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Original Article

Antioxidant and Cytotoxic Attributes of *Paris polyphylla* Smith from Sikkim Himalaya

Dawa Lhendup Lepcha, Abhijit Chhetri, Dhani Raj Chhetri*

ABSTRACT

Introduction: *Paris polyphylla* Smith is a high value medicinal plant available in Sikkim Himalaya which is well known in local traditional medicine system. Scientific study to ascertain its claimed biological activity is lacking. The objective of this work was to determine the antioxidant and anticancer activity of *Paris polyphylla* rhizomes. **Methods:** Phytochemical analysis were carried out by standard methods. Antioxidant activity of the methanolic extract was carried out by DPPH, ABTS, OH-radical and Fe^{2+}chelating activity assays. Cytotoxicity of the extract was determined by MTT assay on three cancer cell lines: HeLa, HepG2 and PC3. **Results:** Of the *P. polyphylla* from two altitudinal zones, *P. polyphylla* from Tholung (PPT), the one from the higher altitude showed higher total phenolic contents in methanolic extracts of rhizomes as compraed to that from the lower altitude i.e., *P. polyphylla* from Uttaray (PPU). PPT also showed a higher content of total falvonoid and total flavonols. Both types of plant were excellent scavenger of DPPH and ABTS radical and Fe^{2+} chelator. A trend of a relatively greater antioxidant activity of PPT was established through these assay methods. In MTT assay, both the extract showed significant dose-dependent inhibition of HeLa cell growth after 72 hrs of treatment, while the extract had a moderately positive effect on the inhibition of PC3 and HepG2 cells growth. **Conclusion**: The study suggested a strong antioxidant activity and appreciable cytotoxic activity of *P. polyphylla* from Sikkim Himalaya. Of the two varieties, PPT was more pronounced in both type of activities.

Key words: *Paris polyphylla*, Sikkim Himalaya, Cytotoxicity, Antioxidant, Anticancer, Phytochemicals.

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INTRODUCTION

The state of Sikkim is situated in the Indian Himalayan Region extending between 27° 5′ - 28° 10′ N latitude and 88° 4′ - 88° 58′ E longitude and lies sandwiched between the kingdom of Nepal in the west and the kingdom of Bhutan in the east. Sikkim is an important component of the biodiversity hot-spot of the Himalayas of India. Due to an wide array of climatic zones, this region is rich in floral diversity, many endemic elements and a number of species which have become rare, threatened or endangered.[1] Ethnomedicinal traditions are very well developed in this part of the world and herbal drugs are prescribed widely because of their effectiveness and relatively low cost.[2] Despite this, a substantially rising number cases of cancer is being reported in Sikkim.[3,4]

Paris polyphylla Smith (locally called 'Satuwa' in Nepali and 'Tuk-tok-bee-sungtee' in Lepcha in Sikkim Himalaya) belongs to the family Melanthiaceae. In traditional medicine, the roots are used as analgesic, antiphlogistic, antipyretic, antispasmodic, antitussive, depurative, febrifuge and narcotic.[5] Furthermore, it has been used to treat liver cancer in China for several decades.[6] In the Indian Himalayan Region, *P. polyphylla*, is used against burn, cut or injury, diarrhea, dysentery, fever, gastritis, skin diseases, stomach pain and wounds.[7-10] In Sikkim Himalaya, the dried rhizome (1.0-1.5 cm) is soaked overnight in a glass of water and the drank in the morning to cure bodyache and as a tonic by the Lepcha people. However, there has not been any report on research about anticancer and antioxidant attributes of this plant from this region.

Cancer is one of the leading causes of death worldwide and about one third of cancer deaths (64.9%) have been reported from developing nations.[11] India is likely to have over 8.8 lakh deaths due to the disease by 2020. Cancer and some of the other chronic diseases share common pathogenesis mechanisms such as DNA damage, oxidative stress and chronic inflammation.[12] Many reports suggest that cancer cells are under a continuous oxidative stress[13,14] due to the generation of ROS that is identified as one of the key molecules involved in the multistage process of carcinogenesis.[15] Research carried out with human tumor cell lines clearly indicates that cancer cells produce ROS at a much higher rate than healthy cells.[16,17] Antioxidants play an important role by inhibiting the initiation step of oxidation of bio-molecules as well as scavenging various free radicals and thereby detoxifying cells.[18] Therefore, natural antioxidants are popular for its therapeutic efficacy which makes prospecting for bioactive plant products an important area of research. Anticancer properties of herbal drugs in most cases are related to their antioxidant activities.

