

**APOPTOSIS MEDIATED CYTOTOXICITY INDUCED BY ISODEOXYELEPHANTOPIN ON NASOPHARYNGEAL CARCINOMA CELLS**FARHA A. K<sup>1</sup>, GEETHA B. S<sup>2</sup>, MANGALAM S. NAIR<sup>3</sup>, DHANYA S.R<sup>3</sup>, LATHA P. G<sup>2</sup> AND REMANI P<sup>1\*</sup>

<sup>1</sup>Division of Cancer Research, Regional Cancer Centre, Thiruvananthapuram, Kerala, India, <sup>2</sup>Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Thiruvananthapuram, Kerala India, <sup>3</sup>National Institute of Interdisciplinary Science and Technology, Thiruvananthapuram, Kerala, India, Email: remanipr@gmail.com.

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**ABSTRACT**

Isodeoxyelephantopin (IDOE), a sesquiterpene lactone isolated from chloroform extract of *Elephantopus scaber* has been shown anticancer effects in various cancer cell lines. In the present study, we investigated the cytotoxic effect of IDOE on human nasopharyngeal epidermoid carcinoma (KB) cells and its different mode of action. Data from MTT viability assay indicated that IDOE inhibited viability of KB cells in a dose and time dependent manner. IC<sub>50</sub> obtained by IDOE was 11.45 μM for 48h exposure. The clonogenic assay showed a dose-dependent inhibition of colony formation in IDOE-treated cells. The results obtained from morphological analysis of apoptosis by AO/EtBr and Hoechst staining revealed the typical morphological features of apoptosis such as chromatin condensation and nuclear fragmentation. In addition, DNA fragmentation assay confirmed apoptosis by showing ladder pattern of DNA in IDOE treated cells. Flow cytometric analysis of annexin V-propidium iodide staining demonstrated that treatment of KB cells with IDOE increased apoptotic cell population in a dose dependent manner. G2/M phase cell cycle arrest and subG1 peak of DNA, characteristics of apoptosis, was observed in IDOE treated cells. These results indicated that isodeoxyelephantopin exhibited apoptosis mediated cytotoxicity in Nasopharyngeal carcinoma cells. This might provide a potential therapeutic option in the management of Nasopharyngeal carcinoma.

**Keywords:** Nasopharyngeal carcinoma, KB cells, *Elephantopus scaber*, Isodeoxyelephantopin, apoptosis, sesquiterpene lactones.

**INTRODUCTION**

Nasopharyngeal carcinoma is one of the malignant tumors with high incidence in the South East Asia region [1]. The main treatment of Nasopharyngeal carcinoma is radiotherapy, usually given in combination with chemotherapeutic drugs [2]. The currently available anticancer drugs are usually toxic to normal cells, often resulting in adverse side effects such as temporary hair loss, nausea and vomiting [3]. Therefore it is essential to search for a new effective chemotherapeutic agent with potent activity and minimal side effects. Sesquiterpene lactones, most widely distributed within the Compositae family, have received considerable attention for their cytotoxic and antitumor properties in recent years [4]. *Elephantopus scaber* (Compositae) reported to possess several sesquiterpene lactones [5, 6, 7]. Isodeoxyelephantopin (IDOE) is a sesquiterpene lactone isolated from chloroform extract of whole plant of *Elephantopus scaber* [8,9].

Previous studies indicated that IDOE possessed potential anti-tumor effect on several cancer cell lines such as cervical carcinoma-HeLa, colon carcinoma-Caco-2, hepatocarcinoma-SMMC7721 [10] Earlier reports from our laboratory showed that IDOE could inhibit the viability of L-929 cells in a dose and time dependent manner. In the present study, anti-proliferative effect and apoptosis inducing efficacy of IDOE against Nasopharyngeal carcinoma (KB) cells have been investigated.

