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Research Article

Evaluation of Anticancer Activity of *Pinus roxburghii* Sarg. Against IMR-32 Human Neuroblastoma Cancer Cell Line.

Pawan Kaushik, Sukhbir Lal Khokra, A.C Rana, Dhirender Kaushik*

Institute of Pharmaceutical Sciences, Kurukshetra University, Kurukshetra – 136119, Haryana, India.

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ABSTRACT

To study the anticancer activity of petroleum ether, ethyl acetate, chloroform and ethanol extract of *Pinus roxburgii* Sarg. by SRB assay method on IMR-32 Human Neuroblastoma cancer cell line. Anticancer activity of different extracts of *Pinus roxburgii* Sarg. was performed on IMR-32 Human Neuroblastoma cancer cell line by the Advanced Centre for Treatment Research and Education in Cancer (ACTREC) Mumbai, India. Out of the four extracts petroleum ether and chloroform extract have showed anticancer activity on IMR-32 Human Neuroblastoma cancer cell line. Cell line were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2mM L-glutamine. Present study implicit the observation that petroleum ether and chloroform extract of the plant *Pinus roxburghii* Sarg. showed a promising anti-cancer activity against only one cell line and may be active against the other cell lines. Further, all these plants extract need to be screened against different cell lines apart from the selected cell line to confirm the activity.

Keywords: *Pinus roxburgii* Sarg., anticancer, IMR-32, SRB assay, human neuroblastoma cancer cell line

INTRODUCTION

Cancer is one of the most fatal diseases in human population and one of the most frequent causes of death worldwide[1]. Cancer is one of the most life threatening diseases and possess many health hazard in both developed and developing countries[2]. By 2015 cancer morbidity may climb to around nine million world-wide. Existing chemotherapy and treatment leads to painful side effects. Hence there is a need to explore new alternative and complementary medicine with anticancer activity. It has been observed that ethanomedicinal plants frequently serve as a source of new drugs with little or no side effects. Phytochemicals have always been sought after because of their inherent potential to cure disease, as established by ancient medicinal practices[3-6]. Furthermore, several plants have been shown to be sources of therapeutically vital agents, valuable in the treatment of cancer. For instance, there are very efficient cancer chemotherapeutic drugs that have been derived from natural origin[7]. These embrace plant derived agents, such as the vinca alkaloids vinblastine and vincristine, isolated from the *Catharanthus roseus*[8]; Taxol, from *Taxus brevifolia*, and the analogue, docetaxel[9]; etoposide and teniposide, derived semi-synthetically from epipodophyllotoxin, an epimer of podophyllotoxin, isolated from roots of Podophyllum species[10]; and camptothecin, from the bark of *Camptotheca acuminata*, a precursor to the semisynthethetic drugs topotecan and irinotecan[11].

Pinus roxburghii Sarg. named after William Roxburgh, is a pine inhabitant to the Himalaya[12]. *Pinus roxburghii* Sarg. has been attributed to many pharmacological activities like hepatoprotective[13], anti-inflammatory, analgesic[14], anticonvulsant[15], antimicrobial[16], antibacterial activities[17]. Ethnopharmacologically *Pinus roxburghii* Sarg. has been used to treat diabetes in India and Africa[18]. *Pinus roxburghii* Sarg. is known to be a rich source of terpenoids, flavanoids and there is interest among the scientist to use this for therapeutic purposes. Almost all the parts of the plant (bark, leaves and root) are found to contain active principles[19-22]. However, the literature indicates that there is no scientific evidence to support the anticancer effect of *Pinus roxburghii* Sarg. In the present study, we investigate the possible anticancer potential of *Pinus roxburghii* Sarg. extracts in IMR-32 Human Neuroblastoma cancer cell line**.**

MATERIALS AND METHOD

Collection of plant material: The bark of *Pinus roxburghii* Sarg. were collected from the hilly region of Morni, District Panchkula, Haryana, in the month of February and was authenticated by Dr. A.K Sharma, Sr. Scientist at Department of Natural Product, FRI, Dehradun, Uttarakhand, India, where a voucher specimen no. 129 FHH was deposited for future reference.

Preparation of extract: Shade dried coarse powdered bark of *Pinus roxburghii* Sarg. in a quantity sufficient as per the volume of the extractor was packed in a thimble (made of filter paper sheet) and sequentially extracted with petroleum ether, chloroform, ethyl acetate and ethanol. A sufficient volume of solvent was added to the reservoir, and hot continuous extraction process in a Soxhlet extractor was started. This extraction process was continued for about 48 hours or until solvent coming down the siphoning tube became colorless. The over abundance of solvent was distilled under reduced pressure using a rotatory vacuum evaporator. (Heidolph Laborota 4011, digital). All the four extracts obtained are stored at 2-4°C

