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Evaluation of anticancer activity of *Pinus roxburghii* Sarg against A549 human lung cancer cell line

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Abstract
In the previous study, extracts prepared from the leaves of *Pinus roxburghii Sarg.* (Pinaceae) were evaluated for extraction, identification and characterization of phytochemicals. Now the present investigation was carried out its anticancer activity using SRB based assay against A549 human lung cancer cell line. Anticancer activity of different extracts and compounds of *P. roxburgii* was performed on A549 human lung cancer cell line by the Advanced Centre for Treatment Research and Education in Cancer (ACTREC) Mumbai, India. The 50% ethanolic and aqueous extracts of *P. roxburghii* showed 112.7 µg/ml and 111.2 µg/ml anticancer activities respectively. Both compounds showed -20.9µg/ml and 94.4 µg/ml anticancer activities. The extracts showed the presences of secondary metabolites which are responsible for the therapeutic effects. Thus, aiming to discover new alternative *P. roxburghii* may be a new hope for the prevention of lung cancer.

Keywords: A549 human lung cancer cell line, anticancer activity, *Pinus roxburghii* Sarg, SRB assay

Introduction
Now a day, lung cancer is commonly identified problem all over the world. According to world health organization estimation, it causes high risk of morbidity with approximately 1.3 million new cases and 300,000 deaths each year [1-3]. According to GLOBOCAN 2012, there is 13% contribution of lung cancer among the sum of newly diagnosed other cancer cases. About 1.8 million latest belongings of lung cancer are recorded per year. A high occurrence rate of lung cancer has raised a major quantity of concern for the health care specialists and alarms the researchers to pay their extra efforts to fight for these types of health ailments.

Earlier cytology states that lung cancer contains two types of cell that is large and small so it is categorized into small non-small cell lung cancer and cell lung cancer. There are various advanced diagnostic and operative techniques besides this many other therapies like radio therapy and chemotherapy are involved to improve the survival rate from cancer. Sometimes it includes the combination of surgical, chemo and radiation therapy that have known side effects.

The invention of higher ability drugs from natural extracts that is least in toxicity with fewer side effects and more potential in anticancer activity are obligatory to be discovered. This might work as referee in physical functions to stop the cancer cell development at its different stages [4].

Pinus roxburghii Sarg. (Family: Pinacae) is a kind of pine tree occupant to the Himalaya [5]. *P. roxburghii* has reported to show diverse pharmacological actions like anti-inflammatory, analgesic [6] anticonvulsant [7], antimicrobial [8] and anticancer [9] activities. Indian and African are also used its bark and leaf to treat diabetes [10]. It is branded of terpenoids [11], flavonoids, tannins [12], xanthones, saponins, phenolic compounds [5], triterpenes and steroids [13] from all the parts of plant.

However, its bioactive cancer preventives potential remain to be recognized. The anticancer action of *P. roxburghii* is explained in hereby present study. Therefore, we concluded scientific results that anticancer action of extracts and isolated phytocompounds from the leaves of *P. roxburghii* against A549 human lung cancer cell line.

Material and Methods
The leaves of *P. roxburghii* were collected from Kumaun district of Nainital in the month of February 2015. It was authenticated by Botanical Survey of India (BSI), Allahabad, as well as the herbarium was submitted there under voucher accession number 100230.

Extraction of plant material (50% ethanolic and aqueous extracts)

AR grade solvents as well as purified water were used in all process of extraction and isolation which were purchased from S.D. Fine chemicals, Mumbai, India. The leaves were washed with distilled water in order to eliminate dirt and impurities, dried away from the sunlight. Air and shed dehydrated leaves from *P. roxburghii* was grinded and overwrought in the route of 30 meshes (0.5 mm).

In the preparation of 50% ethanolic extract, thinly crused plant material (100 gm) was stimulated in a percolator in addition to treated with ethanol: water (1:1 v/v; 500 ml) at 26-30°C for the night. The marc was extracted three times by cold percolation (1:1 v/v; 500 ml×3) and the pooled percolate (1000 ml) was evaporated at 45-50°C underneath vacuum evaporator to get 5-7% dry extract. The finely grinded leaves (100 gm) were treated by 500 ml MilliQ water at 55-70°C for 6-8 h. This procedure was continual further for three times. The warm water extract was filtered by Whatman filter paper number 1. The total filtrate (1300 ml) was distilled at 50-55°C beneath vacuum pressure to get concentrate 300 ml extract. The extract was followed by lyophilized at -20 to -40°C to afford 6-8% dehydrated extract. Both the extracts were stored at -20 to - 40º C or further analysis [14].