**MATERIALS AND METHODS****Isodeoxyelephantopin (IDOE) and drug preparation**

IDOE was purified from the chloroform extract of *Elephantopus scaber* as previously described [9]. Stock solution was prepared by dissolving IDOE in DMSO at a concentration of 1mg/mL and was stored at 4°C. The stock was diluted to the required concentration immediately before use with DMEM media supplemented with 10% FBS.

**Cell culture**

The Nasopharyngeal carcinoma (KB) cell line was purchased from the National Centre for Cell sciences (NCCS) Pune. The cell culture was maintained in DMEM medium supplemented with 10% fetal

bovine serum, 100 U/ml penicillin and 100μg/ml streptomycin at 37°C in humidified atmosphere with 5% CO<sub>2</sub>.

**Cell viability assay**

Effect of IDOE on cell viability was detected by a MTT assay [11]. 5x10<sup>3</sup> cells/well were seeded in a 96-well-plate and allowed to adhere by overnight incubation. The cells were exposed to varying concentrations of IDOE ranging from 5-60μM and incubated further for 24, 48 and 72 h. Cisplatin was used as positive control and DMEM with 0.1% DMSO was used as negative control. Subsequently, 20μL MTT reagent (5mg/mL in sterile PBS) was mixed with 80μL DMEM and added to each well. The plates were incubated for 2h in dark. After incubation, 100μL lysis buffer (20% SDS in 50% dimethyl formamide) was added to dissolve purple formazan crystals and further incubated for 4 h. The absorbance was then determined at 570nm using a microplate reader (Power wave XS, Bio-Tek).

The % of cell growth = (Absorbance of treated cells/ Absorbance of control) x 100

A graph was plotted using Easy plot software to obtain the IC<sub>50</sub>. IC<sub>50</sub> is defined as concentration required inhibiting 50% of cancer cell growth.

**Colony formation assay**

Clonogenic survival of KB cells exposed to IDOE for 2h was assayed by the colony formation assay. Briefly, 200 cells of KB were treated with IDOE of varying concentrations, incubated at 37°C for 2h and then washed thoroughly with medium to remove IDOE. The cells were placed in a 6 well plate and allowed to grow for 14 days. After 14 days, colonies were fixed and stained with 0.03% crystal violet in 10% ethanol and colonies estimated to be greater than 50 cells were counted. The survival was expressed relative to controls.

**Apoptosis detection****Acridine orange –ethidium bromide (AO/EtBr) dual staining**

Morphological analysis of apoptosis by AO/EtBr dual staining procedure was performed [12]. AO is a vital dye that stains both live and dead cells; EtBr only stains cells that have lost membrane

integrity. Early apoptotic cells stain green and contain bright dots in the nuclei. Late apoptotic cells also incorporate EtBr and show condensed and often fragmented nuclei. Necrotic cells also stain in orange, but present nuclear morphology resembling that of viable cells. Briefly,  $2 \times 10^4$  cells per well was seeded in 96 well plate and treated with three concentrations of IDOE (5.72, 11.45 and 22.9  $\mu\text{M}$ ) for 48h. After incubation, the plates were centrifuged. 10  $\mu\text{l}$  of 1mg/ml AO and EtBr mixture was added to each well. Nuclei were visualized and photographed under the fluorescent microscope.

#### Hoechst 33342 staining

Hoechst staining was used to evaluate the condensation and fragmentation of DNA of the apoptotic cells. This dye stains the DNA and distinguishes brightly stained, condensed apoptotic nuclei from dimly stained healthy nuclei [13]. The KB cells were treated with the different concentrations of IDOE for 48h. After incubation, the medium was removed and then 50  $\mu\text{L}$  Hoechst working solution (1  $\mu\text{L}$  Hoechst in 50  $\mu\text{L}$  distilled water) was added to cells with a final concentration 1 mg/mL and incubated for 30 minutes. The stained nucleic DNA was visualized under inverted fluorescence microscopy (Olympus IX 51) at a magnitude of 20 $\times$ .

