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Anti-cancer activity of *Indoneesiella echioides* (L.) Nees leaves using KB cells

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ABSTRACT

Indoneesiella echioides (L.) Nees is a well-known herb used in ethnomedicine. Flavonoids and alkaloids are major constituents of *Indoneesiella echioides* (L.) Nees. In this study, we investigated the anticancer activity of KB and normal HGF-1 cells. We demonstrated the effects of ethanolic extract of *Indoneesiella echioides* (L.) Nees on the cell growth and apoptosis in KB and normal HGF-1 cells were analyzed by the generation of reactive oxygen species (ROS), the level of mitochondrial membrane potential ($\Delta\psi M$) and apoptotic morphological changes were analyzed by AO/EtBr, AO and Hoechst staining. Our results indicated that the ethanolic extracts of *Indoneesiella echioides* (L.) Nees shows better anticancer activity through it's induces increased cell death, ROS generation, alteration of mitochondria membrane potential and apoptosis.

***Keywords*:** *Indoneesiella echioides* (L.) Nees, Anticancer activity, KB, normal HGF-1 cell, ROS generation, Mitochondria membrane potential

1. INTRODUCTION

Indoneesiella echioides (L) Nees (Acanthaceae), also known as *Andrographis echioides* (L) Nees. This is commonly known as False Water Willow, is an abundantly growing in south India. *Indoneesiella echioides* (L) Nees is highly medicinal important.

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The genus of *Indoneesiella* is used in goiter, liver diseases [1], fertility problems, bacterial [2], malarial and fungal disorders. The leaf juice of this plant is used to treating fever [3]. Several *Indoneesiella* species (about 40 species) has been used in treatment of influenza, malaria, dyspepsia and respiratory diseases. The *Indoneesiella* species also used to antidote for poisonous stings of some insects [4,5]. The leaf juice is mixed and boiled with coconut oil used to control falling and greying of hair [6]. Phytochemistry of *Indoneesiella echioides* has been investigated and reported to contain several flavonoids [7,8] and labdane diterpinoids [9-14]. In previous literatures are reported to only flavonoids as a major component in *Indoneesiella echioides* (L) Nees extracts [15-18]. It has been reported that variety of phytoconstituents like phenols, coumarins, lignans, essential oil, monoterpenes, carotenoids, glycosides, flavonoids, organic acids, lipids, alkaloids and xanthene's [19]. In this study, the anticancer activity of ethanolic extracts of *Indoneesiella echioides* (L) Nees in KB cells analyzed by cytotoxicity, ROS measurement, mitochondrial membrane potential and apoptotic morphological changes [24].

2. MATERIALS AND METHODS

2. 1. Collection of plant materials

The leaves of *Indoneesiella echioides* (L) Nees was collected from Poondi village, Thanjavur District, Tamil Nadu. The botanical identity (Voucher No: A.A.R 001 on 04-02-2015) of the plant was confirmed by Dr. S. John Britto, Rapinat Herbarium, St. Joseph's College, Tiruchirappalli.

2. 2. Preparation of Extracts

The fine powder (5 kg) was extracted with 95% ethanol at room temperature for ten days. The extract were filtered and concentrated under reduced pressure in a rotary evaporator and extracted for various solvents in increasing order of polarity from using n-hexane, chloroform, ethyl acetate, acetone,ethanol, butanol and methanol. After that the extract was taken in a beaker and kept in a water bath and heated at 30-40 °C till all the solvent got evaporated. The dried extracts were subjected to preliminary phytochemicals. The preliminary phytochemical analysis of various extract of *Indoneesiella echioides* (L) Nees leaves revealed the following phytochemicals (Table 1).

The dried ethanolic extracts of *Indoneesiella echioides* (L) Nees leaves extract were weighed (10 mg/mL) and dissolved in sterile distilled to prepare appropriate dilution to get required concentrations of 70 and 90 (µg/mL) of *I.echioides* were used for the anticancer activity experiment.

