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Original Article

Cytotoxic Activity of Antioxidant-Riched *Dendrobium longicornu*

Mukti Ram Paudel*, Mukesh B Chand, Basant Pant, Bijaya Pant

ABSTRACT

Context: *Dendrobium longicornu* is a traditional medicinal plant widely used in Asia. It has many bioactive compounds like bibenzyl, phenanthrenes, phenolic compounds. There has been little research in the cytotoxic and antioxidant effects of *D. longicornu*. **Aims:** The aim of this study was to investigate the cytotoxic and antioxidant activities of this plant. **Settings and Design:** Antioxidant and cytotoxic activity of *Dendrobium longicornu* extracts. **Methods and Material:** The plant extracts were prepared by soxhlet's extractor in organic solvents, acetone and ethanol. The total polyphenol content (TPC) in the extracts was determined spectrophotometrically by the Folin-Ciocalteu method and the total flavonoid content (TFC) by aluminium chloride method. The antioxidant activity was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The cytotoxic activity was evaluated against human brain tumor cells (U251) and cervical cancer cells (HeLa) using MTT assay. **Statistical analysis used:** Regression analysis was done for calculation of IC_{50}. Duncan multiple range test and Dunnett test were done to compare the data. **Results:** The *Dendrobium longicornu* acetonic extract (DLA) showed significantly highest TPC and TFC than *Dendrobium longicornu* ethanolic extract (DLE). The antioxidant activity was also significantly higher in DLA followed by DLE. Highest cytotoxicity (i.e., lowest IC_{50} value) was found for the DLA on U251 cells and DLE on HeLa cells. **Conclusions:** This result concluded that *D. longicornu* is a potential source of antioxidant and cytotoxic agents.

Key words: *Dendrobium longicornu*, DPPH, flavonoid, MTT, Polyphenol.

Mukti Ram Paudel[1*], Mukesh B Chand[1], Basant Pant[2], Bijaya Pant[1]

[1]Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, NEPAL.
[2]Annapurna Research Center, Maitighar, Kathmandu, NEPAL.

Correspondence

Mukti Ram Paudel

Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, NEPAL.

Phone: +97714331322

E-mail: m.paudel47@gmail.com

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INTRODUCTION

Dendrobium longicornu is an important medicinal orchid used to treat fever and coughs.[1] Variety of its chemical constituents such as bibenzyls, phenanthrenes, lignin glycoside and phenolic compounds have been reported.[2,3] Besides these, biological activities of this plant have not been reported yet. Reactive oxygen species and reactive nitrogen species, including singlet oxygen, hydrogen peroxide, superoxide anion, hydroxyl radical and nitrous oxide are often generated in the living organisms as byproducts of biological reaction, are known to play a vital role in oxidative cellular damaged. Oxidative stress resulting from these free-radicals plays an important role in manifesting various disorders, including ageing, diabetes, cancer and cardiovascular diseases.[4] Recently, the natural antioxidant study has become popular by DPPH radical scavenging assay because of its simplicity and high sensitivity. This assay is based on the theory that a hydrogen donor is an antioxidant. The antioxidant effect is proportional to the disappearance of DPPH radical in test samples.[5] Biologically active compounds, include the phenolic compounds, phenanthrenes, bibenzyls, alkaloids, are widely used in medicine.[6,7] The phytochemicals screened for biological activity, bioassay that appears capable of bioactivity present in the crude extracts is the MTT assay. This method provides a front-line screen that can be backed up more specific bioassay. It appears that MTT assay is predictive of cytotoxicity of any phytochemicals.[8]

Dendrobium longicornu produce natural antioxidants, they are potent source of useful new compounds with biological activities. The primary objectives of the present study are to estimate the total polyphenol and flavonoid contents in the crude extracts of this plant, and to evaluate the antioxidant and cytotoxic activities of the crude extracts.

SUBJECTS AND METHODS

Plant materials

The stems of *Dendrobium longicornu* were collected from Chitlang, Makawanpur district, Central Nepal. The collected stems were air-dried in shade at room temperature (20°C), grinded to fine powder and stored in tight-seal dark container until used.

Preparation of plant extracts

Plant extracts were prepared using soxhlet extractor. Grinded stem powder was extracted with two solvents, acetone and ethanol in the ratio of 1:10 (w/v).[9] Solvent evaporated under room temperature to obtain dry extract. Each extract was dissolved in 99% ethanol (analytical grade) to make a concentration of 1 mg/ml stock solution and stored at 4°C.

