**Research Article**

**OMICS International**

**Journal of Cytology & Histology**

**Volume 7 • Issue 4 • 1000432**

**J Cytol Histol, an open access journal**

**ISSN: 2157-7099**

**Research Article Open Access**

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**In vitro Anticancer Activities of Ethanolic Extracts of Dendrobium crepidatum and Dendrobium chrysanthum against T-cell lymphoma**

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Abstract

**Background:** The orchid plants of Dendrobium, belong to the largest family of flowering plants orchidaceae. The genus Dendrobium reported to possess various therapeutic activities including anticancer activity. In the present scenario, cancer is one of the most leading causes of death worldwide. Therefore, the main objective of the present study was to demonstrate the cytotoxic and apoptotic inducing activity of Dendrobium crepidatum and Dendrobium chrysanthum ethanolic extracts in T-cell lymphoma.

**Methods:** MTT assay was done to evaluate the cytotoxic effect of the extracts on lymphoma cells. The reactive oxygen species (ROS) generation by the ethanolic extracts was also examined by DCFH-DA. Fluorescence microscopy studies and DNA fragmentation assay were done to investigate the apoptotic cell death. Flow cytometry was done to check the effect of ethanolic extracts on cell cycle phase distribution.

**Results:** The results revealed that both the extracts led to dose dependent cytotoxic effect in T-cell lymphoma along with increased generation of intracellular reactive oxygen species in a dose and time dependent manner. The fluorescent microscopic studies showed that the extracts induced cellular shrinkage, chromatin condensation and nuclear fragmentation, the major hallmarks of apoptosis, in dose dependent manner. The presence of DNA ladder further confirms the process of nuclear fragmentation, and the cell cycle analysis showed significant delay at G2/M phase of the cell cycle with both the extracts.

**Conclusion:** The whole study suggests that Dendrobium crepidatum and Dendrobium chrysanthum ethanolic extracts induced substantial cytotoxic and apoptotic activity in T-cell lymphoma. Therefore, further studies are needed to establish the detailed mechanism of anticancer activity of both the ethanolic extracts.

**Keywords:** Dendrobium crepidatum; Dendrobium chrysanthum; Anticancer; Dalton's lymphoma; ROS; Apoptosis

**Introduction**

Orchid belongs to the family orchidaceae, one of the largest families of flowering plants with nearly 20,000 species and comprising of more than 850 genera [1]. Dendrobium is a huge genus of orchids and contains about 1,200 species. Most of the Dendrobium species grow in the high and mountainous areas, at mild temperature, and in a humid environment. They are characterized by a broad geographical distribution, with different morphological features and are widely distributed in Asia, Australia and Europe, mainly, in India; Sri Lanka; China; Japan; Korea; New Guinea and New Caledonia [2]. Dendrobium genus was found to possess useful therapeutic activities like anticancer, hypoglycaemic, antimicrobial, immunomodulatory, hepatoprotective, antioxidant, and neuroprotective activities. Different array of chemical compounds were reported in orchids including alkaloids, bibenzyl derivatives, flavonoids, phenanthrenes and terpenoids [1]. Cancer is the main cause of death worldwide. Cancers may be caused due to incorrect lifestyle, genetic predisposition and environment. Environmental carcinogens; including both natural and manmade chemicals, radiation, and viruses are the major cause of human cancers now a days. Discovery of medicinal plant drug continues to provide an important leads against various pharmacological targets including cancer, HIV, Alzheimer’s, malaria, and pain. It has been confirmed by World Health Organisation (WHO) that herbal medicines serve the health needs of about 80 percent of the world’s population; especially for millions of people in the vast rural areas of developing countries [3]. There are various reports of antitumour activity of Dendrobium genus till date. The antitumor effects of inhibitory mechanisms in SNU-484 cells and induced apoptosis through downregulation of Bcl-2 and upregulation of Bax in cancer cells [4,5]. The bibenzyl isolated from Dendrobium dracoins inhibited several cancer cell lines including non-small-cell lung cancer cells [6]. There are also reports of antitumor and antibacterial activities from the extracts of Dendrobium nobile [7]. Moscatilin, a bibenzyl derivative from Dendrobium pulchellum found to inhibit the lung cancer cell motility and invasion through suppression of endogenous reactive oxygen species [8]. We have also earlier reported about the antitumour activity of ethanolic extract of an Indian orchid, Dendrobium formosum against T-cell lymphoma [9]. The medicinal properties reported involving different Dendrobium species intrigue us to examine two other species of Indian orchids, regarding their antitumour activity. The two species are Dendrobium crepidatum and Dendrobium chrysanthum whose antitumour activity has not been reported yet to the best of our knowledge. Polysaccharides isolated from stems of Dendrobium nobile, showed a strong antitumor action in inhibiting sarcoma 180 in vivo and HL-60 cells in vitro [4]. In addition, denbinobin isolated from stems of Dendrobium nobile exhibited antitumor action by inhibition of SNU-484 cells and induced apoptosis through downregulation of Bcl-2 and upregulation of Bax in cancer cells [5]. The bibenzyl isolated from Dendrobium dracoins