Medicinal plants have remained very useful in the discovery of new anticancer drugs.[19] The inhabitants

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of Himalaya use indigenous knowledge for the preparation of various plant based ethnomedicines in order to treat cancer.[20] Identification of traditionally effective anticancer plants and evaluation of their underlying mechanisms could be helpful in development of novel drugs for cancer treatment.[21] Therefore, we undertook this first step to look for antioxidant and anticancer activity of *P. polyphylla* from two altitudinal zones of Sikkim Himalaya.

MATERIALS AND METHODS

Collection of plant material

The rhizomes of *P. polyphylla* were collected from two altitudinal zones: the higher temperate zone of 'Tholung' (2700 m asl approx., 27°39′218′′N and 88°27′435′′E) and lower temperate zone of 'Uttaray'(1900 m asl approx., 27°16′115′′N and 88°08′306′′E) respectively (Figure 1). The samples which were morphologically distinct were collected from its natural habitats in the month of October 2016, India. The samples collected from Uttaray (hereafter called PPU) those from Tholung (hereafter called PPT) were deposited at Botanical Survey of India, Eastern Reginal Circle, Gangtok, India, (VS No. 0187 and 0189). The collected material (rhizomes) were washed thoroughly under running tap water and finally with distilled water and allowed to dry under shade to a constant weight.

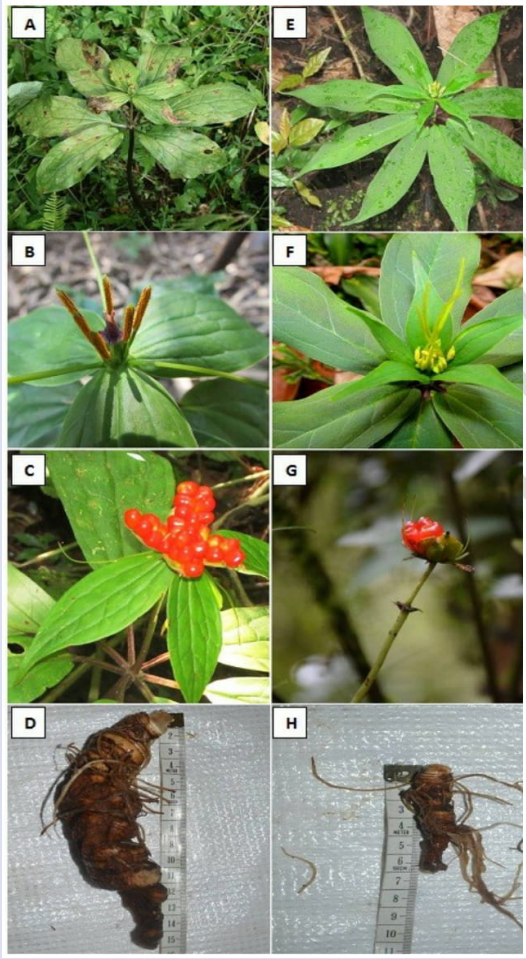

Figure 1: Morphological features of *Paris polyphylla* from Uttray-PPU (A, B, C and D depicting the plant, flower, fruit and rhizome respectively) and *Paris polyphylla* from Tholung-PPT (E, F, G and H depicting the plant, flower, fruit and rhizome respectively).

Solvent extraction of plant material

The completely shade-dried rhizomes of *P. polyphylla* were pulverized using a waring blender, powdered and sieved (0.1 mm mesh). The powdered plant material (10 g) was soaked in 10 vol. (100 ml) of 70% methanol for 24 h at room temperature with intermittent shaking and the supernatant decanted. The extraction was repeated thrice using fresh solvents and the extracts were pooled together and defatted by partitioning with hexanes, the MeOH layer was filtered through a Whatman no. 1 paper, evaporated at low temperature. The residue thus obtained was dissolved in DMSO prior to use.