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Estimation of total polyphenol content

Total polyphenol content (TPC) in the plant extracts was determined using Folin-Ciocalteu's reagent method.[10] Stock solution of each extract (1 mg/ml) was used to estimate total phenolic content. The reaction mixture was prepared by mixing 0.5 ml of stock solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent and 2.5 ml 7.5% $NaHCO_3$. Blank was concomitantly prepared, containing 0.5 ml ethanol, 2.5 ml 10% Folin-Ciocalteu's reagent and 2.5 ml of 7.5% of $NaHCO_3$. The reaction mixtures were thereafter incubated in room temperature for 45 min. The absorbance was measured using Genesys UV-visible spectrophotometer at 765 nm. The estimation of polyphenol content was carried out in triplicate. The same procedure was repeated for the standard solution of gallic acid. The total polyphenol content of extract was expressed as micrograms of gallic acid equivalent (GAE) per milligrams of dry extract.

Estimation of total flavonoid content

Total flavonoid content (TFC) in the plant extracts was determined using Aluminium chloride method.[10] Stock solution of each extract (1 mg/ml) was used to estimate total flavonoid content. The reaction mixture was prepared by mixing 1 ml extract and 1 ml of 2% $AlCl_3$ solution (dissolved in ethanol). The reaction mixtures were incubated for an hour at room temperature. The absorbance was measured using spectrophotometer at 415 nm. This experiment was carried out in triplicate. The same procedure was repeated for the standard solution of quercetin hydrate. The total flavonoid content of extract was expressed as micrograms of quercetin equivalent (QE) per milligrams of dry extract.

Antioxidant activity assay

In vitro antioxidant activity was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay according to the method described earlier.[11] Stock solution of each extract at concentration of 1 mg/ml diluted to prepare the series concentrations (50, 100, 200, 400 and 800 µg/ml) for antioxidant assay. An aliquot of 1.5 ml of 0.25 mM DPPH solution in ethanol and 1.5 ml of extract at various concentrations were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min. Decolourization of DPPH was determined by measuring the absorbance at 517 nm using Genesys UV-visible spectrophotometer. Antioxidant assay was carried out in triplicate for series of concentrations. Antioxidant activity of extracts was expressed as 50% inhibitory concentration of extract (IC_{50} µg/ml of extract). IC_{50} of extracts was calculated using polynomial regression equation where abscissa represents the series concentrations of extracts and ordinate the triplicate percentage antioxidant activity.

Cytotoxic activity assay

Cytotoxic activity of crude plant extracts of *Dendrobium longicornu* was determined by using MTT assay.[8,12] Human brain tumor cells (U251) and cervical cancer cells (HeLa) were cultured in RPMI 1640 medium and incubated under 5% CO_2 at 37°C for 48 hours to reach 80% confluence. The cells were harvested by gentle scraping with a cell scraper and resuspended in the medium. From the suspension, $5x10^3$ cells in 100 µl medium was dispensed into each well of 96-well microtiter cell culture plate and incubated under the same conditions of cell culture for 48 hours to allow adherence and growth of cells. The supernatants were gently aspirated, and 100 µl of *D. longicornu* extracts were added over a range of four cytotoxic concentrations (100 – 800 µg/ml) prepared in medium and incubated for 24 hours. Ten µl of 5 mg/ml MTT was added to every well and the plate was reincubated for further 4 hours. The formazan crystals formed were dissolved in 100 µl DMSO. The plate was then read on a microplate reader (iMarkTM, Bio-Rad) at 595 nm. The number of dead cells per well was calculated as a percentage of the control so measuring cell death after drug exposure. A dose-response curve was plotted for each drug to calculate the inhibition of cell growth by 50%. IC_{50} of extracts was calculated from the regression equation where abscissa represents the series of concentrations of extracts and ordinate the triplicate percentage inhibition of cell growth.

Statistical analysis

Data are expressed as mean of three analyses. Equations for standard gallic acid and quercetin were obtained from linear regression model for calculation of total phenolic and flavonoid contents respectively. IC_{50} of extracts for antioxidant and cytotoxic activities was calculated by using the appropriate linear/non-linear regression equation with F-statistic at $p \leq 0.05$. All the analysis was done using R.[13]

RESULTS

Total polyphenol and total flavonoid contents

Total polyphenol content (TPC) and total flavonoid content (TFC) of *Dendrobium longicornu* were shown in Figure 1. Linear regression equation of gallic acid (y = 0.0154 x – 0.3285) in the range 25 – 100 µg/ml with R^2 value of 0.989 was constructed and linear equation of quercetin hydrate (y = 0.0242 x – 0.1845) was constructed with R^2 value of 0.976 for estimation of TPC and TFC respectively. According to result, the highest TPC and TFC were found in *Dendrobium longicornu* acetonic extract (DLA) showed significant differences with *Dendrobium longicornu* ethanolic extract (DLE) at $p \leq 0.05$.