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Received August 17, 2016; Accepted September 21, 2016; Published October 01, 2016

Citation: Prasad R, Koch B (2016) In vitro Anticancer Activities of Ethanolic Extracts of Dendrobium crepidatum and Dendrobium chrysanthum against T-cell lymphoma. J Cytol Histol 7: 432. doi: 10.4172/2157-7099.1000432

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inhibited several cancer cell lines including non-small-cell lung cancer cells [6]. There are also reports of antitumor and antibacterial activities from the extracts of Dendrobium nobile [7]. Moscatilin, a bibenzyl derivative from Dendrobium pulchellum found to inhibit the lung cancer cell motility and invasion through suppression of endogenous reactive oxygen species [8]. We have also earlier reported about the antitumour activity of ethanolic extract of an Indian orchid, Dendrobium formosum against T-cell lymphoma [9]. The medicinal properties reported involving different Dendrobium species intrigue us to examine two other species of Indian orchids, regarding their antitumour activity. The two species are Dendrobium crepidatum and Dendrobium chrysanthum whose antitumour activity has not been reported yet to the best of our knowledge. Thus, the present study was undertaken to investigate their antitumor property in T-cell lymphoma.

Materials and Methods

Roswell Park Memorial Institute 1640 (RPMI-1640) medium and fetal bovine serum (FBS) were purchased from CellClone, (Genetix Biotech Asia Pvt. Ltd, antibiotic solution (Penicillin 1000 IU and Streptomycin 10 mg/ml), trypsin and MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazoliumbromide dye) were purchased from [Himedia, India], whereas DMSO (dimethyl sulphoxide), RNAase, and proteinase K were obtained from GeNie, Merck, India. Ethidium bromide, Triton X-100, and other chemicals of analytical grades were purchased from Lobachemie Pvt. Ltd., India. Acridine orange was purchased from SRL Pvt. Ltd., India; SDS (sodiumdodecyl sulphate) was obtained from Bio basic Inc., India. Agarose and 2,7-dichlorofluorescin diacetate (DCFH-DA) and Hoechst 33342 were purchased from Sigma, USA and the propidium iodide (PI) dye was obtained from EMD Millipore-Calbiochem, USA.

Plant material collection and preparation of ethanolic extract

Dendrobium crepidatum (D. crepidatum) and Dendrobium chrysanthum (D. chrysanthum) plant was identified and collected from Meghalaya, India. The Leaves of the plants were washed with distilled water and were air-dried. The dried leaves were grinded into fine powder and suspended in absolute ethanol at room temperature for up to 7 days, followed by filtration. Then the filtrate was reduced into the form of residue. Finally this extract was collected and stored at 4°C. The residue (extract) was dissolved in sterile distilled water immediately before use as discussed previously [9].

