

## *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 draconis* 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 draconis*

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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 antitumor 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 antitumor activity. The two species are *Dendrobium crepidatum* and *Dendrobium chrysanthum* whose antitumor 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), RNase, 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'-dichlorodihydrofluorescein 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,  $1 \times 10^6$  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% CO<sub>2</sub> 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.  $3 \times 10^4$  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% CO<sub>2</sub>. On completion of incubation, MTT (5 mg/mL) 10  $\mu$ L was added into each well followed by incubation for 2 h at 37°C. The purple formazan crystal formed was then dissolved in 100  $\mu$ L 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 (IC<sub>50</sub> 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'-dichlorofluorescein diacetate (DCFH-DA), an oxidation-sensitive fluorescent dye. Thus to verify the generation of ROS by the ethanolic extracts this assay was performed.  $0.5 \times 10^6$  DL cells were suspended in complete medium and treatment with 250  $\mu$ g/mL and 325  $\mu$ g/mL of *D. crepidatum* and 350  $\mu$ g/mL, 400  $\mu$ g/mL of *D. chrysanthum* ethanolic extract was done for 16 h and 24 h at 37°C. After the incubation, 10  $\mu$ M of DCFH-DA was added to the cells and incubated further for 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,  $0.5 \times 10^6$  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  $\mu$ g/ml) and acridine orange (100  $\mu$ g/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.  $0.5 \times 10^6$  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  $\mu$ g/ml) and Propidium iodide (15  $\mu$ g/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,  $1 \times 10^6$  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 ( $1 \times 10^6$  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  $\mu$ L 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  $\pm$  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  $\mu$ g/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 IC<sub>50</sub> value of 325  $\mu$ g/mL, while the extract of *D. chrysanthum* was active against DL cells at IC<sub>50</sub> value of 400  $\mu$ g/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  $\pm$  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 IC<sub>50</sub> 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 ethidium 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 ( $\mu$ g/mL) of *D. crepidatum* [A] and *D. chrysanthum* [B] ethanolic extracts at 16 h. [Blue arrow shows live cells, red arrows show late

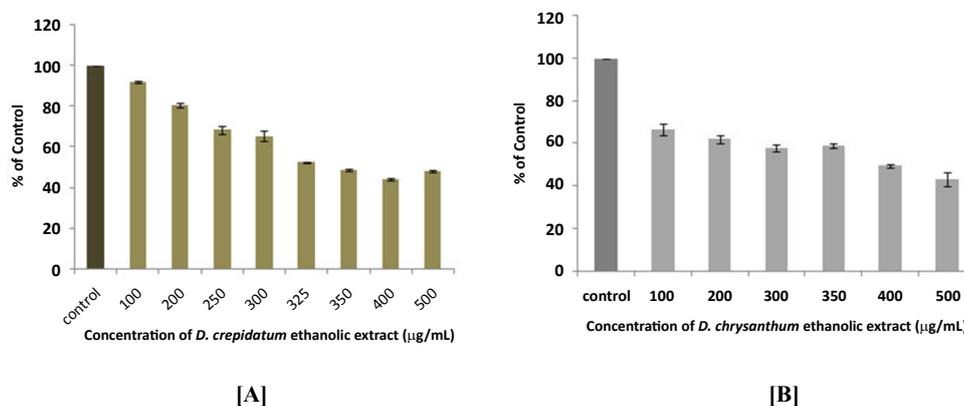


Figure 1: Determination of cell viability by MTT assay.