Antioxidant activity

The antioxidant activity of the extracts has strong relationship with the solvent employed, mainly due to different antioxidant potential of crude extracts with different polarities. Mean percentage DPPH radical scavenging activity of extracts at their different concentrations was shown in Figure 2. *Dendrobium longicornu* acetonic extract (DLA) showed higher DPPH radical scavenging activity. IC_{50} for each extract was calculated using the regression equation from percentage radical scavenging activity. Acetonic extract (DLA) had lowest IC_{50} than ethanolic extract (DLE) means DLA showed highest antioxidant activity (Figure 3). All these presented values were found to be significant different with that of standard ascorbic acid (AA) at $p \leq 0.05$.

Cytotoxic activity

The cytotoxic effect of the *Dendrobium longicornu* extracts against the human cancer cells evaluated using the MTT assay (Figure 5 and 6). The ethanolic extract (DLE) showed the highest percentage inhibitory effect on growth of HeLa cells (Figure 4a) and acetonic extract (DLA) showed on U251 cells (Figure 4b) at the concentrations ranging from 100 – 800 µg/ml. Concentration of extract required for fifty percent cell growth inhibition (IC_{50}) was calculated from the percentage cell growth inhibition at different concentration using appropriate regression equation by F-statistics at $p \leq 0.05$. Lowest IC_{50} showed by DLA on U251 cells is 620.56 µg/ml (y = 18.060 ln(x) – 66.137; F = 28.797; $p = 0.002$) and by DLE on HeLa cells is 294.70 µg/ml (y = 40.257 ln(x) – 178.899; F = 80.935; $p \leq 0.001$). However, acetonic extract showed relatively weak inhibitory effects on the HeLa cells and ethanolic extract on U251 cells.

DISCUSSION

The quantitative analysis of acetonic and ethanolic extracts of *Dendrobium longicornu* for TPC and TFC, revealed the high content of these components, approve and complement other previous works.[14–17] These

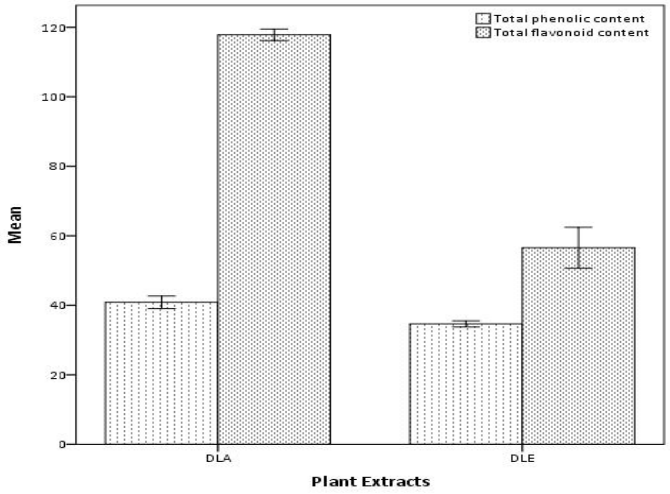

Figure 1: Total polyphenol and flavonoid contents of crude extracts.

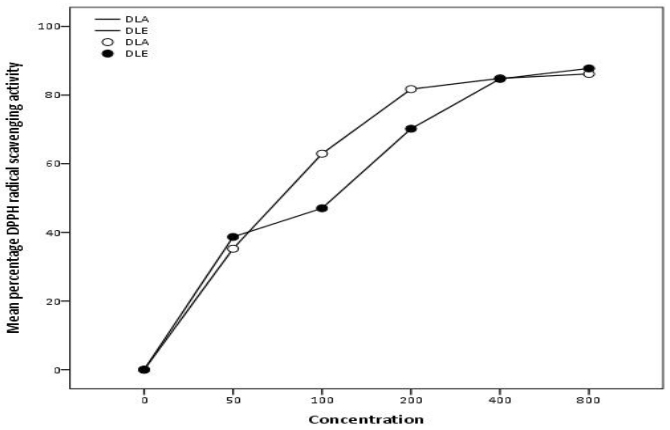

Figure 2: Percentage free radical scavenging activity of crude extracts.