2. 3. Chemicals

a) Dulbecco's Modified Eagles Medium (DMEM),
b) Phosphate Buffered Saline (PBS),
c) Fetal bovine serum (FBS),
d) 0.25% trypsin EDTA,
e) Antibiotics (penicillin, streptomycin),
f) Dimethyl sulfoxide (DMSO),

g) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT),
h) 2,7-diacetyldichloro fluorescein (DCFH-DA),
i) Ethidium Bromide (EtBr),
j) Rhodamine 123,
k) Acridine Orange (AO).

2. 4. Cell culture

The oral carcinoma (KB) cell lines were obtained from NCCS, Pune, India. Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air incubation.

Table 1. Preliminary phytochemical constituents of *Indoneesiella echioides* (L) Nees leaves.

S.N	Phytochemicals	Hexane Extract	Chloroform Extract	Ethyl acetate Extract	Acetone Extract	Ethanol Extract	Butanol Extract	Methanol Extract
1.	Alkaloids	-	-	-	Present	Present	-	-
2.	Flavonoids	-	Present	Present	Present	Present	Present	Present
3.	Terpenes	Present	Present	-	-	-	-	-
4.	Triterpenoid saponins	-	Present	Present	Present	-	-	-
5.	Saponins	-	Present	Present	Present	Present	Present	Present
6.	Glycosides	-	-	-	-	-	-	-
7.	Steroids	Present	Present	Present	Present	-	-	-
8.	Carbohydrates	-	-	-	-	-	-	-
9.	Phenolic compounds	Present	Present	Present	Present	-	Present	Present
10.	Tannins	-	-	-	-	-	-	-
11.	Amino acids	-	-	Present	Present	Present	-	Present

3. EXPERIMENTAL WORK

3. 1. MTT ASSAY

MTT assay is the standard colorimetric assay for detecting cytotoxicity, cell viability, and anticancer activity trialed drugs/compounds. The effect of ethanolic extracts of *I.echioides* on the cell proliferation of KB cells was determined by MTT assay based on the

detection of mitochondrial dehydrogenase activity in healthy cells following the method of Arora *et a. l* [20]. KB cells were seeded in 96- well plates at a density of 5-10^3 cells / well in a final volume of 100 µL with DMEM an incubated up to 24h. The cells were treated with different concentrations (10-100 µL) of ethanolic extracts of *I.echioides*. After 24h, the cells were incubated with 100 µL of MTT solution (1 mg/mL) for 2h at 37 °C. The MTT solution was removed and added 100 µL of DMSO to dissolve the formazan crystals. The plate was read at 570 nm in a Read well touch, ELISA plate reader (Robonic, India).

3. 2. Measurement of intracellular ROS generation

Intracellular ROS was measured by using a non-fluorescent probe, DCFH-DA that can freely penetrate into the intracellular matrix of cells where it is oxidized by ROS to fluorescent dichloro fluorescein (DCF). Thus, the fluorescence intensity is directly proportional to the amount of ROS generation [21]. Cells were seeded (1×106 cells/well) in 6-well plate treated with ethanolic extracts of *I.echioides* different concentrations and kept in a CO_2 incubator for 24 h. After 24 h incubation 1mL of cells were incubated with 100 µL DCFH-DA for 10 min at 37 °C. Fluorescent intensity was measured with excitation and emission filters set at 485 ± 0 and 530 ±12.5 nm, respectively (Shimadzu RF-5301 PC spectroflurometer). The results were articulated as the percentage increase in the % of fluorescence intensity.