The isolation of phytochemicals was passed away by 50% ethanolic extract through column chromatography technique. With this process two compounds were isolated named gallic acid and catechin. This was confirmed by spectroscopic data (UV, IR, NMR and MS) and HPLC [15]. In this research paper, we report the anticancer activity of extracts and isolated compounds- gallic acid & catechin from the leaves of *P. roxburghii*.

Anticancer experimental procedure by Sulforhodamine-B (SRB) assay

The Anticancer activity of samples by *in vitro* SRB assay was studied at Advance Centre for Treatment, Research and Education in Cancer (ACTREC), Mumbai, India. The prepared samples were performed on the A549 human lung cancer cell line and it was grown in RPMI 1640 medium containing 10% Fetal Bovine Serum and 2 mM L-glutamine.

This anti proliferative SRB test was used to calculate growth inhibition. This is a colorimetric examine which calculate cell number ultimately by discoloration entire cellular protein by the SRB stain. The A549 cells (0.5- 1.0 × 105 cells/ml) were inoculated in 96 welled micro-titer plates. To each well 90 µl of diluted cell suspension was afterward added. Earlier to drug action inoculated micro-titer plate was then kept in incubator with 370C temperature, 5% CO2 concentration, 95% air and 100% relative humidity. This incubation was done for 24 hours. Incubation period take 24 hours, afterwards the partial monolayer was produced supernatant was washed. Trial samples were solubilised in DMSO this aliquot was subsequently icy prior to utilize. At the time of addition, the test samples was thawed, and added (10 µl) to respective well containing 90 µl medium at 10, 20, 40 and 80 µg/ml of final concentrations. After the addition of trial drugs, micro-titer plates were incubated for 48 hours. The test was subsequently ended by accumulation of cold TCA to wells. Once completion of 72 hours, 50% cold TCA was added in wells over the trial samples smoothly to in such a mode to form overall concentration of TCA which becomes 10% and then the plate was incubated for an hour at 4°C.

The supernatant was flicked off. The wells were washed 5 times with water to eliminate every trace of medium and air dried. After adding SRB solution [0.4% (w/v) in 1% acetic acid] in all of the wells (50-100 µl), plates were incubated for 30 minutes at room temperature. Later than incubation, plates were washed 4 times through 1% acetic acid to eliminate boundless dye. The plates were then air dried out. To solubilise the bound dye, 10 mM tris base was added, the absorbance was study going on an ELISA plate reader at a wavelength of 540 nm with reference to 690nm. Optical density of drug treated cells was compared with that of control cells and growth inhibition as calculated as percent values.

For each experiment, a known anticancer drug Adriamycin was used like a positive control. All the trials were implemented in triplicate.

Endpoint measurement

Using 6 absorbance measurements, the percentage growth was calculated at each of the drug concentration levels as follows: [time zero (Tz), control growth (C), and test growth in the presence of drug at the 4 concentration levels (Ti)].

Percentage growth inhibition was calculated as: [(Ti-Tz)/(C-Tz)] x 100 for concentrations for which Ti>/=Tz (Ti-Tz) positive or zero [(Ti-Tz)/Tz] x 100 for concentrations for which Ti<Tz. (Ti-Tz) negative.

The dose response parameters were calculated for each test article. Growth inhibition of 50% (GI50) was calculated from [(Ti-Tz)/(C-Tz)] x 100=50, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation.

The drug concentration resulting in Total Growth Inhibition (TGI) was calculated from Ti = Tz. The LC50 (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from [(Ti-Tz)/Tz] x 100 = 50.

Values were calculated for each of these three parameters if the level of activity was reached however, if the effect was not reached or was exceeded, the values for that parameter were expressed as greater or less than the maximum or minimum concentration tested [16].

Statistical analysis

There are three ways to calculate each value. They are as follows:

1. If the level of activity was reached
2. If the effect was not reached
3. If the effect was exceeded

The values for that parameter were expressed as greater or less than the maximum or minimum concentration tested. The results were anticipated *via* linear regression method of plots of the cell viability beside the molar drug concentration of tested compounds. All experiments were performed thrice.