#### Detection of apoptotic cells by Annexin V-FITC and propidium iodide staining

Externalization of phosphatidyl serine is one of the indications of early stage of apoptosis. Apoptotic cells were quantified by using FITC Annexin V -propidium iodide apoptosis detection kit (BD Biosciences, San Diego, CA) according manufacturer's protocol. KB cells ( $2 \times 10^5$ ) were treated with different concentrations of IDOE for 48 hours. Cells trypsinized, washed with PBS and suspended in 100  $\mu\text{L}$  1x binding buffer. Cells were stained simultaneously with 5  $\mu\text{L}$  FITC-conjugated Annexin V and 5  $\mu\text{L}$  PI and incubated for 15 minutes in dark. 400  $\mu\text{L}$  binding buffer was added and analyzed immediately by flow cytometry (FACS Calibur, BD Bioscience).

#### DNA fragmentation assay

The IDOE treated KB cells were harvested and washed twice with PBS. 300  $\mu\text{L}$  DNA extraction buffer (100mM Tris pH-8.5, 5M NaCl, 0.5M EDTA and 10% SDS) was added and mixed well to lyse the cells and kept at 55 $^{\circ}\text{C}$  for 4h. 10  $\mu\text{L}$  Proteinase K was also added. After incubation, equal volume of isopropanol was added and mixed well to precipitate the DNA and kept at 4 $^{\circ}\text{C}$  for 30 minutes. Centrifugation was done at 12000 r.p.m for 20minutes at 4 $^{\circ}\text{C}$ . The supernatant was removed carefully and pellet was dried. The DNA pellet was suspended in TE buffer (10 mmol/L Tris-HCl, 1mmol/L EDTA, pH 8.0) and stored at 4 $^{\circ}\text{C}$ . Then the dissolved DNA was subjected to electrophoresis.

#### Cell cycle analysis

Cells ( $1 \times 10^6$ ) were treated with three concentrations of IDOE for 48h. After incubation, the cells were trypsinized and suspended in ice-cold ethanol (70%) and stored at -20  $^{\circ}\text{C}$  for 4h. The cells washed with PBS, suspended in 250  $\mu\text{L}$  of PBS. 10  $\mu\text{L}$  propidium iodide (1mg/mL) and 10  $\mu\text{L}$  RNase A (10 mg/mL) was added and incubated at room temperature in the dark for 30 min. Cell cycle distribution was analyzed using FACS Calibur (Becton-Dickinson). Data was analyzed using DIVA software program.

#### Statistical analysis

All experiments were done three times and the data were expressed as mean  $\pm$  SD. The significance levels for comparison of differences were determined with one way ANOVA, followed by Tukey Kramer Multiple Comparison Test (GraphPad Software, USA) and P values < 0.001 were considered as highly significant and P < 0.05 was considered statistically significant when compared to control.

## RESULTS

#### Cytotoxic effect of IDOE on KB cells

The results of MTT assay showed that IDOE was able to inhibit

viability of KB cells with a significant increase in a concentration and time dependent manner (Fig. 2A, B and C). The  $\text{IC}_{50}$  value of IDOE for KB cells was 11.45  $\mu\text{M}$  after exposure for 48 hours (Fig. 2D). Three concentrations such as 5.72, 11.45 and 22.9  $\mu\text{M}$  were selected in further experiments based on the  $\text{IC}_{50}$  values determined by MTT assay. The cytotoxicity of IDOE was greater than that of positive control Cisplatin.

#### Effect of IDOE on Clonogenicity of KB cells

Further, the effect of IDOE on colony formation was detected using clonogenic assay. The three concentrations showed significant inhibition of colony formation of KB cells. At 11.45  $\mu\text{M}$  and 22.9  $\mu\text{M}$ , complete absence of colonies indicated that IDOE lethal to KB cells at higher concentrations (Fig.3).

#### Apoptosis induction in KB cells by IDOE

Morphological changes of KB cells occurred by the treatment with IDOE. Cells lost their viability, rounded up and floated in medium (Fig.4).