3. 3. Determination of mitochondrial membrane potential

Alteration of mitochondrial membrane potential has considered being an early sign of cell death or apoptosis. Mitochondrial membrane potential ($\Delta\psi M$) was measured by Rhodamine-123 (Rh-123), lipophilic cationic dye [22]. Cells were cultured in 6 wells plate (1 ×10^6 cells/well) and treated with ethanolic extracts of *I. echioides*. After the 24 h treatment, the cells were incubated with Rh-123 dye for 30 min. The $\Delta\psi M$ was evaluated qualitatively under a floid cell imaging station (Invitrogen, USA). Consequently, cells were trypsinaized and fluorescence intensity was measured at 485/530 nm under Spectrofluorometer (Schimadzu, USA). The graphical results were compared with positive control.

3. 4. Determination of apoptotic morphological changes

Acridine orange (AO) and ethidium bromide (EtBr) staining were used to detect apoptotic cells affirmation [23]. The cells were cultured in 6-well plate (3×10^4 /well) treated with different concentrations of ethanolic extracts of *I. echioides* for 24 h. The cells were fixed in methanol: glacial acetic acid (3:1) for 30 min at 4 °C. The cells were washed in PBS, and stained with 1:1 ratio of AO/EtBr for 30 min at 37 °C. Stained cells were washed with PBS and viewed under a fluorescence microscope. The number of cells showing features of apoptosis was counted as a function of the total number of cells present in the field.

3. 5. Statistical analysis

The results were expressed as the mean ± SD. The statistical analyses were performed using SPSS 11.0 software package. Statistical variances were assessed using ANOVAs. Significant differences ($p < 0.05$) between the means were identified by Duncan's Multiple Range Test (DMRT).

4. RESULTS AND DISCUSSION

4. 1. Cell proliferation inhibition effect of ethanolic extracts of *I. echioides* on KB cells

The cytotoxic effect of ethanolic extracts of *I. echioides* on KB cells was determined by MTT assay. Cells were treated with different concentrations (70 µL and 90 µL) of ethanolic extracts of *I. echioides* for 24 h incubation, which revealed a dose-dependent inhibition of cell proliferation. Maximum cell death was observed at 100 µg/mL concentration. Hence, the inhibitory concentration 50 (IC_{50}) of ethanolic extracts of *I. echioides* for KB cells 60µg/mL apparent from growth inhibition curve, Hence the (IC_{50}) value shown to be 68.5 µg/mL. Therefore 70 and 90 µg/mL doses of ethanolic extract of *I. echioides* were treated with KB cells for further studies.

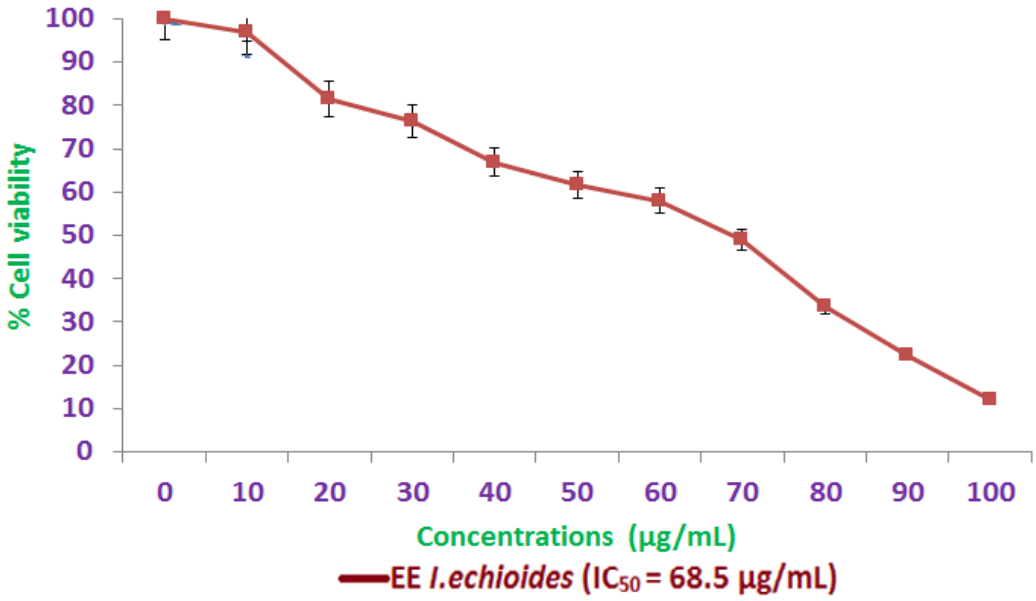
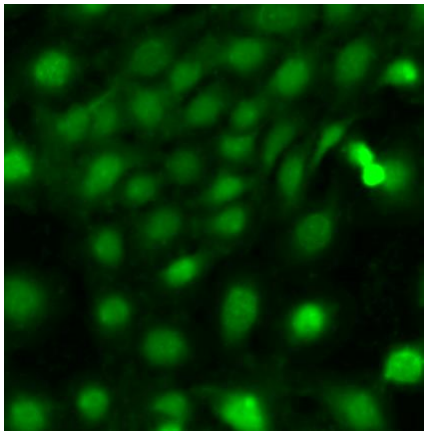


