Bioscience Discovery, 9(3):344-355, July - 2018

Print & Online, Open Access, Research Journal Available on http://jbsd.in
ISSN: 2229-3469 (Print); ISSN: 2231-024X (Online)

Research Article

In vitro Evaluation of Cytotoxic Properties of *Peperomia pellucida* (L.) H.B.K. Against Human Cancer Cell Lines

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Article Info

Received: 04-03-2018,
Revised: 24-05-2018,
Accepted: 30-05-2018

Keywords:
Colon carcinoma, cytotoxicity, tryphan blue, MTT assay, *Peperomia pellucida*

Abstract

Medicinal plants have a long standing history in many indigenous communities and continue to provide useful tools for treating various diseases. Plant derived components have attracted particular attention as a substitute to encounter a number of diseases including cancer. Cancer is one of the most dreadful diseases of the 20th century and spreading continuously with increasing incidence in 21st century. It is world's second killer after cardiovascular disease. Emerging evidence suggests that a number of plants are known to be the source of useful drugs in modern medicine and have been accepted currently as one of the main source of cancer chemoprevention drug discovery and development due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects. With this backdrop, the present study was aimed at determining the cytotoxicity of aqueous and ethanolic extracts of *Peperomia pellucida* against HT-29 (Human, Colon carcinoma) cell lines using Tryphan blue dye exclusion method and MTT assay. The results revealed that the cytotoxic potential of *P. pellucida* increased when the concentration of plant extracts increases. The present study may provide the landmark for further exploration of *P. pellucida* for its potent anticancer constituents.

INTRODUCTION

The usage of plants as supply of medicines for the healing of contagious and non-contagious diseases is an old human custom (Petrovska, 2012), and the practice is now ever-increasing due to amplified global health challenges (WHO, 2002). Medicinal plants are the nature's gift to mankind to make disease free healthy existence (Vasantha *et al.*, 2012). Medicinal plants have received the consideration of the pharmaceutical and scientific communities in recent times and various publications have recognized the therapeutic significance of natural compounds in a bid to authenticate claims of their biological property. Plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Mary and Indira, 2017). The screening of plant extracts has been of great concern to scientists in the quest for new drugs for effectual treatment of numerous ailments (Dimayugu and Garcia, 1991).

Cancer is one among the most progressive distressing disease posing a threat of mortality in humans and at present there is substantial scientific discovery of new anticancer agents from natural products.

Drug discovery from medicinal plants has played a vital role in the healing of cancer and indeed, over the last half century most of the plant bioactive compounds and their derivation have been used towards fighting cancer (Nweman *et al*., 2000; Bulter, 2004; Narayana moorthi *et al*., 2015). The major reason of cancer is: smoking, dietary imbalances, hormones, chronic infections/inflammations.

Many of the discovered phytoconstituents seem to combat diseases such as cancer, heart attack and stroke. At the same time other scientists are conducting epidemiological studies to establish the relationship between the consumption of phytoconstituents and human wellbeing. A large number of studies showed that diets loaded in plants gave lower rates of cancer and heart disease. The principles underlying herbal medicines are relatively trouble-free, although they are quite distinctive from conventional and herbal medicine.

The need of scientific and chemical information in support of improved understandings of the efficiency and security of the herbal drugs has become the major hindrance to the use of traditional herbal preparations. Hence, it is necessary to conduct more chemical and pharmacological studies at molecular level to explore untapped prospective of the herbal drugs. Cancer chemopreventive bioactive compounds would endow with potential opportunities for future design of chemo-preventive agents based on molecular targeting. Cytotoxicity of plant extracts on both plant and animal test material is capturing scientific interest, as it can specify their antitumor and anticarcinogenic potentials, which have at all times been a gateway for the progression of novel anticancer drugs.

Peperomia pellucida is a herbaceous plant also known as shiny bush or silver bush belonging to the family Piperaceae. The leaves and stems are eaten as vegetable (Sheikh *et al*., 2013). As an ethnomedicinal plant, it is been used for treating abdominal pain, abscesses, acne, boils, colic, fatigue, gout, headache, renal disorders and rheumatic joint pain. It also used to treat breast cancer, impotence, measles, mental disorders and smallpox. *P. pellucida* is used in salads or as a cooked vegetable to help in relieving rheumatic joint pain (Idu, 2009).