Dalton’s lymphoma

Dalton’s lymphoma (DL), a murine transplantable T-cell lymphoma was used as a tumour model to study the anticancer activity of the ethanolic extracts. It was originated at the National Cancer Institute, Bethesda, USA, in 1947 in the thymus gland of a DBA/2 mouse and from then it is being serially transplanted in the intraperitoneal cavity from mouse to mouse [10]. For in vitro experiments, 1 × 10⁶ DL cells were transplanted intraperitoneally into Swiss albino mice and Dalton’s lymphoma ascites (DLA) were maintained from mouse to mouse. DL cells harvested were cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotic solution (penicillin 1000 IU and streptomycin 10 mg/ml) in 5% CO₂ at 37°C. The protocols of the animal study were approved by the central animal ethical committee (CAEC) of the university and the ethic number (Dean/12-13/CAEC/210).

In vitro cytotoxicity assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] assay is a quantitative assay, to check viability, toxicity, proliferation and cellular activity of the cells. 3 × 10⁴ DL cells were harvested from DL bearing mice and seeded in culture plates in complete RPMI-1640. The cells were treated with various concentration of D. crepidatum and D. chrysanthum ethanolic extract for 24 h at 37°C and 5% CO₂. On completion of incubation, MTT (5 mg/ml) 10 µl was added into each well followed by incubation for 2 h at 37°C. The purple formazan crystal formed was then dissolved in 100 µl of DMSO at 37°C and left for 30 min. The absorbance was measured in a plate reader at the wavelength of 570 nm [11]. All experiments were carried out in three replicates and the inhibitory concentration (IC₅₀ value) of the extract was estimated as described earlier [9].

Measurement of reactive oxygen species (ROS)

The intracellular ROS level was analyzed using 2’,7’-dichlorofluorescin diacetate (DCFH-DA), an oxidation-sensitive fluorescent dye. Thus to verify the generation of ROS by the ethanolic extracts this assay was performed. 0.5 × 10⁶ DL cells were suspended in complete medium and treatment with 250 µg/ml and 325 µg/ml of D. crepidatum and 350 µg/ml and 400 µg/ml of D. chrysanthum ethanolic extract was done for 16 h and 24 h at 37°C. After the incubation, 10 µM of DCFH-DA was added to the cells and incubated for further 30 min at 37°C. The cells were then mounted in PBS on a clean slide, and were observed under fluorescence microscope. The amount of DCF fluorescence intensity exhibited reveals the amount of ROS generation in the cells. The fluorescence induced by DCF was observed [12] and captured using a fluorescence microscope (Nikon E800, Japan).

Apoptotic detection by acridine orange and ethidium bromide staining

Acridine orange/ethidium bromide (AO/EB) dual staining is used to study the, apoptotic and necrotic cells to visualize the cellular and nuclear changes with respect to live cells morphology. For this assay, 0.5 × 10⁶ DL cells were treated with indicated concentrations of D. crepidatum and D. chrysanthum ethanolic extracts for 16 h at 37°C. The cells were initially washed with PBS and then stained with ethidium bromide (100 µg/ml) and acridine orange (100 µg/ml) as described earlier with some modification [9]. Post staining the cells were washed with PBS and mounted on a clean glass slide. The cells were examined under a fluorescence microscope (Nikon E800, Japan) and photographed.

Apoptotic nuclear morphology analysis by Hoechst 33342 and propidium iodide staining

To detect the altered nuclear morphology during apoptosis Hoechst 33342/Propidium iodide staining was done. The blue fluorescent Hoechst 33342 is a cell permeable nucleic acid binding dye where as the red-fluorescent propidium iodide (PI) is a cell impairment DNA-binding dye, which enters the cells with lost membrane integrity. 0.5 × 10⁶ DL cells harvested were suspended in complete medium, and the treatment was done with D. crepidatum and D. chrysanthum ethanolic extracts at indicated concentration for 16 h at 37°C. The treatment was followed by washing the cells with PBS and staining with Hoechst 33342 (10 µg/ml) and Propidium iodide (15 µg/ml) for 30 min at 37°C [13]. Cells were then studied under a fluorescence microscope and the images got were captured (Evos FL, life technologies).