Fig. 1. Cell proliferation inhibition effect of ethanolic extracts of *I.echioides* on KB cells

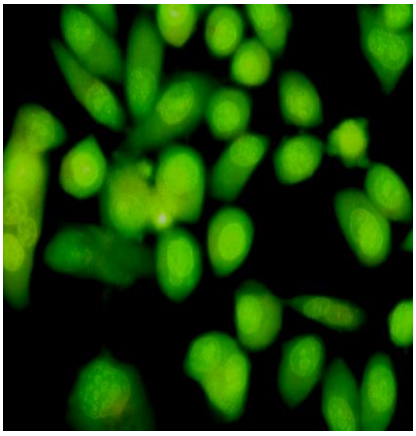
4. 2. Intracellular ROS generation by DCFH-DA staining of *I. echioides* on KB cells

Fig. 2, the effect of ethanolic extracts of *I.echioides* on intracellular ROS generation was evaluated with KB cells by using DCFH-DA staining. The ethanolic extracts of *I. echioides* treated KB cell shows increased ROS generation was indicated by deep DCF fluorescence intensity (ii and iii).

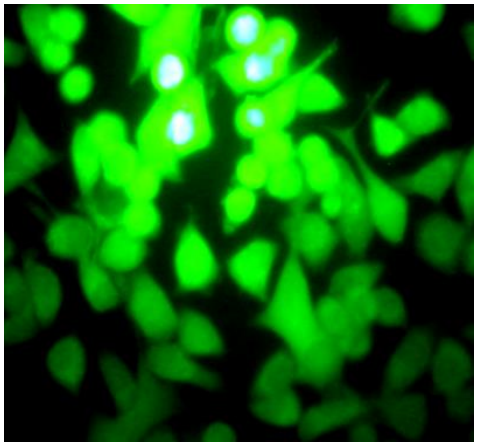
The images were acquired by floid cell imaging station. Cells were treated with different concentrations of ethanolic extracts of *I. echioides* (70 and 90 µg/mL) for 24 h incubation, which revealed a dose-dependent inhibition of cell proliferation. Percentage of ROS generation was detected by spectrofluorometer. All experiments were performed in triplicate and all the values were expressed as mean ± standard deviation of the mean. Statistical significance was determined by a one way ANOVA followed DMRT.



i. Controlii.



ii. EE *I.E* (70 µg/mL)



iii. EE *I.E* (90 µg/mL)