*Author for Correspondence

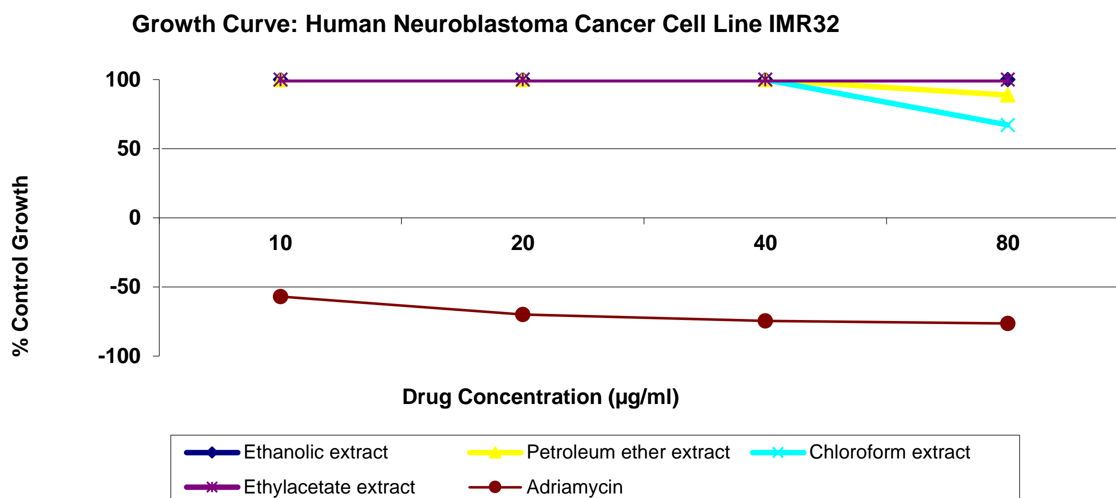

Figure 1. Effect of *Pinus roxburghii* Sarg. extracts on growth of human neuroblastoma cancer cell line IMR32

for further analysis.

Experimental procedure or SRB assay[23]*:* The anticancer activities of extracts were studied at Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Mumbai where 14 cell lines were maintained in ideal laboratory conditions. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates 90µL/well at appropriate plating densities, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37°C, in 5% CO_2, 95% air and 100% relative humidity for 24 hrs prior to addition of experimental drugs. After 24 hrs, cells from one plate of each cell line were fixed in-situ with TCA (trichloro acetic acid), to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental extracts were solubilised in appropriate solvent at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to 10 times the desired final maximum test concentration with complete medium containing test article at a concentration of 100, 200, 400 and 800µg/ml. Aliquots of 10 µl of these different dilutions were added to the appropriate microtiter wells already containing 90µl of cell suspension, resulting in the required final drug concentrations of 10, 20, 40 and 80 µg/ml. For each of the experiments, a known anticancer drug Adriamycin (Doxorubicin) was used as a positive control.

Endpoint measurement: After compound addition, plates were incubated at standard conditions for 48 hrs and assay was terminated by the addition of cold TCA. Cells were fixed in-situ by the gentle addition of 50 µl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4 ℃. The supernatant was discarded; the plates were washed 5 times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing 5 times with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM Trizma base, and the absorbance was read on an Elisa Plate Reader at a wavelength of 540 nm with 690 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100. Using the 6 absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the 4 concentration levels (Ti)]; the percentage growth was calculated at each of the drug concentration levels. 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% (GI_{50}) 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 LC_{50} (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.

Statistical analysis: 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,

Table. 1 Effect of *Pinus roxburghii* Sarg. extracts on Human neuroblastoma cancer cell line IMR32

Extracts	Human Neuroblastoma Cancer Cell Line IMR32 (% Control Growth) Drug Concentrations (μg/ml)															
	Experiment 1				Experiment 2				Experiment 3				Average Values			
	10	20	40	80	10	20	40	80	10	20	40	80	10	20	40	80
Ethanolic extract	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Petroleum ether extract	100.0	100.0	100.0	80.7	100.0	100.0	100.0	88.2	100.0	100.0	100.0	97.7	100.0	100.0	100.0	88.9
Chloroform extract	100.0	100.0	100.0	63.0	100.0	100.0	100.0	61.3	100.0	100.0	100.0	77.5	100.0	100.0	100.0	67.2
Ethyl-acetate extract	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Adriamycin	-55.6	-69.4	-74.1	-76.4	-56.9	-71.3	-76.7	-77.3	-58.1	-69.4	-73.2	-75.8	-56.9	-70.1	-74.6	-76.5

the values for that parameter were expressed as greater or less than the maximum or minimum concentration tested. The experiment data were estimated using linear regression method of plots of the cell viability against the molar drug concentration of tested compounds.

RESULTS AND DISCUSSION

Anticancer activity

The anticancer activity of four extracts of *Pinus roxburghii* Sarg. was conducted against IMR-32 Human Neuroblastoma Cancer cell lines of which only Petroleum ether and Chloroform extracts have shown anticancer activity (Table. 1, Figure. 1). In the present investigation, Adriamycin (Doxorubicin) served as positive control and showed 100% anticancer activity (Table. 1). Further experimental analyses of *Pinus roxburghii* Sarg. extracts are needed, to obtain more detail mechanism of action for development of new drug which may be useful in the treatment and/or prevention of cancer which may be boon for the society. This study will also be useful to the other researchers to take forward the references for further scientific evaluation of anticancer activity.

CONCLUSION

Four plant extracts for studying anti-cancer activity based on available literature have been selected randomly and found that Petroleum ether and Chloroform extracts having activity against neuroblastoma (IMR-32). Present study implicit the observation that Petroleum ether and Chloroform extracts of the plant *Pinus roxburghii* Sarg. showed a promising anti-cancer activity against only one cell line and may be active against the other cell lines. Further, all these plants extract need to be screened against different cell lines apart from the selected cell lines to confirm the activity. The results of the study will also need to be confirmed using in vivo models.

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CONFLICT OF INTEREST

None

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