Results

Our attention was to scientifically estimate 50% ethanolic extract, aqueous extract and isolated compounds of *P. roxburghii* for anti-cancer activity respectively against A549 human lung carcinomous cell line. Here examination, Adriamycin used like positive control and represent 100% anticancer activities (Table-I).

Anti-cancer activity of 50% ethanolic extract of *P. roxburghii* against A549 human lung cancer cell line

According to table no. I, graph no.1 and image no. 1 (c), 50%

ethanolic extract had shown results in a range of 96.6-112.7 and very slight partiality in a graph at maximum concentration level i.e.80 µg/ml. This shows the insignificant activity, yet if the concentration of drug was raised. Therefore, it can be said that 50% ethanolic extract of *P. roxburghii* has nil result on A549 human lung cancer cell line.

Anti-cancer activity of aqueous extract of *P. roxburghii* against A549 human lung cancer cell line

According to table no. I, graph no. 1 and image no. 1 (d), aqueous extract had shown results in range of 103.2-111.2 and very slight partiality in a graph at maximum concentration level i.e. 80µg/ml. This shows the insignificant activity, even if the concentration of drug was raised. Thus, it can be said that aqueous extract of *P. roxburghii* has nil result on A549 human lung cancer cell line.

Anti-cancer activity of isolated compound (A) gallic acid extracted from *P. roxburghii* against A549 human lung cancer cell line

According to table no. I, graph no.1 and image no. 1 (a), gallic acid had shown results in a range of -20.9-44.6 and partiality of line in graph, as the drug concentration raised which indicates good activity at highest drug concentration i.e. 80µg/ml. According to the SRB assay procedure, compound is said to be significantly active if the value gets anticipated within the limit of 80.

Anti-cancer activity of isolated compound (B) catechin extracted from *P. roxburghii* against A549 human lung cancer cell line

According to table no. I, graph no.1 and image no. 1 (b), catechin had shown results in a range of 92.9-98.9 and very slight inclination in a graph at maximum concentration level i.e.80 µg/ml. This indicates the insignificant activity, even if the concentration of drug was raised. Thus, it can be said that catechin has insignificant effect on A549 human lung cancer cell line.

LC50: The Concentration of drug causing 50% cell kill. **GI50:** Concentration of drug causing 50% inhibition of cell growth. GI50 value of ≤ 10µg/ml is considered to demonstrate activity in case of pure compounds. For extracts, GI50 value ≤ 20µg/ml is considered to demonstrate activity. **TGI:** Concentration of drug causing total inhibition of cell growth. **ADR:** Adriamycin, Positive control compound (Table no- II).

Table I: Drug concentrations (µg/ml) and percentage of growth inhibition

Human Lung Cancer Cell Line A549																
% Control Growth																
Drug Concentrations (µg/ml)																
Drug Samples	**Experiment 1**				**Experiment 2**				**Experiment 3**				**Average Values**			
	10	**20**	**40**	**80**	**10**	**20**	**40**	**80**	**10**	**20**	**40**	**80**	**10**	**20**	**40**	**80**
Gallic Acid	54.1	-47.9	-40.4	-22.1	48.8	-56.3	-45.4	-17.5	31.0	-57.3	-53.2	-23.2	44.6	-53.8	-46.4	-20.9
Catechin	95.0	94.7	100.4	90.6	91.2	100.9	104.2	95.3	92.5	87.3	92.1	97.2	92.9	94.3	98.9	94.4
50% Ethanolic Extract	93.5	100.7	102.4	109.2	100.7	101.8	108.3	117.8	95.6	95.3	99.8	111.1	96.6	99.3	103.5	112.7
Aqueous Extract	102.0	100.1	95.1	99.4	109.1	113.2	110.5	118.6	103.1	108.8	103.9	115.6	104.7	107.4	103.2	111.2
ADR	-2.2	-5.6	-29.4	-42.6	-5.3	-4.7	-23.1	-32.6	0.7	-8.0	-21.3	-34.9	-2.3	-6.1	-24.6	-36.7