After staining with AO/EtBr, control untreated cells appeared green in color with intact nuclei. After IDOE treatment, early apoptotic cells showed condensed nuclei and appeared bright green in color. Late apoptotic cells appeared in red color with condensed and fragmented nuclei. In necrotic cells, Ethidium bromide penetrated into the membranes of dead cells and stains their nuclei which appear red. From the data it was clear that with increasing concentration of IDOE, the number of apoptotic cells increased tremendously.

Hoechst 33342 staining was also carried out to investigate the apoptosis induction of IDOE on KB cells treated with 5.72, 11.45 and 22.9  $\mu\text{M}$  for 48 hours. After treatment with Hoechst 33342, control cells were seen with uniformly light blue nuclei under fluorescence microscope; while IDOE treated KB cells exhibited bright blue color because of chromatin condensation (Fig.4).

The nature of the cytotoxicity was also evaluated with FITC Annexin V-PI staining and analyzed by flow cytometry. Cells in the upper left quadrant represent necrotic cells, cells in the upper right quadrant represent late apoptotic cells, cells in the lower left quadrant represent viable cells and cells in the lower right quadrant represent early apoptotic cells. The apoptotic cell percentage was 13.11% in untreated KB cells. After treatment with IDOE at 5.72, 11.45 and 22.9  $\mu\text{M}$ , the percentages of apoptotic cells were 18.7%, 20.82% and 50.84% respectively (Fig.5). Furthermore, DNA fragmentation analysis by agarose gel electrophoresis showed typical DNA ladder during treatment with 11.45 and 22.9  $\mu\text{M}$  IDOE. All these data indicated that cell death occurred by IDOE treatment is primarily through apoptosis (Fig. 6).

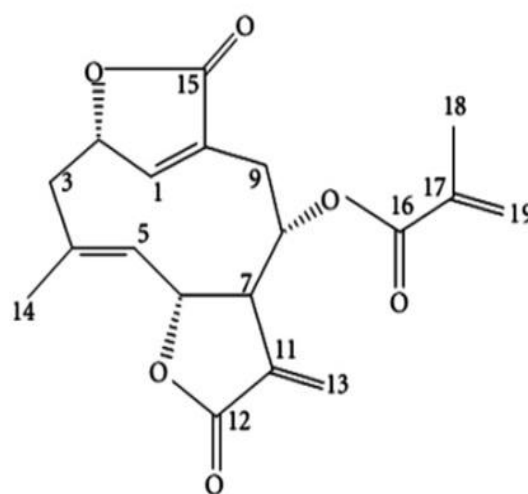


Figure 1: Structure of Isoodeoxyelephantopin isolated from chloroform extract of *Elephantopus scaber*

