Antioxidant and Cytotoxic Attributes of Paris polyphylla Smith from Sikkim Himalaya

Dawa Lhendup Lepcha, Abhijit Chhetri, Dhani Raj Chhetri*

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. 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. Despite this, a substantially rising number cases of cancer is being reported in Sikkim. 

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. Furthermore, it has been used to treat liver cancer in China for several decades. In the Indian Himalayan Region, P. polyphylla, is used against burn, cut or injury, diarrhea, dysentery, fever, gastritis, skin diseases, stomach pain and wounds. 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. 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. Many reports suggest that cancer cells are under a continuous oxidative stress due to the generation of ROS that is identified as one of the key molecules involved in the multistage process of carcinogenesis. Research carried out with human tumor cell lines clearly indicates that cancer cells produce ROS at a much higher rate than healthy cells. 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. 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. The inhabitants...
of Himalaya use indigenous knowledge for the preparation of various plant based ethnomedicines in order to treat cancer. Identification of traditionally effective anticancer plants and evaluation of their underlying mechanisms could be helpful in development of novel drugs for cancer treatment. 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 Regional 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.

**Phytochemical analysis**

**Determination of total flavonoid content**

The total phenolic contents was determined from the extract according to the method of Lin et al. Rutin was used as standard and the flavonoid contents were expressed as rutin equivalents (RtE).

**Determination of total flavonols**

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

**Determination of antioxidant activity**

**DPPH radical scavenging activity**

DPPH scavenging activity of the rhizome extracts was determined according to Yu et al. 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:

\[
\text{DPPH radical scavenging activity (\%)} = \frac{(\text{Abs control}-\text{Abs sample})}{\text{Abs control}} \times 100
\]

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\text{50} value which is the concentration of extracts at which DPPH radicals are scavenged by 50%. The lower IC\text{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. 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 \( \text{H}_2\text{O}_2 \). Absorbance was measured at 560 nm after 5 mins incubation at room temperature. The relative hydroxyl radical scavenging activity (%) was calculated as:

\[
\text{Hydroxyl radical scavenging activity (\%)} = \frac{A_{control}-A_{sample}}{A_{control}} \times 100
\]
Scavenging Activity (%) = \frac{(A_{blank} - A_{sample})}{(A_{blank})} \times 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 Fe^{2+} was measured according to the method of Dinis et al.\textsuperscript{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\textsubscript{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 Fe^{2+} was calculated using the formula mentioned below:

Chelating activity (%) = \frac{(A_c - A_s)}{A_c} \times 100

where A\textsubscript{c} = absorbance of the control in the reaction system and A\textsubscript{s} = absorbance of the sample.

The IC\textsubscript{50} value was calculated as before from the calibration curve obtained.

Determination of ABTS radical scavenging activity

ABTS radical cation (ABTS\textsuperscript{+}) discoloration test is a spectrophotometric method widely used for assessment of the antioxidant activity of various substances. The ABTS\textsuperscript{+} scavenging activity was measured by the method of Re et al.\textsuperscript{26} Ascorbic acid was used as a positive control.\textsuperscript{27}

Percentage of inhibition was calculated using the formula:

\text{Inhibition} (%) = \frac{(A_c - A_s)}{A_c} \times 100

where A\textsubscript{c} is the absorbance of control and A\textsubscript{s} is the absorbance of sample. IC\textsubscript{50} value was also determined.

Anti proliferative activity of methanolic extract of Paris polyphylla

The human carcinoma cells, HeLa (cervical cancer), PC3 (prostate 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\textsubscript{2} incubator (Figure 2).

Under the influence of a methanolic extract, cell viability was assessed by the MTT assay as described by Mosmann.\textsuperscript{28} For the experiment, exponentially growing cells were trypsinized and aseptically collected, counted and adjusted to a final concentration of 3 x 10\textsuperscript{4} 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\textsubscript{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:

\text{Mean OD of untreated cells (control)} \times 100\%

\text{% Inhibition} = \frac{\text{Mean OD of untreated cells (control)} - \text{Mean OD of treated cells (sample)}}{\text{Mean OD of untreated cells (control)}} \times 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\textsubscript{50} values, linear regression analysis was performed.
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 flavonoid content of 30.46 ± 0.06 mg QE/g DW respectively. Higher content of total flavonol was obtained from the rhizomes of *P. polyphylla* obtained from the higher altitude. Thus, PPT exhibited a flavonol content of 23.08 ± 0.03 mg RE/g DW as against 13.74 ± 0.02 mg RE/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<sub>50</sub> values. The methanolic rhizome extracts from both types of the herb proved to be an excellent scavenger of DPPH with an IC<sub>50</sub> 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 scavenging activity was quite high (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 activity (IC<sub>50</sub> = 1.97 μg/ml) as compared to that from the higher altitude (2.35 μg/ml) which is opposite to the trend in other types of assay. As expected, the opposite result was observed in the Fe<sup>2+</sup> chelating activity with PPT extract giving IC<sub>50</sub> 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 cell 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 up to 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.