ADR stands for Adriamycin, used as a positive control

Table II: Drug concentrations (µg/ml) calculated from graph

A-549	LC50	TGI	GI50
Gallic Acid	NE	NE	<10
Catechin	NE	NE	NE
50% Ethanolic Extract	NE	NE	NE
Aqueous Extract	NE	NE	NE
ADR	NE	3.0	<10

- **LC50** is the Concentration of drug causing 50% cell kill.
- **GI50** is the Concentration of drug causing 50% inhibition of cell growth. GI50 value of ≤ 10µg/ml is considered to demonstrate activity in case of pure compounds. For extracts, GI50 value ≤ 20µg/ml is considered to demonstrate activity.
- **TGI** is the Concentration of drug causing total inhibition of cell growth.
- **NE** means non- evaluable data.

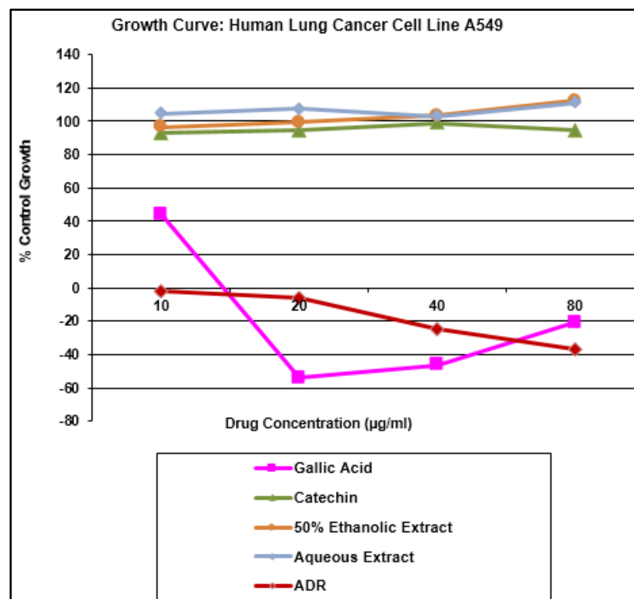

Fig 1: The Plot of percentage control growth vs. molar drug concentration shows the effective drug concentration on the human lung cancer cell line A549

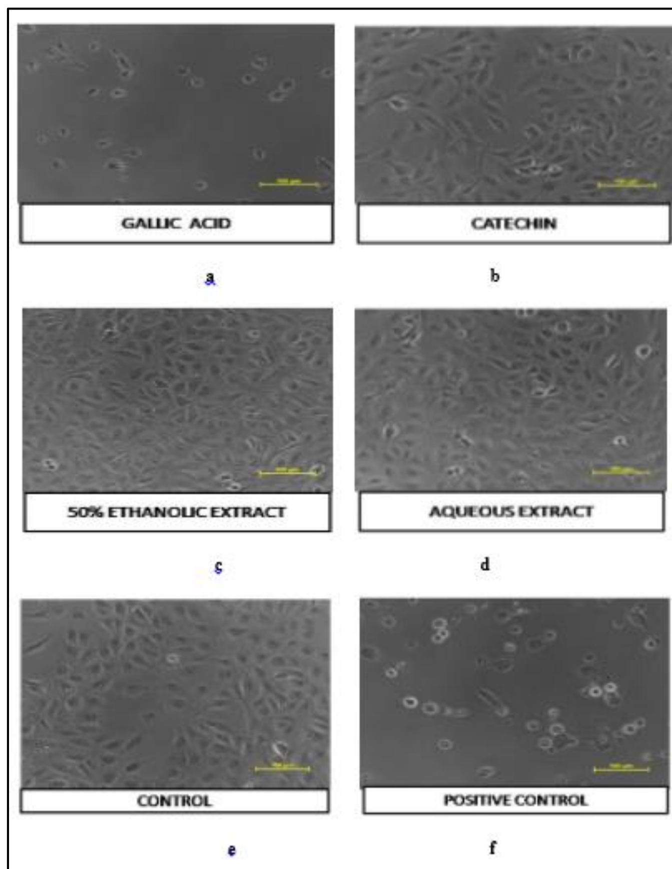

Fig 2: *P. roxburghii* leaves extracts, gallic acid and catechin treatment to human lung cancer cell line A549

- **Image (a)** - Effect of gallic acid on human lung cancer cell line A549.
- **Image (b)** - Effect of catechin on human lung cancer cell line A549.
- **Image (c)** - Effect of 50% ethalonic extract on human lung cancer cell line A549.
- **Image (d)** - Effect of aqueous extract on human lung cancer cell line A549.
- **Image (e)** - Effect of control on human lung cancer cell line A549.
- **Image (f)** - Effect of positive control (Adriamycin) on human lung cancer cell line A549.