Phytochemical analysis

Determination of total phenolic content

The total phenolic contents was determined from the extract according to the method of Lin *et al.*[22] using Folin-Ciocalteau reagent. Gallic acid (GA) was used as a standard. Total phenol contents were calculated as milligrams of gallic acid equivalent (GAE) per gram of dry weight (mg GAE/g).

Determination of total flavonoid content

The flavonoid contents of the extract was measured using a modified colorimetric method described by Lin *et al.*[22] Rutin was used as standard and the flavonoid contents were expressed as rutin equivalents (RtE).

Determination of total flavonols

Total flavonol contents of the extract was measured using the method given by.[23] Quercetin was used as standard and the flavonoid contents were calculated and expressed as mgQuercetin equivalents/g DW.

Determination of antioxidant activity

DPPH radical scavenging activity

DPPH scavenging activity of the rhizome extracts was determined according to Yu *et al.*[24] with slight modification. This method is based on the ability of an antioxidant to scavenge the DPPH cation radical. Briefly, 1.0 mL of the sample extract (containing 50, 100, 200, 300 and 500 µg/ml of lyophilized extract) or standards was added to the 3 mL of 0.5 mM DPPH solution and vortexed vigorously; then incubated in dark for 30 mins at room temperature and the decolourization of DPPH was measured against blank at 517 nm. The ability of sample to scavenge DPPH radical was calculated by the following equation:

$$DPPH\ radical\ scavenging\ activity\ (\%) = [(Abs\ control - Abs\ sample)]/(Abs\ control) \times 100$$

Where, Abs control is the absorbance of DPPH radical +methanol; Abs sample is the absorbance of DPPH radical + extract/ standard.

The radical scavenging activity of extracts was determined by IC_{50} value which is the concentration of extracts at which DPPH radicals are scavenged by 50%. The lower IC_{50} value indicates higher radical scavenging capacity and vice versa.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity each extracts were calculated as described by Yu *et al.*[24] with slight modification. This assay is based on Fenton reaction. Briefly 2 mL of 0.2 M phosphate buffer (pH 7.2), 0.04 mL ferrous sulphate (0.02 M), 2 mL of extract (of 200, 400, 600, 800 100 µg/ml concentration) and 1 mL of 1, 10-phenanthroline (0.04 M) were delivered into the test tube. The Fenton reaction was initiated by addition of 0.1 mL of 7 mM H_2O_2. Absorbance was measured at 560 nm after 5 mins incubation at room temperature. The relative hydroxyl radical scavenging activity (%) was calculated as:

Scavenging Activity (%) = $(A_{blank}-A_{sample})/(A_{blank})$x 100

IC_{50} value was determined using the same method mentioned above.

Fe^{2+} chelating activity

The chelating activity of the extracts for ferrous ions Fe2+ was measured according to the method of Dinis *et al.*[25] with slight modification. To 0.4 ml of extract (containing 200, 400,600,800 and 1000 µg/ml of extract powder), 1.6 ml of methanol was diluted and mixed with 0.04 ml of $FeCl_2$ (2 mM). After 30s, 0.8 ml ferrozine (5 mM) was added. After 10 min at room temperature, the absorbance of the Fe^{2+}–Ferrozine complex was measured at 562 nm. The tube without the sample extract served as control. The chelating activity of the extract for Fe2+ was calculated using the formula mentioned below:

Chelating activity (%) = (Ac - As)/Ac × 100

where Ac = absorbance of the control in the reaction system and

As = absorbance of the sample.

The IC_{50} value was calculated as before from the calibration curve obtained.

Determination of ABTS radical scavenging activity

ABTS radical cation (ABTS•+) discoloration test is a spectrophotometric method widely used for assessment of the antioxidant activity of various substances. The ABTS·+ scavenging activity was measured by the method of Re *et al.*[26] Ascorbic acid was used as a positive control.[27] Percentage of inhibition was calculated using the formula:

Inhibition (%) = $(A_0 - A_1 / A_0) \times 100$

Where, A_0 is the absorbance of control and A_1 is the absorbance of sample. IC_{50} value was also determined.

Anti proliferative activity of methanolic extract of *Paris polyphylla*

The human carcinoma cells, HeLa (cervical cancer), PC3 (prostrate cancer) and HepG2 (hepato carcinoma) were used to investigate the cytotoxic activity evaluation of the methanolic extract of the sample. The cells were cultured to reach confluence in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 unit/ml penicillin and 100 µg/ml streptomycin and maintained at 37°C in a humidified atmosphere with 5% CO_2 incubator (Figure 2).