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. 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<sup>19</sup> 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 polyphenolic compounds in plant parts and vegetables<sup>31</sup> having potential health benefits as antioxidant, antiproliferative and chemopreventive agents. 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.<sup>15</sup>

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).<sup>16</sup> The methanolic root extract of *Hypochoeris radiata* contains about one fifth and one fourth quantity of total flavonoids as compared to that in PPT and PPU respectively.<sup>24</sup> 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 slightly higher scavenging activity (IC<sub>50</sub> = 2.01 μg/ml). The IC<sub>50</sub> 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. In the ABTS assay also the methanolic extract of both varieties of *P. polyphylla* exhibited strong scavenging activity (IC<sub>50</sub> = 2.22-2.57 μg/ml). Some compounds which show DPPH scavenging activity may not show ABTS scavenging activity.<sup>25</sup> However, in the present case, extracts were found effective in both the assays possibly due to the action of altogether different metabolites.

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

<table>
<thead>
<tr>
<th>Phytochemical contents</th>
<th>PPT</th>
<th>PPU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Phenolic content (mg GAE/g DW)</td>
<td>16.29 ± 0.03 mg</td>
<td>5.96 ± 0.02 mg</td>
</tr>
<tr>
<td>Total Flavonoid content (mg QE/g DW)</td>
<td>30.46 ± 0.06 mg</td>
<td>23.99 ± 0.01 mg</td>
</tr>
<tr>
<td>Total Flavonol content (mg RE/g DW)</td>
<td>23.08 ± 0.03 mg</td>
<td>13.74 ± 0.02 mg</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Assay</th>
<th>PPU</th>
<th>PPT</th>
<th>Standard (Ascorbic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH radical Scavenging Activity</td>
<td>2.55 ± 0.04</td>
<td>2.01 ± 0.16</td>
<td>2.91 ± 0.05</td>
</tr>
<tr>
<td>Hydroxyl radical scavenging activity</td>
<td>1.97 ± 0.03</td>
<td>2.38 ± 0.04</td>
<td>3.25 ± 0.06</td>
</tr>
<tr>
<td>Fe&lt;sup&gt;2+&lt;/sup&gt; Chelating Activity</td>
<td>2.35 ± 0.06</td>
<td>1.97 ± 0.23</td>
<td>5.92 ± 0.04</td>
</tr>
<tr>
<td>(ABTS•+) radical scavenging activity</td>
<td>2.57 ± 0.06</td>
<td>2.22 ± 0.08</td>
<td>2.47 ± 0.03</td>
</tr>
</tbody>
</table>
Among the reactive oxygen species, hydroxyl radical is considered to be one of the most potent oxidants which can easily cross cellular 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\textsubscript{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\textsubscript{50} = 310 µg/ml.\textsuperscript{48}

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\textsubscript{50} = 0.37 mg/ml).\textsuperscript{49}

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 up to 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.\textsuperscript{50} Similar cytotoxicity against HeLa cell proliferation as that of Paris polyphylla was also exhibited by the ethanolic extract of *Rumex nepalensis* rhizome.\textsuperscript{48} The ethanolic extract (50 µg/ml) of the rhizomes of *Bergenia ciliata* showed 27.14% inhibition of HepG2.\textsuperscript{47} 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.\textsuperscript{50} 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.

**ACKNOWLEDGEMENTS**

University Grants Commission, Government of India is thankfully acknowledged for research fellowship to DLL, Forest, Environment and Wildlife Management Department, Government of Sikkim is acknowledged for the samples. The authors are thankful to the traditional healers of North Sikkim for sharing their knowledge.

**CONFLICT OF INTEREST**

None.

**ABBREVIATIONS**

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

**REFERENCES**


Lepcha, et al.: Paris polyphylla from Sikkim Himalaya


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 Fe²⁺-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.

About Authors

Dawa Lhendup Lepcha has completed M.Sc. in Ethnobotany & Social Medicine studies and presently pursuing Ph.D programme in Botany in the Department of Botany, Sikkim University, India. Himalayan biodiversity, ethnomedicinal and Phytochemistry are his fields of interest. So far he has published 4 research articles in various international and national Journals.

Abhijit Chhetri did his Bsc. and Msc. in Microbiology and currently pursuing his Ph.D in Botany from Sikkim University, India. He has published five research articles in international journals. Bioprospecting for anticancer and anti-diabetic plants from Himalayan flora is his field of interest.
Dhani Raj Chhetri is an Associate Professor in the Department of Botany, Sikkim University, India. His research interest includes Himalayan medicinal plants, ethnopharmacology, plant biochemistry and biotechnology. Dr. Chhetri is a recipient of National Merit Scholarship and he has completed 8 research projects funded by national and international agencies. He has more than 50 research publications in international journals, authored two books and has an edited book to his credit. Besides academics, Dr. Chhetri takes keen interest in literary works and writes popular articles.