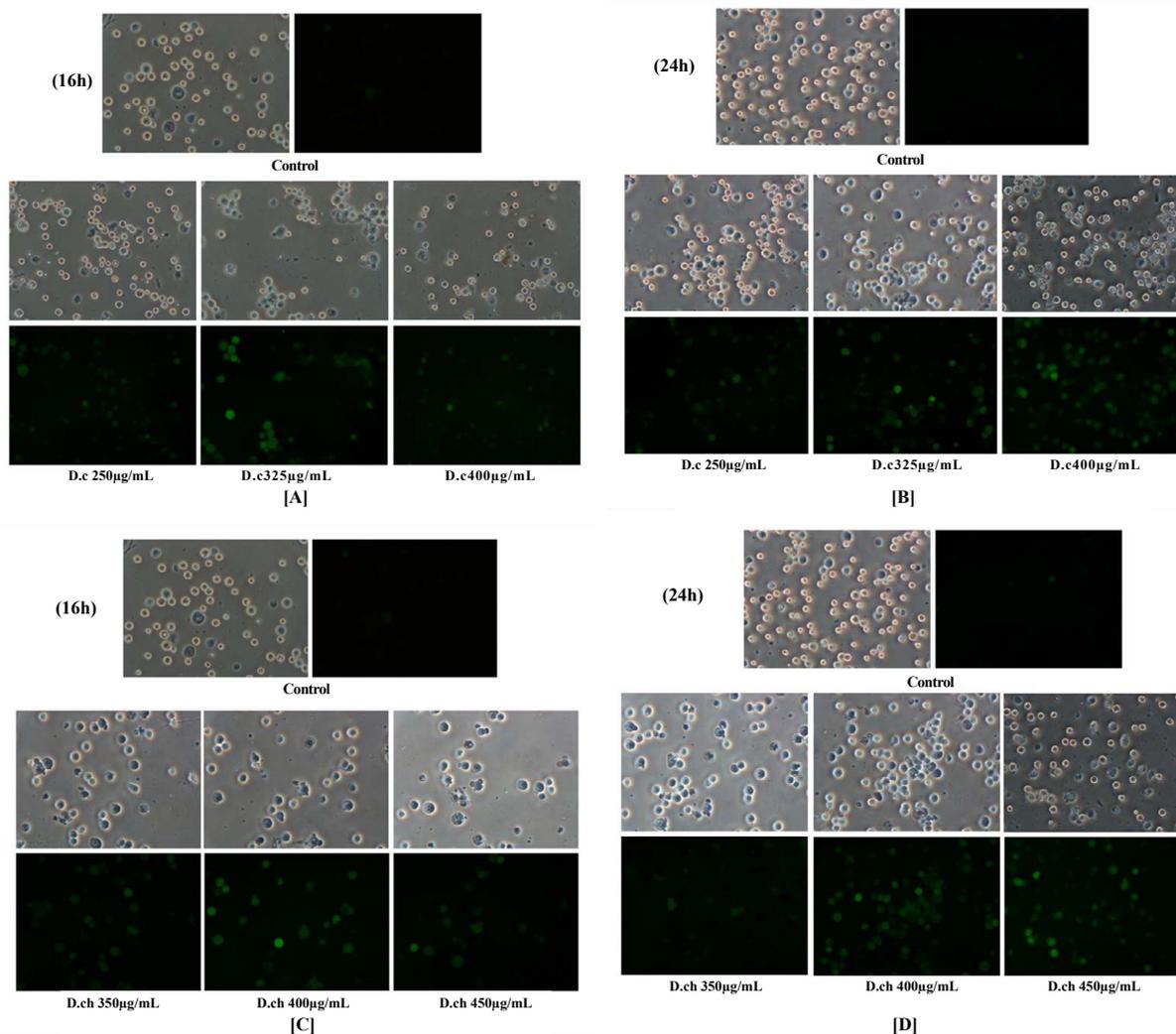


Figure 2: Measurement of intracellular ROS production.

apoptotic cells, dotted red arrow shows membrane blebbing in early apoptotic cells and yellow arrows shows necrotic cells].

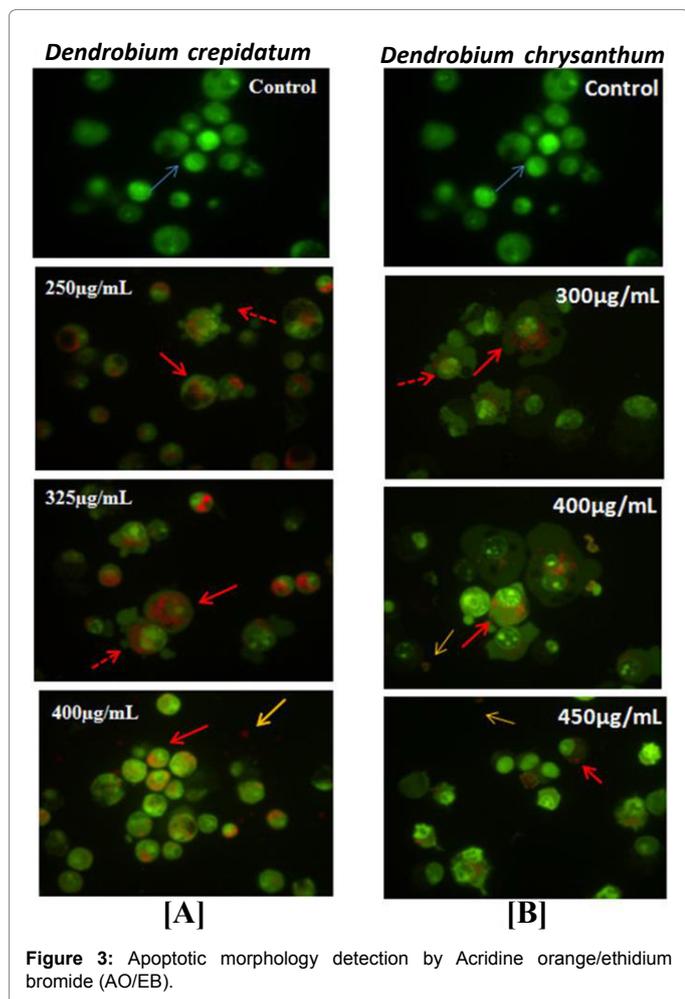
### Hoechst 33342/ propidium iodide staining