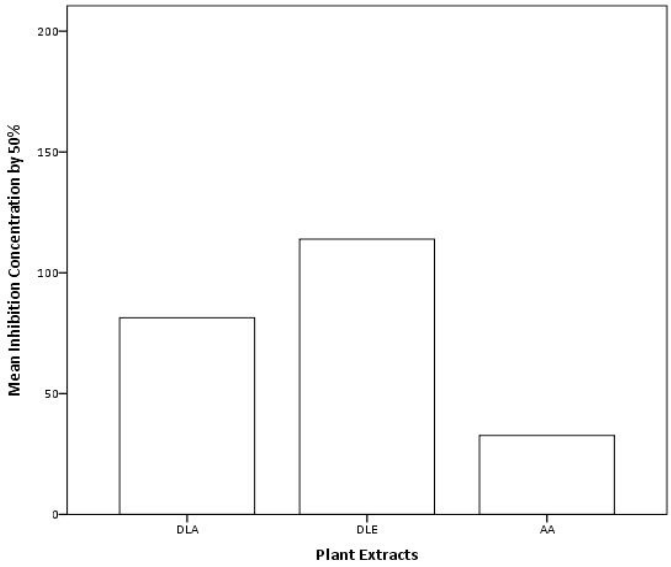

Figure 3: IC_{50} of crude extracts.

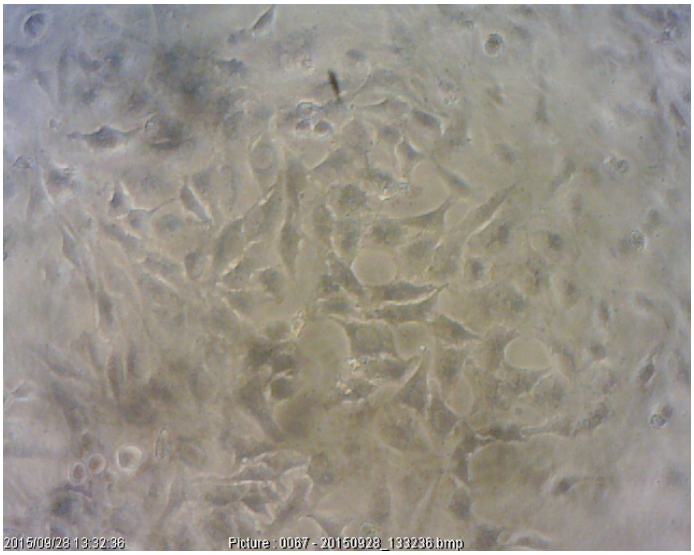

Figure 5 (a): Living human cervical cancer cells (HeLa) before addition of crude ethanol extract.

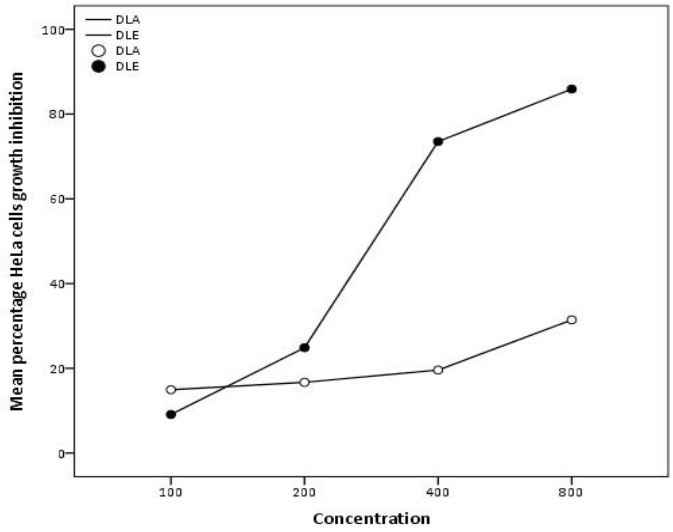

Figure 4 (a): Percentage inhibition of growth of human cervical cancer cells (HeLa) by crude extracts.