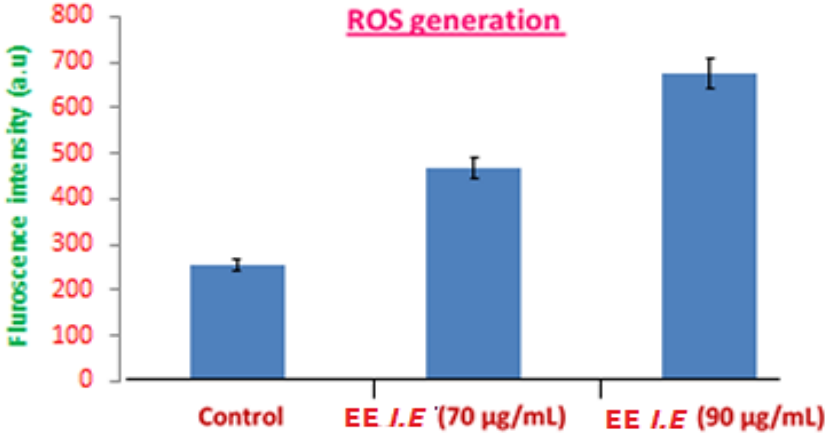
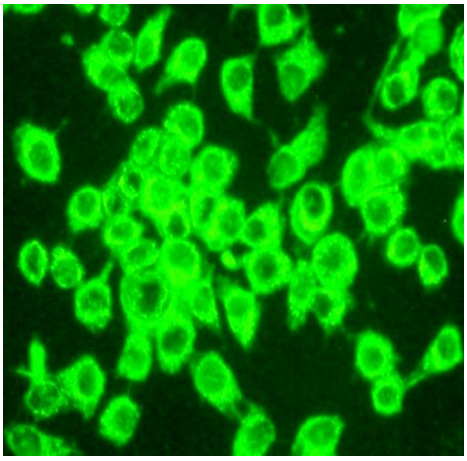
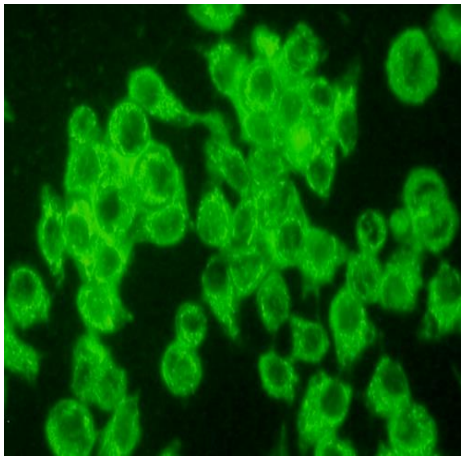


Fig. 2. The levels of ROS generation in control and ethanolic extracts of *I. echioides* treated cells.

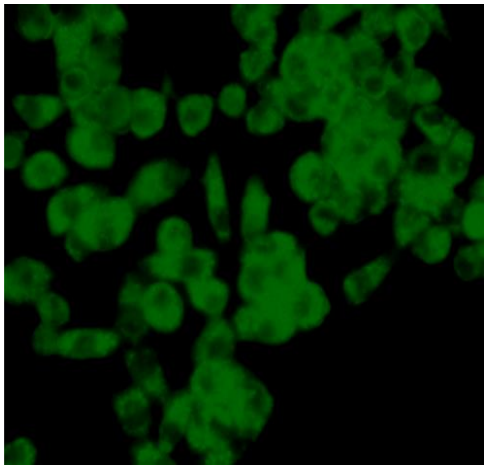
4. 3. Mitochondrial membrane potential by Rhodamine 123 staining of *I.echioides* on KB cells.



i. Control



ii. EE *I.E* (70 µg/mL)



iii. EE *I.E* (90 µg/mL)

Early stage of apoptosis is triggered by alteration of mitochondrial membrane potential were assessed by lipophillic cationic dye, Rhodamine-123. The effect of ethanolic extracts of *I. echioides* on mitochondria membrane potential ($\Delta\psi M$) damage was evaluated with KB cells using the Rhodamine 123.Untreated KB (control) cells show high fluorescence which indicate polarized mitochondria membrane (imge. i). Image (ii and iii) shows KB cells were treated with different concentration of ethanolic extracts of *I. echioides* (75 and 90 µg/mL) for 24 h and fluorescence intensity was decreased as indicated by collapsed mitochondria matrix. The images were acquired by floid cell imaging station. The depicted fluorescence intensity was detected by spectrofluorometer. All experiments were performed in triplicate and all values were expressed as mean ± standard deviation of the mean. Statistical significance was determined by a one way ANOVA followed DMRT.