DNA Fragmentation Assay

DNA fragmentation assay was done to ascertain nuclear fragmentation during apoptosis. Briefly, 1 × 10⁶ DL cells were exposed...
with indicated concentrations of *D. crepidatum* and *D. chrysanthum* ethanolic extracts for 16 h at 37°C. The cells were washed followed by cell lysis using lysis buffer (20 mM TrisHCl, pH 8.0, 5 mM EDTA, 40 mM NaCl and 1% SDS) at 0°C for 15-30 min. The lysate obtained was spun at 3000 rpm for 8 min. The supernatant was transferred in a tube followed by incubation with Proteinase K (20 mg/ml) for 2 h at 37°C. The DNA obtained was precipitated with absolute ethanol and 5 M NaCl at -20°C overnight. To pellet out the DNA, centrifugation was done and the pellet was washed with 70% ethanol. The DNA was air dried and dissolved in distilled water, as discussed earlier [9]. Now to check the DNA fragmentation, agarose gel (1.8%) was run and the DNA band pattern was observed in a gel doc (G: Box, Syngene).

**Cell cycle analysis**

Flow cytometry was done to analyze the cell cycle distribution of DL cells on exposure to both the ethanolic extracts. Briefly, DL cells (1×10^6/ mL/6 well plate) were treated with the ethanolic extracts at indicated concentrations for 24 h. On completion of incubation, cells were harvested after washing with PBS. Now the cells were fixed with 70% ethanol overnight at -20°C followed by washing with cold PBS. The cells were then subjected to staining with 500 μL PI-RNase solution (1 mg/mL PI solution, Triton X-100 (0.1% v/v), and 10 mg/mL RNase) at 37°C for 30 min in dark [14]. Finally, the cells were analyzed for DNA contents on a flow cytometer FACScan (Becton Dickinson, NJ, USA). The cell cycle histograms were analyzed using Cell Quest software (Becton Dickinson).

**Statistical analysis**

Data obtained were presented as mean ± standard error mean (SEM) of at least three independent experiments. The statistical analysis of data was performed with one-way analysis of variance (ANOVA) followed by Bonferroni t-test and *P* values < 0.05 were considered significant using Sigma Stat 2.0 version.

**Results**

**In vitro cytotoxicity**

The cytotoxic activity of the two plant extracts were measured using the MTT assay against Dalton’s lymphoma. The cytotoxicity was assessed at 24 h following treatment with different concentrations of the ethanolic extracts in the range of 100-500 μg/mL. The findings showed that, the extracts inhibited the growth of DL cells in a dose dependent manner (Figure 1A and 1B). The extract of *D. crepidatum* was most active against DL cells with IC₅₀ value of 325 μg/mL, while the extract of *D. chrysanthum* was active against DL cells at IC₅₀ value of 400 μg/mL. Thus, we can conclude that both the extracts induce cytotoxic effect against DL cells. (Figure 1). The histogram represents the cytotoxic activity of *D. crepidatum* [A] and *D. chrysanthum* [B] ethanolic extracts against DL cells at various concentrations for 24 h. Results are expressed as a percentage of control ± SEM from at least three independent experiments.

**Measurement of generation of ROS by DCFH-DA**

To investigate whether the extracts of *D. crepidatum* and *D. chrysanthum* inhibit the DL cells through the generation of ROS, we revealed the redox status of the DL treated cells by the oxidation sensitive fluorescent dye DCFH-DA at 16 h and 24 h. As shown in Figure 2, the level of ROS generated after the treatment of DL cells varies with the different concentration of the extracts at different time interval. The higher ROS levels were found on 16 h and 24 h treatment mainly with IC₅₀ concentration in case of both the extracts compared to control. (Figure 2A-2D). DCFH-DA staining test for detection of induced intracellular ROS level was done by observing the fluorescence intensity of DL cells on treatment with indicated concentrations of *D. crepidatum* [A and B] and *D. chrysanthum* [C and D] ethanolic extract for 16 h and 24 h respectively and the images were captured under the fluorescence microscope (Nikon E800).