Under the influence of a methanolic extract, cell viability was assessed by the MTT assay as described by Mosmann.[28] For the experiment, exponentially growing cells were trypsinized and aseptically collected, counted and adjusted to a final concentration of 3×10^3 cells/well, to be inoculated on 96-well plates. After 24 hr adherence, the cells were treated with various concentration of *P. polyphylla* rhizome extract for 72 hr. After the incubation 1:10 volume of MTT solution (5 mg/mL) was added to each well and incubated for 4 hr in dark. Then the medium was carefully removed and the formazan formed in the wells was dissolved for homogenous measurement in 150 µl of dimethyl sulfoxide, the plates were kept for 5 min on a plateshaker. The absorbance was measured at 570 nm using a microplate reader. For the control, SDYB medium (pH 7.4) and Dulbecco's PBS were used in place of the rhizome extract.

Cytotoxicity was calculated as the percent reduction in absorbance relative to the control (DMSO). Unless stated otherwise, all experiments were performed in quadruplicate (n=4). Half maximal inhibitory concentration (IC_{50}) was determined graphically by plotting percentage of inhibition against the concentration of drug. The percentage inhibition is calculated, from, the data using the formula:

Mean OD of untreated cells (control)–Mean OD of treated cells (sample)

% Inhibition =**x 100**

Mean OD of untreated cells (control) x 100%.

Statistical analysis

The data obtained from the phytochemical estimation and antioxidant assays were presented as means of triplicate determinations ± standard deviation (SD). Cytotoxic activity of the sample extracts were determined from quadruplicate observations and values are presented as Mean ± SE. To calculate IC_{50} values, linear regression analysis was

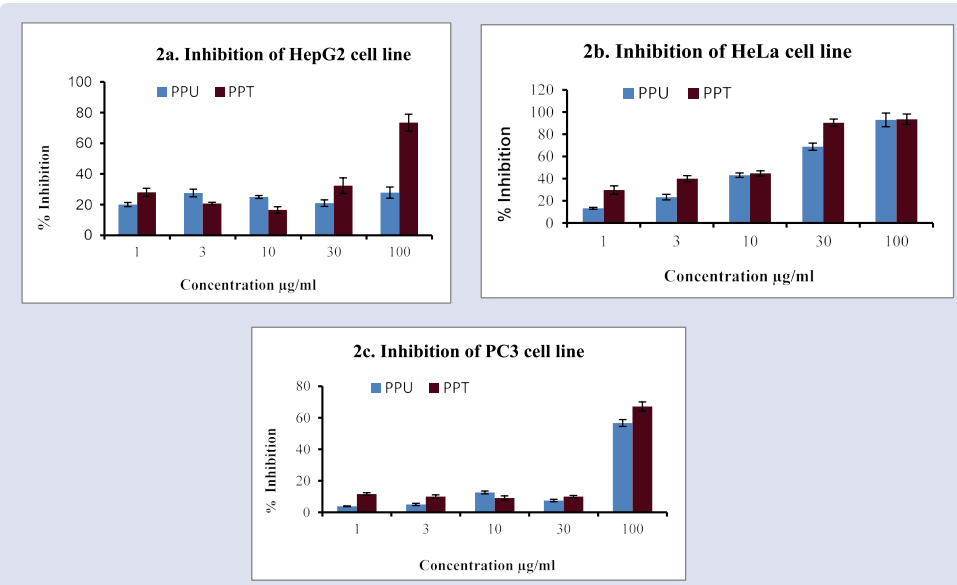

Figure 2: Percentage inhibition in growth of **a)** HepG2, **b)** HeLa and **c)** PC3 cancer cell lines in presence of different concentration of methanolic rhizome extracts of PPU and PPT as determined by MTT assay. Values were expressed as Mean ± SEM of quadruplicate independent observations. The % inhibition was calculated as percent difference between growth in DMSO (control) and growth in presence of extracts at 72 h of incubation.

carried out using Microsoft Excel 2010 (Microsoft Corporation, WA, USA). Data were analysed by SPSS 15.0 software (SPSS Inc, IL, USA) software and differences with $p \leq 0.05$ were considered significant.