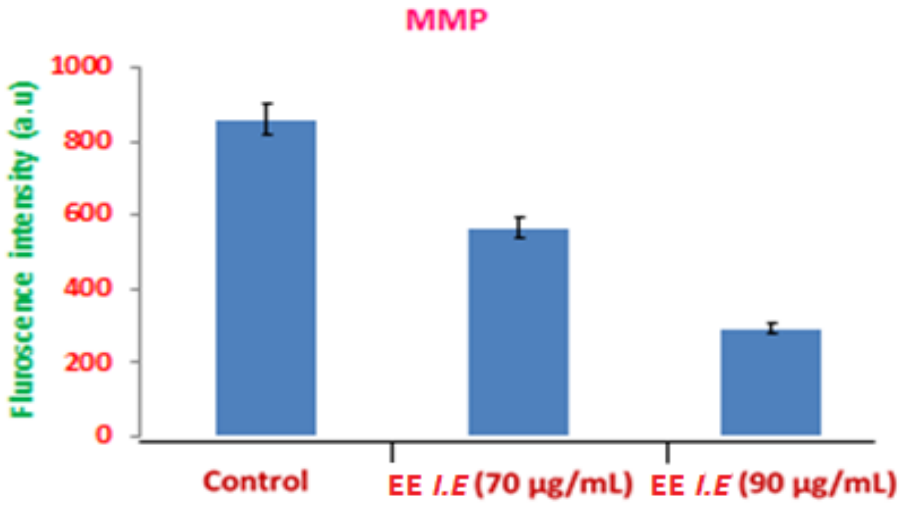


Fig. 3. The levels of MMP in control and ethanolic extracts of *I.echioides* treated cells.

4. 4. Apoptotic Morphological changes by Acridine orange and ethidium bromide staining of *I. echioides* on KB cells.

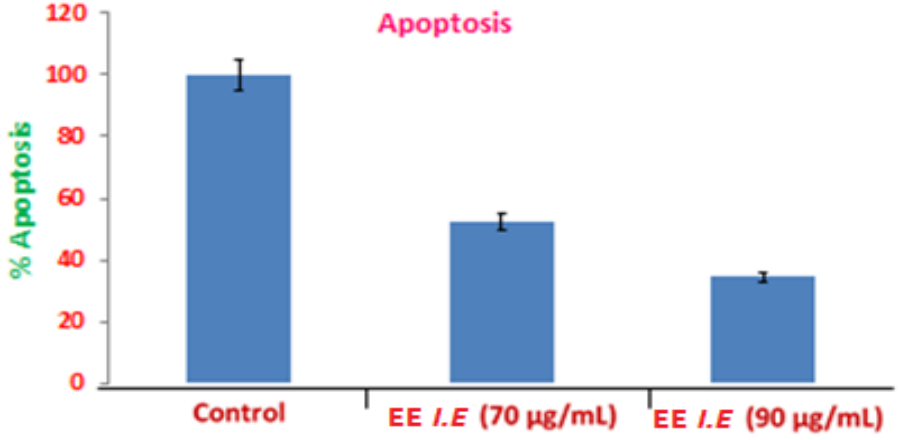
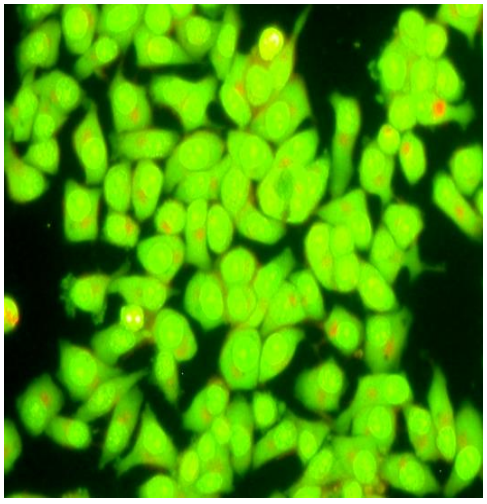