**Acridine orange/ethidium bromide staining**

Acridine orange/ethidium bromide (AO/EB) staining is performed to observe the morphological changes associated with apoptotic cell death. Acridine orange (AO) is a vital dye that could stain nuclear DNA with an intact cell membrane and etidium bromide (EB) could only stain cells that had lost membrane integrity [15]. Untreated (control) cells displayed normal green nucleus with intact membrane. The cells treated with indicated concentrations of *D. crepidatum* and *D. chrysanthum* for 16 h showed marked changes in morphology such as irregular shape, membrane blebbing, and the early apoptotic cells displayed greenish yellow nucleus with condensed chromatin whereas the late apoptotic cells showed orange red nucleus with chromatin condensation and nuclear fragmentation. Our data also showed a dose dependent increase in apoptotic cell death when compared to control in case of both the extracts (Figure 3). AO/EB staining was performed to study the morphology of DL treated cells at different concentrations (μg/mL) of *D. crepidatum* [A] and *D. chrysanthum* [B] ethanolic extracts at 16 h. [Blue arrow shows live cells, red arrows show late

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**Figure 1:** Determination of cell viability by MTT assay.
Propidium iodide staining. After treatment with the ethanolic extract at indicated concentrations (µg/mL) for 16 h, cells were stained and observed under fluorescence microscope (EVOS, FL). [Yellow arrow shows live cells whereas red arrow represents the early apoptotic cells with altered nuclear morphology, condensed and fragmented nuclei (blue) and white arrows show late apoptotic cells (pink nucleus) with condensed or fragmented nuclei].

DNA ladder assay

The ladder like DNA fragmentation is qualitative indicator of apoptosis. Therefore, to elucidate further, whether the plant extracts decreased cell survival by the induction of DNA fragmentation during apoptosis, DNA ladder assay was done. In the present study, no ladder like pattern was observed in control cells whereas typical ladder like pattern was observed on treatment with the extracts. On treatment for 16 h treatment it showed typical ladder like pattern as visible with later stage apoptosis (Figure 3). This implied that, incubation of DL cells treated with both the extracts for 16 h induced cell death which was markedly accompanied by the presence of DNA fragments. This observation also validates the results from the study of apoptotic cell death by fluorescence staining which showed nuclear fragmentation in apoptotic cells, dotted red arrow shows membrane blebbing in early apoptotic cells and yellow arrows shows necrotic cells].

Hoechst 33342/ propidium iodide staining

The morphological changes in cell nuclei were determined by fluorescence microscope by staining the DL cells with Hoechst 33342 and propidium iodide dye. The morphological observation in the cell nuclei of DL cells after treatment with D. crepidatum and D. chrysanthum ethanolic extracts for 16 h has been shown in Figure 4. The live cells appeared have intact and round shaped nuclei which were stained with a blue fluorescence. Whereby cells treated with the ethanolic extracts showed evidence of early and late apoptotic cells. In early apoptosis stage, cell shrinkage and high chromatin condensation with bright blue fluorescence in nuclei was observed whereas later stage of apoptosis showed condensed and fragmented nuclei with pink fluorescence (Figure 4). Both the ethanolic extract showed similar dose dependent apoptotic cell death as observed on staining with acridine orange and ethidium bromide. Thus, this substantiates the apoptosis inducing property of the two ethanolic extracts (Figure 4). Nuclear morphological changes by D. crepidatum [A] and D. chrysanthum [B] ethanolic extract in DL cells were detected by Hoechst 33342/
later stage of apoptosis (Figure 5). A 1.8 % agarose gel was run to separate DNA fragments after exposure of DL Cells with various concentrations (µg/mL) of the ethanolic extracts for 16 h with D. crepidatum [A] and D. chrysanthum [B]. DNA fragments were visualised under UV light. M: 200 bp DNA ladder marker, C: control for 16 h.

