*., 2009). At the given conditions, an exposure of 500 µg mL^{-1} PWA for 24 h has down regulated the expression of HIF-1α and VEGF to 0.54-fold and 0.31 folds (Figure 4b), respectively.

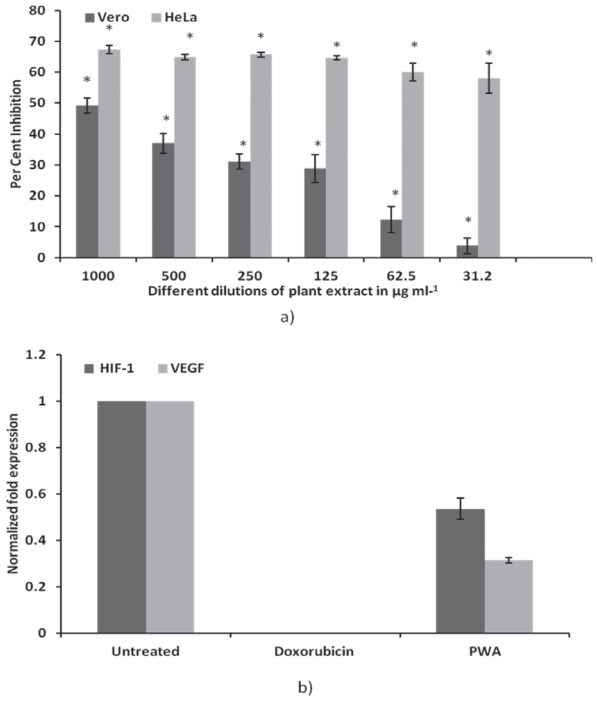

Fig. 4. (a) Antiproliferative-cell proliferative activity, Per cent inhibition of HeLa and Vero cell line by aqueous extract of *P. wallichiana*. (b) Normalized fold expression of VEGF and HIF-1α in presence of control and aqueous extract of PWA: *Pinus wallichiana* extract. * indicates P value< 0.05.

Discussion

Antioxidants are very important compounds that neutralize free radicals and therefore are used in treating various diseases related to oxidative stress such as cardiovascular diseases, cancer, neurodegenerative diseases, Alzheimer's disease and inflammatory diseases (Gerber *et al*. 2002; Matteo and Esposito 2003). Plants have been the natural source of various antioxidants like quercetin, ascorbic acid, citric acid, gallic acid and hesperidin etc. and these compounds are well known for their antioxidant properties and anticancer properties (Sourani *et al*., 2016; Ganzalez and Miranda – Massari, 2014; Lakhanpal, 2007; Devi *et al*., 2015).

In the present study the *P. wallichiana* needle extract showed significant phenolic and flavonoid content and also exhibits substantial antioxidant properties in different *in vitro* assays of radical scavenging and reducing potential (Table 2). In XTT assay, PWA inhibited ≥ 50% cancer cells at a concentration of 31.25 µg mL^{-1}, in contrary, the inhibition of Vero cells by PWA was 3.84%, respectively at a concentration of 31.2 µg mL^{-1}. The results indicated that PWA is more toxic against cancerous cell than normal cells, which is expected from an effective anticancer drug with least side effects. The present result of anti-proliferative assay is fully in harmony with the guidelines of National Cancer Institute, USA. According to the National Cancer Institute, USA, if crude extract, at concentration of < 30-40 µg mL^{-1}, is able to inhibit ≥ 50% cancer cell lines than it may be considered as anti-proliferative drugs (Oskoueian *et al*., 2011). The high ability of extract to inhibit HeLa cells can be related with the presence of phenols (gallic acid) and flavonoids (quercetin).

Conclusion

The aqueous extract of *Pinus wallichiana*, prepared by pressurized liquid extraction, was found enriched with phenolics and flavonoids and contained high antioxidant potential. The extract showed promising anti-proliferative activities against human cervical cancer cell line, HeLa, without causing any significant toxicity to non-cancerous cells. The extract down regulated the mRNA levels of two most important factors responsible for angiogenesis in cancerous tissues, HIF-1α and VEGF in HeLa cells. Plant extract have the potential to be developed as natural anticancer agent.

Recommendations for Farmers

Keeping in the view the importance and prospective usage of this plant, following are the recommendations for the farmers:

1. Farmers may explore the opportunities in growing and harvesting the *Pinus* from natural forests for their new indications as raw material for anticancer medications.
2. Farmers may also be indulged in growing *Pinus* in identified forest areas with the support of state governments.
3. Extraction units may be setup in clusters to provide mean of livelihood to the farmers in the hilly areas to uplift the socio-economic face of villagers.

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Disclosure Statement

The authors declare no conflict of the interest.

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