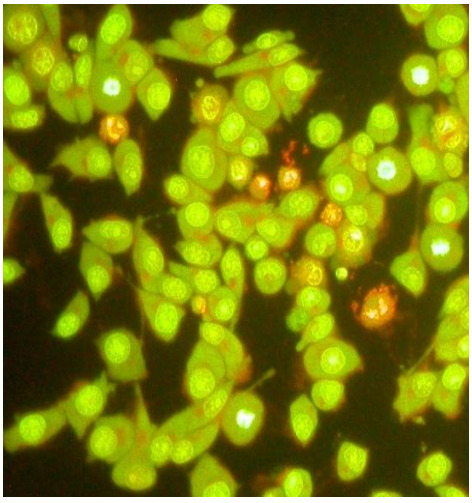
Fig. 4. The levels of Apoptosis in control and ethanolic extracts of *I. echioides* treated cells.

Fig. 4 Illustrated fluorescence microscopy images of apoptotic morphology by dual staining (AO/EtBr). Image i, untreated KB (Control) cells. Images ii, iii show different concentrations of ethanolic extracts of *I. echioides* (70 and 90 µg/mL) treated KB cells which shows increased % of apoptotic cells in a concentration dependent manner. The red fluorescence dye of EtBr was selectively penetrated into condensed nuclei of apoptotic cells, while the AO (green) had only taken up healthy cells. The observed results show untreated

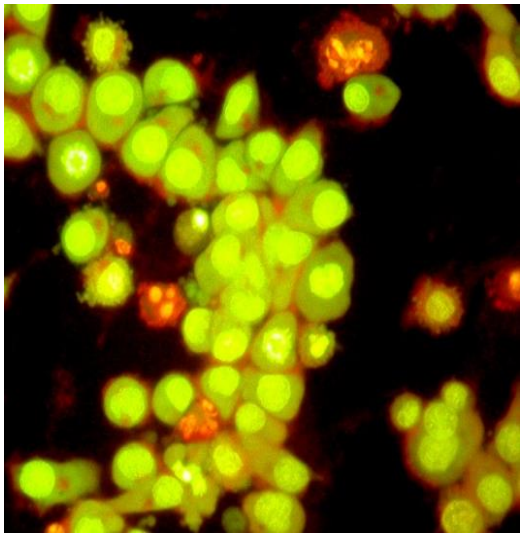
KB (control) cells which has highly green fluorescence nucleus that indicates live cells (image. i). The ethanolic extracts of *I. echioides* (70 and 90 µg/mL) treated cells showed orange color point out early apoptotic and red stained fragmented nuclei indicates late apoptosis at concentration depended manner for 24 h represented in (image ii and iii).



i. Control



ii. EE *I.E* (70 µg/mL)



iii. EE *I.E* (90 µg/mL)

5. CONCLUSION

The preliminary phytochemical analysis of *Indoneesiella echioides* (L) Nees leaves contains many bioactive chemicals like flavonoids, alkaloids, terpenoids, triterpenoids saponins, saponins, phenolic compounds, sterols and amino acids. The cytotoxicity effect of

ethanolic extract of *I.echioides* on KB and normal HGF-1 cells measured by MTT assay, the inhibitory concentration 50 (IC_{50}) of *I.echioides* is 68.5 µg/mL. The ethanolic extract of *I.echioides* shows anticancer activity through its induces increased cell death, ROS generation, alteration of mitochondria membrane potential and apoptosis. The anticancer activity of *I.echioides* might be oxidative damage through proxidant mechanisms. These results indicated that we concluded that ethanolic extract of *I.echioides* could be used as a novel therapeutic agent for the prevention of cancer.

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