Discussion

The vital need for new anticancer drugs is an overall distress owing to high rate of mortality and morbidity caused by the same. Also adding an obvious evident of economical and viable barriers, cancer patients also have to meet with miscellaneous problems related with the current scientifically approved drugs. The aim of this study was to screen out leaf extracts of *P. roxburghii* for possible effects on lung cancer. *P. roxburghii* was originated to show important effect on cancer cell line. This significant inhibition of cancer in our study could be due the presence of major secondary metabolites, e.g.-detection of alkaloids, tannins, saponins, steroids, phenols, terpenoids, flavonoids, glycosides. In numerous methods ranging from expansion and stress responses, apoptosis is chief outline of cell demise that plays an essential role. The central objective of cancer is the inactivation of apoptosis. Thus, induction of apoptosis can be effective strategy against tumor progression. Our outputs are in harmony among some of the previous studies on this plant. Kaushik *et al.* (2015) found that chloroform extract from the bark of *P. roxburghii* exhibits a challenging anti-cancer doings next to IMR-32 human neuroblastoma cancer cell line [9].

The present results, together with previous studies, suggest that *P. roxburghii* extract possess anti-oxidant, analgesic, anti-inflammatory, anticonvulsant, hepatoprotective and cytotoxicity activity. We found in research that the cytotoxicity of drugs using SRB assay (Table -I, Graph-1 and Image-1) against A549 human lung carcinomous cell line. In moderate acid conditions, SRB attaches stoichiometrically to basic amino acids. The growth of cells was controlled after the treatment of compound (A), gallic acid secluded from plant extracts as compared to ADR. Gallic acid is a phenolic compound and it is chemically called as 3, 4, 5-trihydroxybenzoic acid. Gallic acid has a structure which contains phenolic groups which is a real source of hydrogen atoms. Therefore, radicals produce can be delocalized over the phenolic structure [17]. It has pharmacological activity as radical scavengers. It has been determined to contain possible defensive and curative outcomes in various diseases, where the oxidative stress has been concerned, including cardiovascular diseases, cancer, neurodegenerative disorders and in aging [18, 19]. The inhibition of cancer by gallic acid at different concentrations is recorded (Table- II). In current affairs, we have noticed that clogging effect of gallic acid is more impulsive at the concentration of 80µg/ml. Therefore, could be handy as anticancer agent. Thus, overall % of growth inhibition of gallic acid is greater than (10µg/ml) positive control ADR. So we anticipate that gallic acid may of great hope as an anti-cancer agent. And the 50% inhibition of cell growth value of gallic acid was greater than 10µg/ml which was also equal to positive control (ADR).

Thus, overall observations from present and previous studies support theory of anti-cancer activity of *P. roxburghii*. Furthermore, there is much kind of key limitations in phytochemical analysis such as types of extracts and techniques used. Since we have used crude extract, the definite phyto-chemical cause might be in minute concentration in this extract. Different techniques used in the analysis of extracts may be able to effect on concluding concentration of these compounds in crude extract. Constant heating, evaporation procedures may be responsible for the reduction in concentration. Also, one aspect that needs to be considered is the geographical area. Hence, both the extracts show negligible effect on cancer cell line. While the isolated pure compound show good anticancer activity due to its higher concentration. This paper depicts gallic acid to have significant activity on cancer cell line but the catechin does not illustrate any important activity. Such explanations are cheering for additional security and efficacy studies on other human cancer cell lines and *in vivo* trials.

Conclusions

Our studies show that gallic acid activates cell death in A549 lung cancer cells in a dose dependent manner. Further experimental analysis of the plant extracts is needed. So as to isolate many more active anticancer compounds and obtain more detailed mode of action for the development of new drug which may be useful in the treatment or prevention of cancer. This may be bonus for the society.

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Conflicts of interest

The authors declare no conflicts of interests.

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