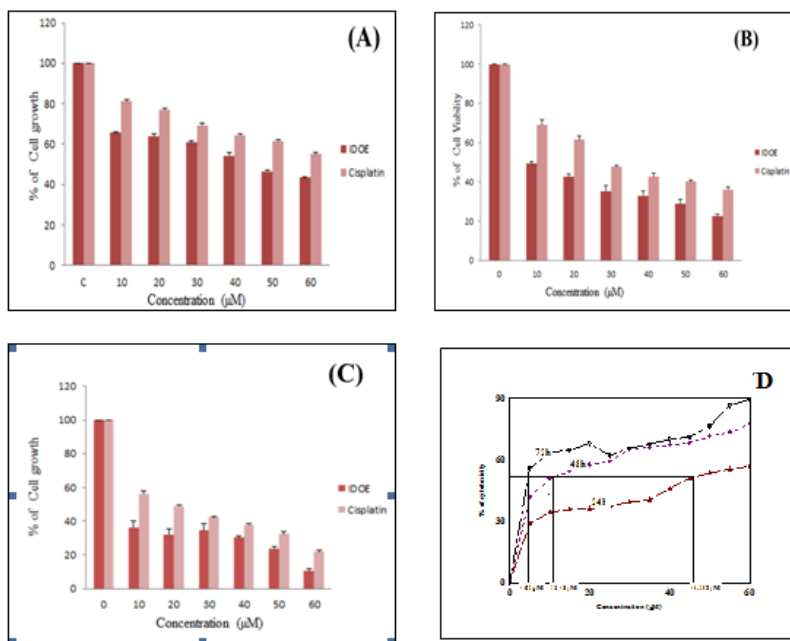


Figure 2: Cytotoxic effect of IDOE on KB cells. Cell viability was evaluated using MTT assay for (A) 24h; (B) 48h; (C) 72 h; (D) IC<sub>50</sub> value of IDOE for 48h; The assay was performed in triplicate, and data are the mean  $\pm$ SD.

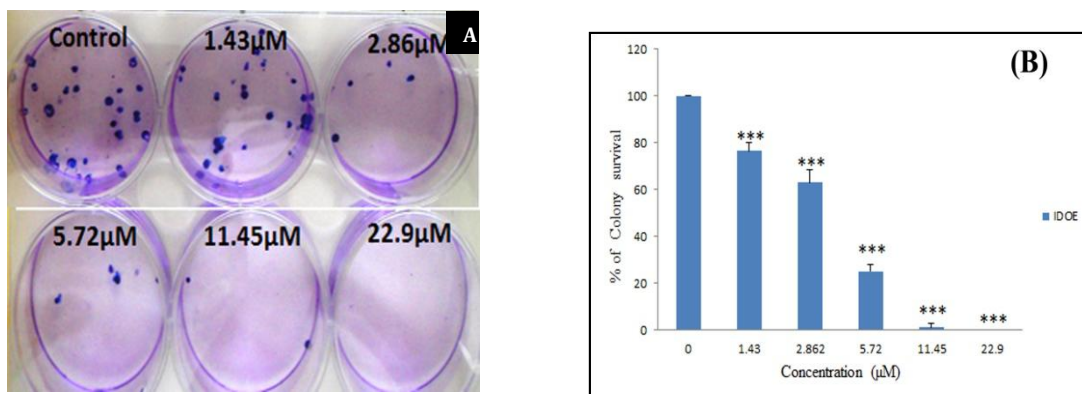


Figure3: Effect of IDOE on clonogenicity in KB cells. KB cells were treated with different concentrations of IDOE for 2h and allowed to form colonies for 14 days. (A) A representative plate showing colony formation of KB cells after treatment with IDOE; (B) IDOE treated KB cells exhibited fewer colonies than control KB cells. Values are expressed as mean values  $\pm$  SD from three independent experiments and the asterisk indicates statistical significance compared to the control \*\*\*P<0.001.

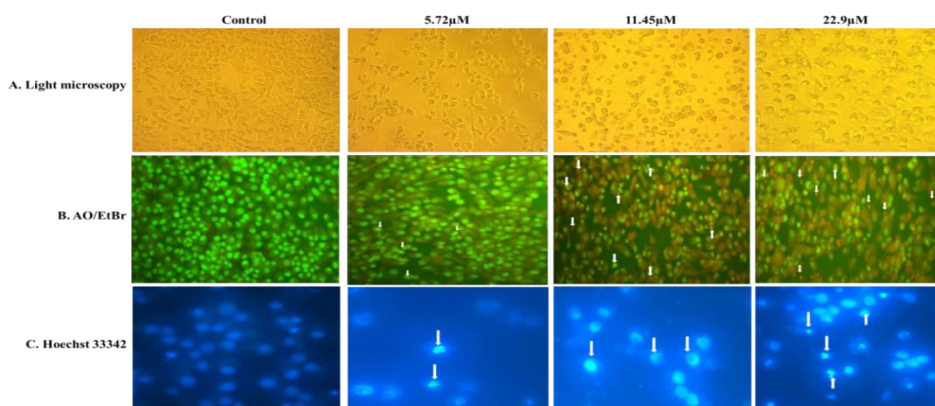


Figure 4: Apoptotic effect of IDOE on KB cells. Cells were treated with IDOE of indicated concentrations. (A) Morphological changes in KB cells after treatment with different concentrations of IDOE for 48h exposure at 20 $\times$ magnification; (B) AO/EtBr staining: Control KB cells showed green normal nucleus. IDOE treated KB cells showed early apoptotic cells with green bright nucleus and late apoptotic cells showed condensed red nucleus; (C) Hoechst 33342 staining: Nuclear morphology of KB cells stained with Hoechst stain 33342.