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/

Propidium iodide staining. After treatment with the ethanolic extract at indicated concentrations ( $\mu\text{g/mL}$ ) for 16 h, cells were stained and observed under fluorescence microscope (EVOS, FL). [Yellow arrow shows live cells where as 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

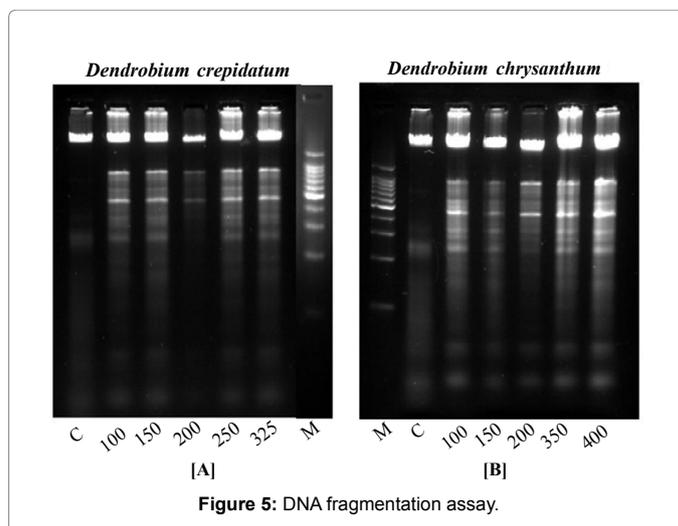
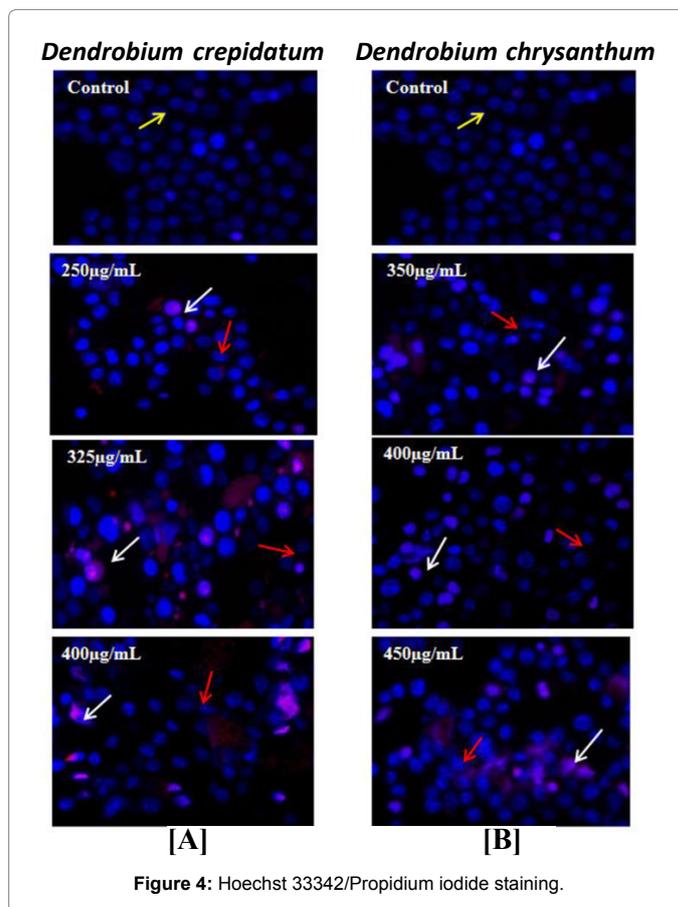
The ladder like DNA fragmentation is qualitative indicator of apoptosis. Therefore, to elucidate further, whether the plant extracts decreased cell survival by the induction of DNA fragmentation during apoptosis, DNA ladder assay was done. In the present study, no ladder like pattern was observed in 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