RESULTS

Total phenolic, flavonoid and flavonol content

Phenols, ubiquitous secondary metabolites that could play a vital antioxidant role against different free radicals. The total phenolic contents of methanolic extracts of rhizomes of *P. polyphylla* from Tholung(PPT) and *P. polyphylla* from Uttaray (PPU), the higher and lower altitude samples contained 16.29 ± 0.03 mg GAE/g DW and 5.96 ± 0.02 mg GAE/g DW respectively. Substantial quantity of flavonoids were present in both the samples from high and low altitudes with PPT and PPU showing total falvonoid content of 30.46 ± 0.06 mg QE/g DW respectively. Higher content of total flavonol was obtainded from the rhizomes of *P. polyphylla* obtainded from the higher altitude. Thus, PPT exhibited a flavonol content of 23.08 ± 0.03 mg RtE/g DW as against 13.74 ± 0.02 mg RtE/g DW for that from PPU (Table 1).

Antioxidant activity assay

Free radical scavenging activity in the extracts of *P. polyphylla* rhizome extracts were measured in terms of DPPH assay by determining its IC_{50} values. The methanolic rhizome extracts from both types of the herb proved to be an excellent scavenger of DPPH with an IC_{50} value of 2.01 µg/ml and 2.55 µg/ml for PPT and PPU respectively. Moreover, when compared to standard ascorbic acid, the DPPH scavanging activity of was quite high (Table 2).

ABTS assay exhibited that the methanolic extract giving the IC_{50} value (2.22 µg/ml and 2.57 µg/ml for PPT and PPU respectively. The IC_{50} value of the methanolic extract was comparable to that of the standard, ascorbic acid. In other words, these extracts showed potent antioxidant activity as compared to the standard (Table 2).

The hydroxyl radical scavenging activity of the rhizome extracts of *P. polyphylla* showed that the plant from the lower altitude showed strong scavenging avtivity (IC_{50}: 1.97 µg/ml) as compared to that from the higher altitude (2.38 µg/ml) which is opposite to the trend in other types of assay. As expected, just the opposite result was observed in the Fe^{2+} chelating activity with PPT extract giving IC_{50} value of 1.97 µg/ml and 2.35 µg/ml *PPT* and PPU respectively (Table 2).

Inhibition of cell proliferation

The effect of methanolic rhizome extract on the inhibition of cell growth was investigated by the MTT assay in a dose dependent manner. Among the three cancer cells lines studied, the effect of the methanolic extract had an extremely high dose-dependent inhibition of HeLa cell growth reaching >90% inhibition at 100 µg/ml concentration within 72 hrs of treatment. Both the extracts, PPT and PPU had a relatively moderate effect on HepG2 cells growth upto 30µg/ml concentration, however, PPT showed its significant inhibition (73.47%) at 100 µg/ml concentration. Similarly, PC3 cell line was resistant to both PPU and PPT upto a certain extent, but again both the extracts showed appreciable inhibition (>50%) at a high concentration of 100 µg/ml. All the anticancer activities of methanolic extracts were significantly higher than those of control, suggesting the role of extracts in enhancing these attributes.

Table 1: Total Phenolic, flavonoid and flovonol contents of methanolic extract of *Paris polyphylla* rhizome from two altitudinal zones (Values are expressed as the mean±SD (n=3)).

Phytochemical contents	PPT	PPU
Total Phenolic content (mg GAE/g DW)	16.29 ± 0.03 mg	5.96 ± 0.02 mg
Total Flavonoid content (mg QE/g DW)	30.46 ± 0.06 mg	23.99 ± 0.01 mg
Total Flovonol content (mg RtE/g DW)	23.08 ± 0.03 mg	13.74 ± 0.02 mg

GAE= gallic acid equivalent, QE= quercetin equivalent, RtE= Rutin

Table 2: Antioxidant activity in methanolic extract of the rhizomes of PPU and PPT as determined by different assay in terms of IC_{50} values (mg/ml) (Values are mean ± SD, (n = 3)).