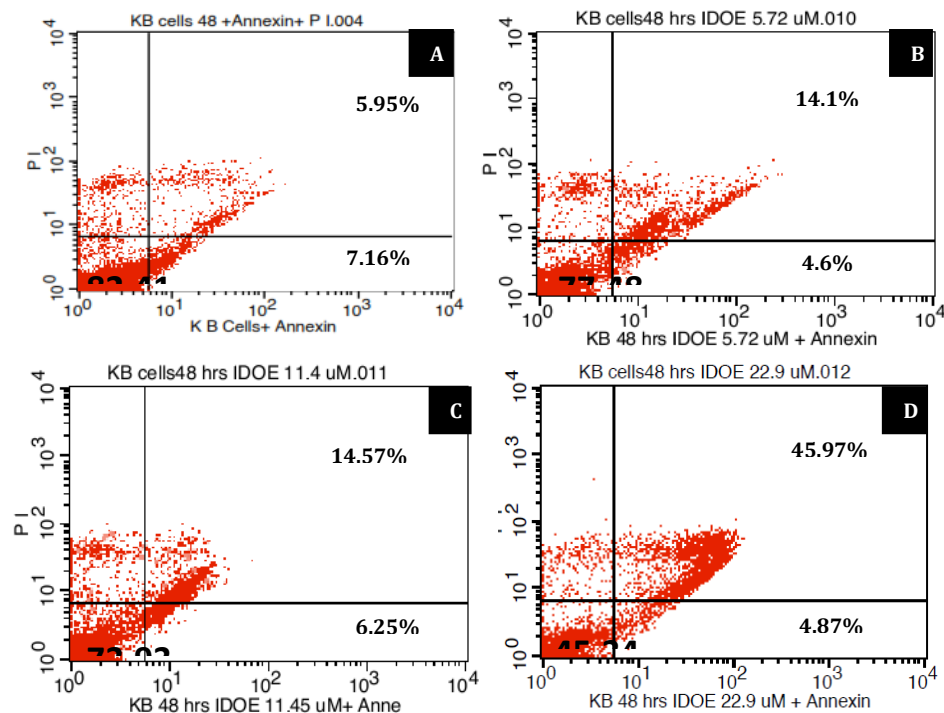


Figure 5: Flow cytometric detection of apoptosis by annexin V-PI staining of KB cells treated with IDOE. (A) Untreated control; (B) IDOE-5.72  $\mu\text{M}$ ; (C) IDOE -11.45  $\mu\text{M}$ ; (D) IDOE -22.9  $\mu\text{M}$ .

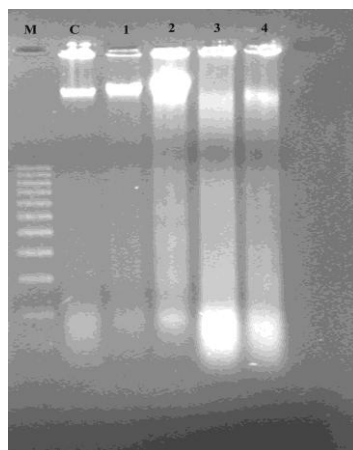


Figure 6: DNA fragmentation of KB cells induced by IDOE of varying concentrations for 48h. Cisplatin was used as positive control. M- Marker; C-untreated control cells; Lane 1- 5.72  $\mu\text{M}$ ; Lane 2-11.45  $\mu\text{M}$ ; Lane 3 - 22.9  $\mu\text{M}$ ; Lane 4-Cisplatin 20  $\mu\text{M}$ .