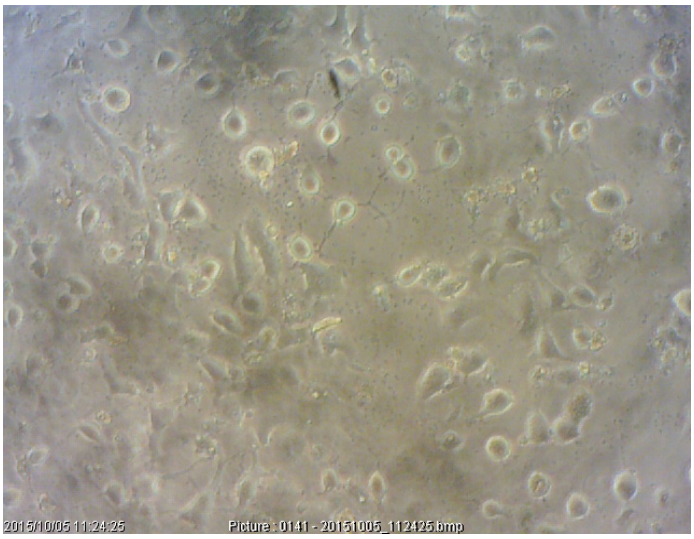

Figure 5 (b): Dead human cervical cancer cells (HeLa) after addition of crude ethanol extract.

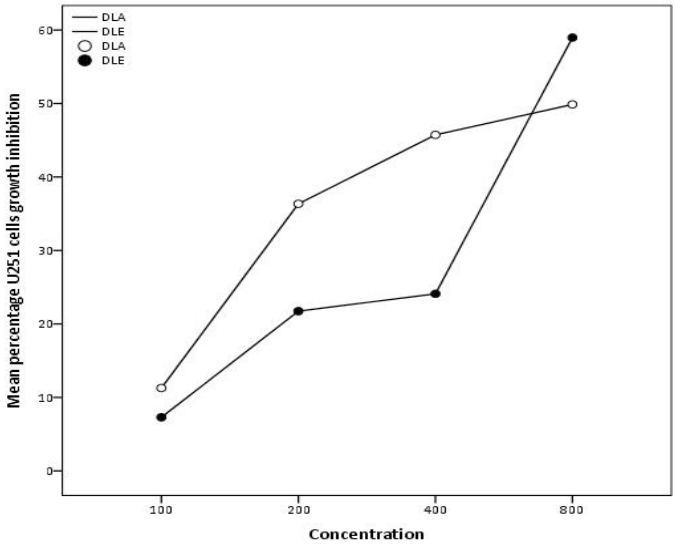

Figure 4 (b): Percentage inhibition of growth of human brain tumor cells (U251) by crude extracts.

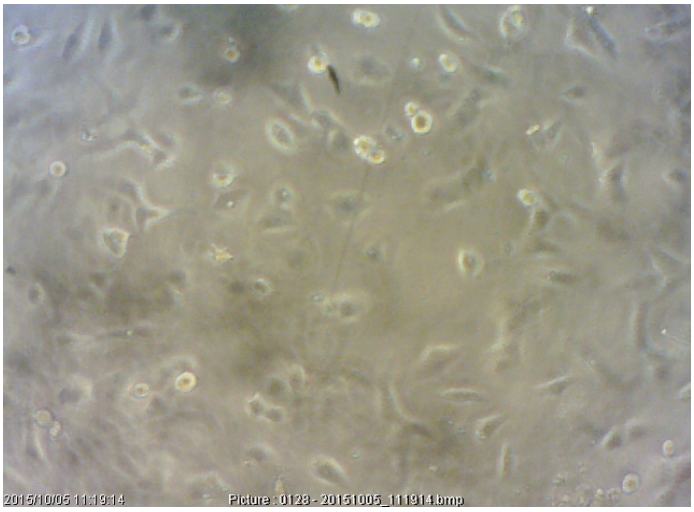

Figure 6 (b): Dead human brain tumor cells (U251) after addition of crude acetone extract.

phytoconstituents play a significant role in the medicinal properties. The polyphenol and flavonoid compounds are mainly found in acetonic extract could be attributable to the observed high antioxidant properties.[18–20] This is manifested by strong free radical scavenging activity; acetonic extract (DLA) superior with lowest IC_{50} as similar with the standard ascorbic acid (AA).[21–23]

The role of antioxidants is their interaction depends on the oxidative free radicals. The summary of DPPH method is that antioxidants react with the stable free radical, 2,2-diphenyl-1-picrylhydrazyl (deep violet colour) and convert it to 2,2-diphenyl-1-picrylhydrazine with discolouration. The discolouration indicates the scavenging potential of the extract with high polyphenol and flavonoid contents.[5,24]