Assay	PPU	PPT	Standard (Ascorbic acid)
DPPH radical Scavenging Activity	2.55 ± 0.04	2.01 ± 0.16	2.91 ± 0.05
Hydroxyl radical scavenging activity	1.97 ± 0.03	2.38 ± 0.04	3.25 ± 0.06
Fe^{2+} Chelating Activity	2.35 ± 0.06	1.97 ± 0.23	5.92 ± 0.04
(ABTS•+) radical scavenging activity	2.57 ± 0.06	2.22 ± 0.08	2.47 ± 0.03

DISCUSSION

Phenolic compounds contribute to the quality and nutritional value in terms of modifying color, taste, aroma and flavor and also in providing health beneficial effects. They are effective antioxidants as free radical scavenger, chelator of metals ions and absorber of oxygen radicals.[29] The total phenolic contents of PPT and PPU were found to be quite high, but the content was almost 3 times higher in case of *Paris* obtained from higher altitude (PPT). The methanolic extract of tuber of anticancer plant *Plectranthus rotundifolius* contains 5.49 mg GAE/g DW[30] which is much lower than the total phenolic content of PPT 16.29 ± 0.03 mg GAE/g DW but similar to that of PPU (5.96 ± 0.02 mg GAE/g DW).

Flavonoids are therapeutic, water soluble polyphenloic compounds in plant parts and vegetables[31] having potential health benefits as antioxidant, antiproliferative and chemopreventive agents.[32] They exhibit anti-proliferative activities in numerous cancer cell lines and inhibit tumor growth in certain animal models. Kaempferol, quercetin and kaempferol-3-(60-coumaroyl) glucoside, isolated from herbal extracts showed anti-proliferative activities in cervical, colon, prostate and oral cavity cancer cell lines.[33]

Flavonoid content in *P. polyphylla* (T) was significantly high and as much as that found in *Plectranthus rotundifolius* which contains 22.59 mg QE/g DW. However, *P. polyphylla* (U) contains much similar quantity of flavonoids (23.99 mg QE/g DW).[30] The methanolic root extract of *Hypochaeris radiata* contains about one fifth and one fourth quantity of total flavonoids as compared to that in PPT and PPU respectively.[34] The amount of flavonol in methanolic extract of both varieties of *P. polyphylla* was quite appreciable with PPT showing almost twice the activity as compared to that of PPU.

DPPH assay revealed reasonably high antioxidant activity in both the extracts *P. polyphylla* with rhizome extract of PPT showing slighghtly higher scavenging activity (IC_{50} = 2.01 µg/ml). The IC_{50} value for different extracts of *Valeriana hardwickii* as determined by DPPH Assay varied between 15.60-25.05 mg/ml. High scavenging activity of these extracts may be due to its high flavonoid contents.[34] In the ABTS assay also the methanolic extract of both varieties of *P. polyphylla* exhibited strong scavenging activity (IC_{50} = 2.22-2.57 µg/ml). Some compounds which show DPPH scavenging activity may not show ABTS scavenging activity.[35] However, in the present case, extracts were found effective in both the assays possibly due to the action of altogether different metabolites.

Among the reactive oxygen species, hydroxyl radical is considered to be one of the most potent oxidants which can easily crosscell membranes and readily react with most biomacromolecules, such as carbohydrates, proteins, lipids and DNA. Therefore, scavenging hydroxyl radicals is extremely important for the protection of living systems. Scavenging effects of *P. polyphylla* rhizome extracts on hydroxyl radical were determined based on the principle of Fenton reaction and the effects increased with concentrations. The scavenging activity was higher in case of PPT at IC_{50} = 1.97 µg/ml. In contrast, leaf polysaccharide purified from *P. polyphylla* had shown an extremely low scavenging effect on hydroxyl radical at IC_{50} = 310 µg/ml.[36]

Metal chelating capacity is important because it reduces the concentration of transition metals that catalyze lipid peroxidation. The decrease in concentration dependent color formation in this case in the presence of *P. polyphylla* extracts indicated its iron chelating activity. Ferrous chelation activity was appreciable in both the extracts with higher activity shown by *P. polyphylla* (T). The ferrous chelating activity of another root drug, *Withania somnifera* was much lesser (IC_{50} = 0.37 mg/ml).[37]