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 ( $\mu\text{g/mL}$ ) 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  $\text{IC}_{50}$  concentration of both the extracts [*D. crepidatum* - (325  $\mu\text{g/mL}$ ) and *D. chrysanthum* - (400  $\mu\text{g/mL}$ )] about 20% cells were at G2/M phase vs. 13% in case of control. At concentration lower to  $\text{IC}_{50}$  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* ethanolic 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, 400 $\mu\text{g/mL}$  of *D. crepidatum* and 350, 400, 450 $\mu\text{g/mL}$



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

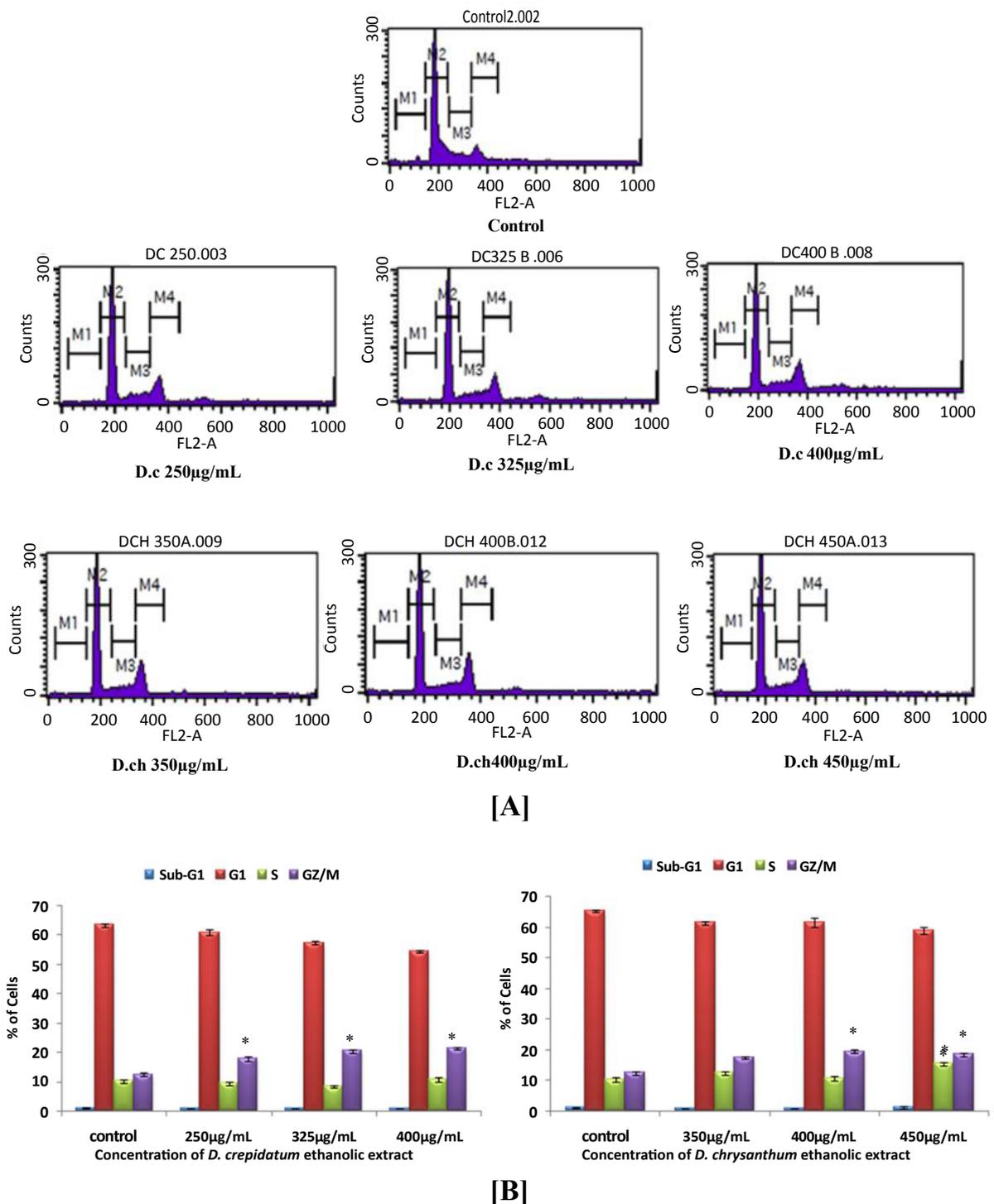


Figure 6: Effect of ethanolic extracts of *D. crepidatum* and *D. chrysanthum* on cell cycle phase distribution.