#### Effect of IDOE on cell cycle

Cell cycle analysis showed that after treatment with IDOE, the percentage of cells in the sub-G1 fraction was increased significantly. The cell cycle distribution of control KB cells was 74.7%, 15.4%, 8.3% and 0.9% at G1/G0, S, G2/M and Sub G1 phase respectively. In 22.9  $\mu\text{M}$  IDOE treated KB cells the cell cycle distribution was 56.7% at G1/G0, 12.9% at S, 13.6% at G2/M and 13.6% at SubG1 phase. As shown in Fig.7 after 48 h of treatment, an increase in G2/M-phase was observed from 8.3% in control to 13.6% in cells treated with 22.9  $\mu\text{M}$  IDOE. The cell cycle analysis by flow cytometer showed that G2/M arrest occurs at higher concentration of IDOE (22.9  $\mu\text{M}$ ).

#### DISCUSSION

Recently, attention has been given by researchers in worldwide to develop more effective and less toxic anticancer drugs from natural plant products. Due to their antitumor and antiinflammatory activity, sesquiterpene lactones have attracted the considerable

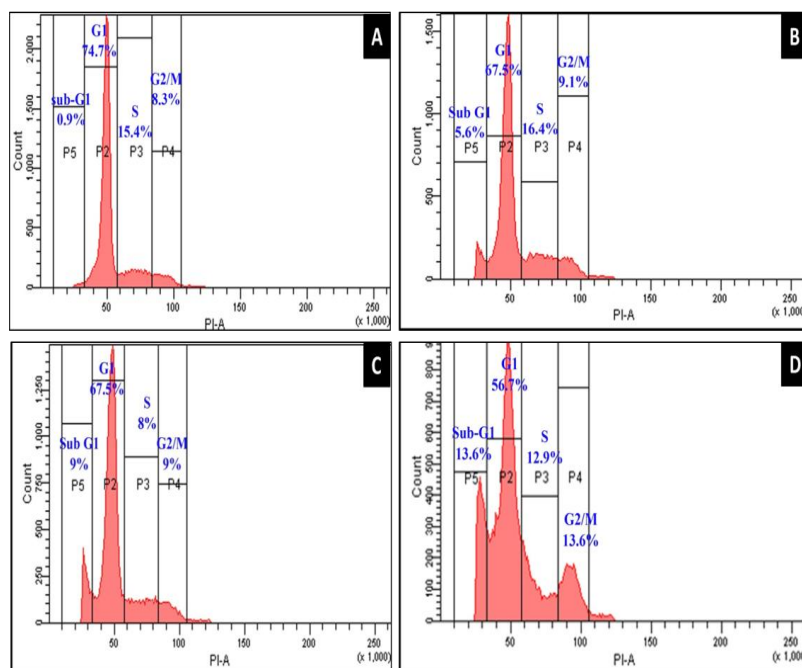
attention in pharmacological research [14, 15]. Several studies demonstrated the apoptosis inducing efficacy of

isodeoxyelephantopin, a sesquiterpene lactone from *Elephantopus scaber* in various cell types. No reports available regarding the apoptosis inducing property of Isodeoxyelephantopin in Nasopharyngeal carcinoma cells. Therefore, we evaluated the cytotoxic effects of IDOE on KB cells and its apoptosis inducing activity by different methods. It was observed that IDOE inhibited the growth of KB cells in a dose and time dependent manner with  $\text{IC}_{50}$  value of 11.45  $\mu\text{M}$  at 48h. The highly electrophilic  $\alpha,\beta$ -unsaturated carbonyl structures, such as the  $\alpha$ -methylene- $\gamma$ -lactone ring and the  $\alpha,\beta$ -unsaturated cyclopentenone, are considered as the general bioactive functional groups in sesquiterpene lactones as they allow the structures to interact rapidly with the nucleophilic sites of biological molecules in a Michael-type addition[4]. Cytotoxicity might be due to the presence of  $\alpha$ -methylene- $\gamma$ -lactone ring.