The result of cytotoxicity of the acetonic extract of *D. longicornu* showed significant cell growth inhibition against U251 cell lines, whereas ethanolic had no significant cytotoxic activity against the U251 cells. The cytotoxicity of acetonic extract may be due to the presence of high content of flavonoid compounds. Similarly, cytotoxic activity of the ethanolic extract of *D. longicornu* showed signicant result against the HeLa cell lines

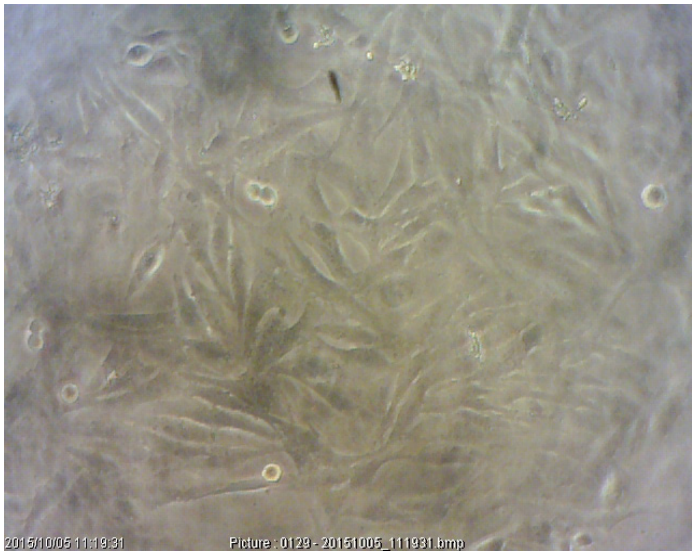

Figure 6 (a): Living human brain tumor cells (U251) before addition of crude acetone extract.

due to presence of high amount of flavonoid compounds. The flavonoids have reported for their cytotoxic activity by earlier researchers.[25–27]

The extracts of *Dendrobium longicornu* have been shown to exert antiproliferative effects on different human cancer cell lines. The ethanolic extract showed cytotoxic actitvity on HeLa cells whereas acetonic extract on U251 cells. The conventional cancer treatments like chemotherapy and radiotherapy are costly and also cause adverse effects. Hence, it is necessary to identify another anticancer drugs, which are more potent, selective and less toxic than conventional treatment. This research on plant based compounds has been efficacious in the field of anticancer drugs discovery.

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CONFLICT OF INTEREST

None

ABBREVIATIONS USED

AA: Ascorbic acid; DLA – Dendrobium longicornu acetone extract; DLE: Dendrobium longicornu ethanol extract; DPPH: 2,2-diphenyl-1-picrylhydrazyl; GAE: Gallic acid equivalent; HeLa: Human cervical cancer cells; IC_{50} – 50% inhibitory concentration; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; QE: Quercetin equivalent; TFC: Total flavonoid content; TPC: Total polyphenol content; U251: Human brain tumor cells.

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SUMMARY

- *Dendrobium longicornu* is widely used traditional medicinal orchids, had many valuable secondary metabolites. The biological activities of this orchid had not been explored. However, the present study explores the biological activities such as antioxidant, cytotoxicity of the crude extracts of this orchid. This study proved baseline for the pharmacological uses of this orchid.

ABOUT AUTHORS

Mukti Ram Paudel: He is PhD scholar of the Tribhuvan University at Central Department of Botany, Kathmandu, Nepal. He has been doing the research on bioactivity of secondary metabolites isolated from the different Dendrobium species. He has published more than half dozen of papers in national and international peer-review academic journals and has presented his papers in national and international conferences.

Mukesh Babu Chand: He is free-lancer researcher of plant science. He did research on antioxidant activities of some wild orchids of Nepal. He has published his research in national and international peer-review journals and has presented his papers in national conferences.

Basant Pant: He is Professor of neurosurgery and Chairman of Annapurna Research Center. He has published various papers in brain tumors in reputed peer-reviewed academic journals. Notably has worked on angiotensin-II induced hypertensive chemotherapy.

Bijaya Pant: She is Professor of Tribhuvan University at Central Department of Botany, Kathmandu, Nepal. She has been doing research on tissue culture and bioactivity of some medicinal orchids of Nepal. She has published more than hundred papers on tissue culture and bioactivity of orchids in national and international peer-review academic journals and has presented her papers in national and international conferences.

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