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 350µg/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

[16]. Thus, our result advocates that both the ethanolic extracts were capable of inducing cytotoxicity in Dalton's lymphoma. It has been reported that ROS metabolism play an important role in all types of programmed cell death [17]. It has also been reported that low and intermediated levels of ROS can protect cell from apoptotic cell death by activating antioxidant mechanisms, on the contrary, high levels of intracellular ROS results in a breakdown of the transmembrane mitochondrial potential ( $\Delta\Psi_m$ ), commonly occurring during early apoptosis, which is followed by DNA fragmentation regardless the type of cell and the apoptotic signalling [18]. Thus, to investigate the level of ROS in ethanolic extract treated cells; DCFH-DA dye was used, which showed increased ROS generation in DL cells compared to untreated cells (control). It has been reported that HeLa cells exposed to methanolic extract of *B. racemosa* bark (MEBR) and then exposed with DCFH-DA showed bright green fluorescence due to induced ROS production and its intracellular accumulation [19]. Thus our result suggests that an increase in the intracellular levels of ROS might lead to cellular damage and could induce apoptotic cell death by the ethanolic extracts in DL cells. Whether the ethanolic extracts could induce apoptosis, the morphological character changes in DL cells were investigated using the AO/EB staining and Hoechst 33342/PI staining under fluorescence microscope. We observed that treatment for 16 h showed presence of early as well as late apoptotic cells. Early apoptotic cells showed membrane blebbing with greenish/yellow condensed chromatin and late apoptotic cells showed more condensed and fragmented chromatin (orangish/red); at higher concentrations few necrotic cells were also observed with intact deep orange/red nucleus. We have earlier reported similar observations with *D. formosum* ethanolic extract treated Dalton's lymphoma with a dose and time dependent apoptotic cell death [9]. Induction of apoptosis is the key to success of plant products as anticancer agents [20,21]. On staining with Hoechst 33342/PI, after 16 h of treatment with *D. crepidatum* and *D. chrysanthum* ethanolic extracts, also showed dose dependent apoptotic cell death with presence of characteristic apoptotic nuclei. Cells which are undergoing apoptosis typically exhibit fragmentation of the DNA into small oligonucleosomal fragments, a hallmark of apoptosis [22]. Hence, to further validate the nuclear fragmentation occurring during apoptotic cell death as observed with fluorescent staining, analysis of DNA fragmentation was done. The results obtained confirmed that both the ethanolic extracts induced DNA ladder formation, which is the characteristic feature of apoptosis. In our previous study, similar DNA ladder pattern was observed on treatment of DL cells with *D. formosum* ethanolic extract [9]. Typical ladder pattern due to induction of apoptosis was also reported with aqueous ethanol seed extract of *Ziziphus mauritiana* in HL-60 cells in a concentration and time-dependent manner [23]. The cell cycle studies were done to rule out whether the ethanolic extracts affects the cell cycle progression or not and our analysis showed that they might be inhibiting the growth of DL cells by arresting them at G2/M phase. In our previous study with *D. formosum* ethanolic extract, we have reported similar results where the extract is found to arrest the DL cells at G2/M phase [9]. In another report, it was shown that moscatilin from Indian orchid *Dendrobium moschatum* and *Dendrobium loddigesii* also induces arrest of HCT-116 cells at G2/M phase of cell cycle [24]. Thus our present data are in corroboration with the above findings and they further substantiate the inhibition of lymphoma cells by antitumor activity of the ethanolic extracts.

## Conclusion

The results from our study evidently demonstrated the cytotoxic 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.