Clonogenic ability is considered to be a gold standard test for determining the antitumor activity of isolated compounds from plant origin [16]. Clonogenic survival of KB cells decreased in a dose-dependent manner and lost their viability at 11.45  $\mu\text{M}$  and its higher concentration suggested the antitumor activity of IDOE.

Apoptosis is characterized by morphological changes such as membrane blebbing, cell shrinkage, chromatin condensation and DNA degradation followed by rapid engulfment of cell debris by neighbouring cells [17]. Induction of apoptosis in cancer cells is one

of the useful strategies for anticancer drug development [18]. Therefore, we examined whether IDOE induced cytotoxicity on KB cells through the induction of apoptosis.



**Figure 7: Cell cycle analysis by flow cytometry. KB cells were treated with three indicated concentrations of IDOE for 48h. Accumulation of cells in Sub G1 peak appeared after the treatment with IDOE indicated the formation of apoptotic cells. (A) Untreated control; (B) IDOE-5.72 µM; (C) IDOE -11.45 µM; (D) IDOE -22.9 µM.**

Morphological features of IDOE treated KB cells after staining with AO/EtBr and Hoechst 33342 revealed the morphological features of apoptosis such as membrane blebbing, cell shrinkage and chromatin condensation after 48h of treatment. The percentage of apoptotic cells increased dose dependently. These findings are in agreement with those obtained in DLA and HCT-116 cancer cells treated with 3µg/mL Isodeoxyelephantopin [9]. The translocation of phosphatidyl serine molecules to the outer surface of the cell allows determination of apoptosis, when Annexin-V labeled with FITC is applied to the cell environment [19]. We subsequently quantified the number of apoptotic cells formed with IDOE treatment by FITC annexin V- propidium iodide staining. Treatment with all three concentrations resulted in a time-dependent increase in both annexin V and PI positive cells. The nuclear DNA of apoptotic cells shows a characteristic laddering pattern of oligonucleosomal fragments, the hall mark of apoptosis [20].

There is an intimate relationship between cell cycle and apoptosis, and together play an important role in the sensitivity of cancer cells to chemotherapy [21]. Cell cycle analysis of KB cells treated with IDOE at 48 hours blocked cells in G2/M-phase with concomitant increase in Sub G1-phase population of cells in a dose-dependent way. Accumulation of cells in subG1 phase was an indication of apoptosis. Our results are in agreement with those of Ichikawa who reported the apoptosis inducing efficacy of isodeoxyelephantopin in various cell types by inhibiting NF-kB activity and its downstream target genes such as IAP1, IAP2, Bcl-2, Bcl-x<sub>L</sub> and survivin [22]. COX-2 inhibitors are known to enhance the toxic action of anti-tumor drugs against cancer cells [23]. Isodeoxyelephantopin has also been reported to be a COX-2 inhibitor, thus it might play a key role in IDOE-induced apoptosis in KB cells which expresses high level of COX-2.

In summary, we have demonstrated isodeoxyelephantopin induced cytotoxic effect in Nasopharyngeal carcinoma (KB) cells. Both AO/EtBr and Hoechst staining exhibited the morphological features of apoptosis such as chromatin condensation and membrane blebbing in IDOE treated KB cells. DNA fragmentation analysis

revealed the intranucleosomal cleavage of DNA, a hall mark of apoptosis. Cell cycle analysis confirmed the apoptosis induction by accumulating cells in Sub G1 phase and G2/M cell cycle arrest in a concentration dependent manner. Further experimental research is necessary to better define antitumor mechanism of IDOE. Moreover, these data suggest the possibility of utilizing isodeoxyelephantopin as a potential new chemotherapeutic drug for Nasopharyngeal carcinoma.

#### Conflict of interest statement

We declare that we have no conflict of interest.

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