The effect of methanolic extract on the inhibition of cell growth was investigated by the MTT assay on the three cancer cell lines studied which showed that both the extracts had an extremely high dose-dependent inhibition of HeLa cell growth within 72 hrs of culture, while the same had moderate effect on HepG2 cells growth. At a concentration of upto 30 µg/ml, the PC3 cells were resistant to the drug, which showed dramatic inhibition at 100 µg/ml concentration. These results also suggest that at higher concentration, methanolic extract had an inhibitory effect on cell viability, a trend which was observed in the case of antioxidative assay. The result obtained for cytotoxic effects of methanolic extracts of the rhizome are in accordance with the similar observations. 80% inhibition of HeLa cell growth reportedly took place by the influence of methanolic extract (10 µg/ml) of a well known anticancer plant, *Taxus cuspidata* needles and twigs.[38] Similar cytotoxicity against HeLa cell proliferation as that of Paris polyphylla was also exhibited by the ethanolic extract of *Rumex nepalensis* rhizome.[39] The ethanolic extract (50 µg/ml) of the rhizomes of *Bergenia ciliata* showed 27.14% inhibition of HepG2.[39] On the other hand, methanolic extract (200 µg/ml) of *Allanblackia floribunda* and *Lannea nigritana* caused inhibitions of PC3 cells by 92% and 21% respectively.[40] Thus, our results, in terms of cytotoxic properties of *P. polyphylla* was very promising but within the expected range. This is the first such report involving *P. polyphylla* from Sikkim Himalaya. This study will provide baseline information for further research.

CONCLUSION

The methanolic extracts of *P. polyphylla* rhizomes showed an appreciable content of total phenols, flavonoids and flavonols. The extracts exhibited remarkable antioxidant properties through different assays. In MTT assay, significant dose-dependent inhibition of growth was observed after 72 hrs of treatment especially in case of HeLa cells. Moderate inhibition of growth was also seen in PC3 and HepG2 cells. Of the two varieties, PPT was more pronounced in both antioxidant and cytotoxic activities.

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CONFLICT OF INTEREST

None.

ABBREVIATIONS

ABTS: 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); DPPH: 2, 2-diphenyl-1-picrylhydrazyl; DMSO: Dimethyl Sulfoxide; GAE: Gallic Acid Equivalents; RtE: Rutin Equivalents; QE: Quercetin Equivalents.

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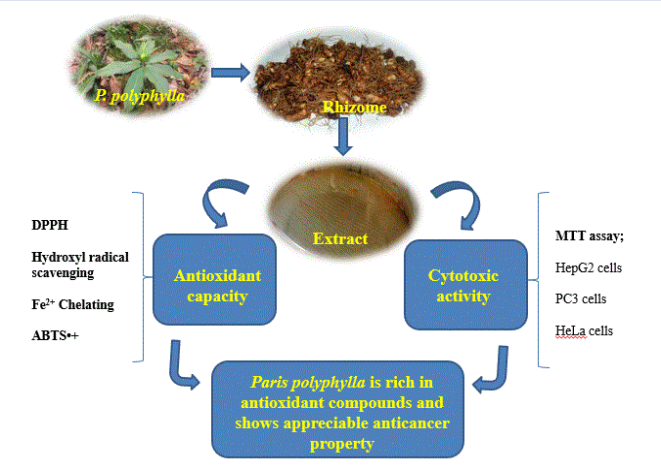
GRAPHICAL ABSTRACT



SUMMARY

Methanolic extracts of rhizomes of *Paris polyphylla* from two altitudes of Sikkim Himalaya viz., Tholung (PPT) and Uttaray (PPU) were analyzed to explore its antioxidant activity as well as anticancer property. Total phenolics, flavonoids and flavonols were determined from the extract and the antioxidant capacity was tested by DPPH assay, ABTS assay, OH-radical scavenging assay and Fe^{2+}chelating capacity methods. Similarly, cytotoxicity of the extract was determined by MTT assay on three cancer cell lines: HeLa, HepG2 and PC3. The extracts were rich pools of phenolic compounds and presented a high antioxidant potential in all the tests carried out. Moreover, potent anticancer activity was the hallmark of the extracts as exemplified by *in-vitro* growth inhibition of human cancer cell lines especially, HeLa cells. The activity of PPT was more intense in both the counts of antioxidant and cytotoxic attributes as compared to that of PPU.

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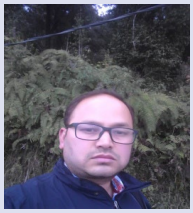

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