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## References

1. Martha R, Gutiérrez P (2010) Orchids: a review of uses in traditional medicine, its phytochemistry and pharmacology. J Med Plants Res 4: 592-638.
2. Wood HP (2006) The Dendrobiums, Timber Press, Portland, Ore, USA.
3. Kong JM, Goh NK, Chia LS, Chia TF (2003) Recent advances in traditional plant drugs and orchids. Acta Pharmacol Sin 24: 7-21.
4. Wang JH, Luo JP, ZhaXQ, Feng BJ (2010) Comparison of antitumor activities of different polysaccharide fractions from the stems of *Dendrobium nobile* Lindl. Carbohydr Polym 79: 114-118.
5. Song JI, Kang YJ, Yong HY, Kim YC, Moon A (2012) Denbinobin, a phenanthrene from *Dendrobium nobile*, inhibits invasion and induces apoptosis in SNU-484 human gastric cancer cells. Oncol Rep 27: 813-818.
6. Charoenrungruang S, Chanvorachote P, Sritularak B, Pongrakhananon V (2014) Gigantol, a bibenzyl from *Dendrobium draconis*, inhibits the migratory behaviour of non-small cell lung cancer cells. J Nat Prod 77: 1359-1366.
7. Uma Devi P, Selvi S, Devipriya D, Murugan S, Suja S (2009) Antitumor and antimicrobial activities and inhibition of in-vitro lipid peroxidation by *Dendrobium nobile*. Afr J Biotechnol 8: 2289-2293.
8. Kowitdamrong A, Chanvorachote P, Sritularak B, Pongrakhananon V (2013) Moscatilin inhibits lung cancer cell motility and invasion via suppression of endogenous reactive oxygen species. Biomed Res Int.
9. Prasad R, Koch B (2014) Antitumor activity of ethanolic extract of *Dendrobium formosum* in T- cell lymphoma: an in vitro and in vivo study. Biomed Res Int 2014: 753451.
10. Chakrabarti S, Chakrabarti A, Pal AK (1984) Chromosome analysis of Dalton's lymphoma adapted to the Swiss mouse: clonal evaluation and C-heterochromatin distribution. Cancer Genet Cytogenet 11: 417-423.
11. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63.
12. Karlsson M, Kurz T, Brunk UT, Nilsson SE, Frennesson CI (2010). What does the commonly used DCF test for oxidative stress really show? Biochem J 428: 183-190.
13. Rahman SNSA, Wahab NA, Abd Malek SN (2013) In Vitro morphological assessment of apoptosis induced by antiproliferative constituents from the rhizomes of *Curcuma zedoaria*. Evid Based Complement Alternat Med.
14. Lim JT, Piazza GA, Han EK, Delohery TM, Li H, et al. (1999) Sulindac derivatives inhibit growth and induce apoptosis in human prostate cancer cell lines. Biochem Pharmacol 58: 1097-1107.
15. Xu X, Gao X, Jin L, Bhadury PS, Yuan K, et al. (2011) Antiproliferation and cell apoptosis inducing bioactivities of constituents from *Dysosma versipellis* in PC3 and Bcap-37 cell lines. Cell Div 6: 14.
16. Azadmehr A, Hajjaghaee R, Mazandarani M (2013) Induction of apoptosis and G2/M cell cycle arrest by *Scrophularia striata* in a human leukaemia cell line. Cell Prolif 46: 637-643.
17. Temkin V, Karin M (2007) From death receptor to reactive oxygen species and c-Jun Nterminal protein kinase: the receptor-interacting protein 1 odyssey. Immunol Rev 220: 8-21.

18. Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, et al. (1995) Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J Exp Med* 182: 367-377.
19. Rahman MA, Akhtar J, Sahabjada, Arshad M (2016) Evaluation of cytotoxic potential and apoptotic effect of a methanolic extract of *Bauhinia racemosa* Lam. against a human cancer cell line, HeLa. *Eur J Integr Med*
20. Pieme CA, Santosh KG, Ngamegni B, Ngogang J, Shashi B, et al. (2013) Ethyl acetate fraction of *Garcinia epunctata* induces apoptosis in human promyelocytic cells (HL-60) through the ROS generation and G0/G1 cell cycle arrest: a bioassay-guided approach. *Environ Toxicol Pharmacol* 3: 865-874.
21. Hsiao WL, Liu L (2010) The role of traditional Chinese herbal medicines in cancer therapy-from TCM theory to mechanistic insights. *Planta Med* 76: 1118-1131.
22. Thatte U, Bagadey S, Dahanukar S (2000) Modulation of programmed cell death by medicinal plants. *Cell Mol Biol* 46: 199-214.
23. Mishra T, Khullar M, Bhatia A (2011) Anticancer potential of aqueous ethanol seed extract of *Ziziphus mauritiana* against cancer cell lines and Ehrlich ascites carcinoma. *Evid Based Complement Alternat Med*.
24. Chen TH, Pan SL, Guh JH, Liao CH, Huang DY, et al. (2008) Moscatilin induces apoptosis in human colorectal cancer cells: a crucial role of c-Jun NH2-terminal protein kinase activation caused by tubulin depolymerization and DNA damage. *Clin Cancer Res* 14: 4250-4258.