Cell cycle analysis

To study whether the ethanolic extract of D. crepidatum and D. chrysanthum could stimulate antitumor activity due to delay or arrest in the cell cycle phases, cell cycle distribution was analysed by FACS in asynchronous DL cells. Flow cytometry analysis exhibited an increase in the percentage of cells in G2/M phase treated with the ethanolic extracts compared to control (Figure 6A and 6B). The results shows that at IC50 concentration of both the extracts [D. crepidatum - (325 µg/mL) and D. chrysanthum - (400 µg/mL)] about 20% cells were at G2/M phase vs. 13% in case of control. At concentration lower to IC50 there was also an increase in the population of G2/M cells with about 17-18% cells while at a higher concentration there is further increase in G2/M phase cell (21.3%) with D. crepidatum extract where as the D. chrysanthum extract did not show further increase. This implied that the extracts inhibited the growth of DL cells by arresting them at G2/M cell cycle phase (Figure 6). Ethanolic extract treatment shows significant increase in the proportion of G2/M phase cells with decrease in G1 phase cells. [A] Cell cycle analysis by flow cytometry, after exposure of DL cells to 250, 325, 400µg/mL of D. crepidatum and 350, 400, 450µg/mL of D. chrysanthum ethanolic extracts for 24 h, cells were harvested and stained with propidium iodide. [B] Flow cytometry histograms are representative of percentage of cell populations in different phases of the cell cycle. Data were analyzed by one way - ANOVA followed by Bonferroni t test. *p<0.05 vs. control.

Discussion

Natural products play an important role in remedial of several ailments. The main objective of the present study was to evaluate the
anticancer efficacy of the two ethanolic extract of D. crepidatum and D. chrysanthum in DL cells, a murine transplantable tumor. Our study revealed that both the ethanolic extracts reduce the lymphoma growth in vitro. We observed that the D. crepidatum and D. chrysanthum ethanolic extract exert cytotoxicity in dose dependent manner with IC_{50} value at 325 µg/mL and 400 µg/mL respectively. In our earlier study we have shown that the ethanolic extract of D. formosum induced cytotoxicity in DL cells with an IC_{50} value of 350 µg/mL [9]. In another report, cytotoxicity of moscatilin from Dendrobium pulchellum to human lung H23 cells was checked by MTT assay which showed significant cytotoxic effect of moscatilin at 24 h [8]. Similar work was reported with 400 µg/ml of Scrophularia striata extract which induced cytotoxic effect on Jurkat human leukaemia cells on 48 h incubation.
and apoptosis inducing activity of the ethanolic extract of *D. crepidatum* and *D. chrysanthum* against Dalton’s lymphoma. Thus, both the ethanolic extracts seem to possess profound cytotoxic activity against cancer cells. As apoptosis as well as delay in cell cycle are regarded as a main target in discovery of anticancer agents, therefore results obtained above confirm the potential of *D. crepidatum* and *D. chrysanthum* as a novel chemotherapeutic agent. But the molecular mechanistic aspects of its effect are still unidentified. Thus, further research work is needed to establish the exact anticancer activity of both the ethanolic extracts.

**Acknowledgement**

The authors would like to acknowledge University Grant Commission (UGC), New Delhi, India (F. No. 41-162/2012 (SR)) to carry out this research work. The authors also like to acknowledge Interdisciplinary school of Life Sciences (ISLS), Institute of Science, Banaras Hindu University, Varanasi, for providing flow cytometry facility. The author is also thankful to UGC, New Delhi for RFSMS as senior research fellowship.

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