It was claimed by the local community that decoction of the plant was helpful to treat bone aches and pains. The leaf was also used in headache, fever, eczema, abdominal pains and convulsions treatments. Elsewhere, this plant served multi-function, including mental disorder treatment in Bangladesh; haemorrhages treatment in Bolivia, cholesterol reduction in Brazil and renal problem and uric acid reduction in Guyana and Philippines (De Padua *et al*., 1999). Some biological properties of *P. pellucida* such as antibacterial, anti-inflammatory, analgesic and antifungal have been studied. Whole plant or parts of plant are used for different purposes. In spite of its wide range of folk medicinal uses in India sub-continent, there is very little scientific documentation available on its pharmacological and biological activities as well as its chemical constituents (Gutierrez *et al*., 2016). In this view point, an attempt is made to investigate the cytotoxicity of aqueous and ethanolic extracts of *Peperomia pellucida* against HT-29 (Human Colon carcinoma) cell lines.

MATERIALS AND METHODS

Collection of Plant Sample

In the present study, *Peperomia pellucida* (L.) H.B.K. plants are collected from a garden in Senthil Nagar, Coimbatore, Tamil Nadu and authenticated by Botanical Survey of India (The voucher number is BSI/SRC/5/23/2015tech-1169 dt. 22.5.2015).

Preparation of Plant Extracts

The whole plant of *P. pellucida* was cut into small pieces, washed, dried at room temperature; the dried samples were powdered in a Wiley mill. Hundred grams of powdered plant samples were separately packed in a Soxhlet apparatus and extracted with ethanol and water. The ethanol and aqueous extracts was concentrated in a rotary evaporator. The concentrated extracts were used for *in vitro* cytotoxicity studies.

Cell lines and Culture Condition

HT-29 (Human, Colon carcinoma) cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated. Fetal Bovine Serum (FBS), Penicillin (100 IU/ml), Streptomycin (100 µg/ml) and Amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25cm culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

***In Vitro* Assay for Cytotoxicity Activity:**

Tryphan Blue Dye Exclusion Method (Sheeja *et al*., 1997)

Tryphan blue dye exclusion test is used to determine the number of viable cells present in a cell suspension. It acts as an exclusion test, not penetrating live cells with intact membrane structures (Demirci *et al*., 2001). It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as tryphan blue, Eosin, or propidium, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. The protocol presented here, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm (Shapiro, 1988). Short- term *in-vitro* cytotoxicity was assessed using HT-29 cell lines cells by incubating different concentrations of the aqueous and ethanolic extracts of the selected plant drugs at 37°C for 3h. For the cytotoxicity assay, different concentrations of the extracts (25-500µg/ml) were added to each tubes and the final volume was adjusted to one ml with normal saline. Control tubes were kept with the saline, tumor cells and without the drugs. All the tubes were incubated at 37°C for 3h. After incubation 0.1ml of 0.4% tryphan blue dye in isotonic saline was added to each tube and the number of viable (unstained) and dead (stained) cells were counted using haemocytometer.

$$\% \text{ of Dead cells} = \frac{\text{Total Cells Counted} - \text{Total Viable Cells}}{\text{Total Cells Counted}} \times 100$$

***MTT Assay* (Francis and Rita, 1986)**

MTT assay is based on the ability of viable cells with active mitochondria to produce succinate dehydrogenate enzyme which cleave the tetrazolium rings of MTT (Mosmann, 1983) where the optical density (OD) obtained was proportional to the number of healthy viable cells.

Preparation of Test Solutions

For cytotoxicity studies, each weighed test samples were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1mg/ml concentration and sterilized by filtration. Serial dilutions were prepared from this for carrying out cytotoxic studies.

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to $1x10^6$cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere and microscopic examination was carried out and observations were noted every 24h interval. After 72h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3h at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540nm. The percentage of growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ of Growth Inhibiton} = \frac{\text{Mean OD of Individual test group}}{\text{Mean OD of Control group}} \times 100$$

RESULTS AND DISCUSSION

In vitro Cytotoxicity and Anticancer Activity

From the results of quantity analysis of phytochemicals, free radical scavenging activity and antimicrobial activity, it was identified that aqueous extract (*Pp*-Aq) and ethanol extract (*Pp*-Et) has good antioxidant and free radical scavenging property than the other three (hexane, chloroform, ethyl acetate) extracts of *Peperomia pellucida. H*ence *Pp*-Aq and *Pp*-Et were selected for *in vitro* anticancer studies. The *in vitro* anticancer activity of the *Pp*-Aq and *Pp*-Et were evaluated using Tryphan Blue Method and MTT assay (Human Colon colorectal cell lines (HT-29)).

Tryphan Blue Method

To assess time dependent cytotoxicity, direct counting of living and dead cells after exposure to cytotoxic concentration of *Pp*-Aq and *Pp*-Et against HT-29 were carried out using tryphan blue dye exclusion assay and the untreated cells served as control. Resultant cell suspension was then admixed with 0.4% Trypan blue dye and counted in Neubauer chamber and viable cells were examined for *Pp*-Aq and *Pp*-Et (Figure 1 & 2 and Plate I & II).

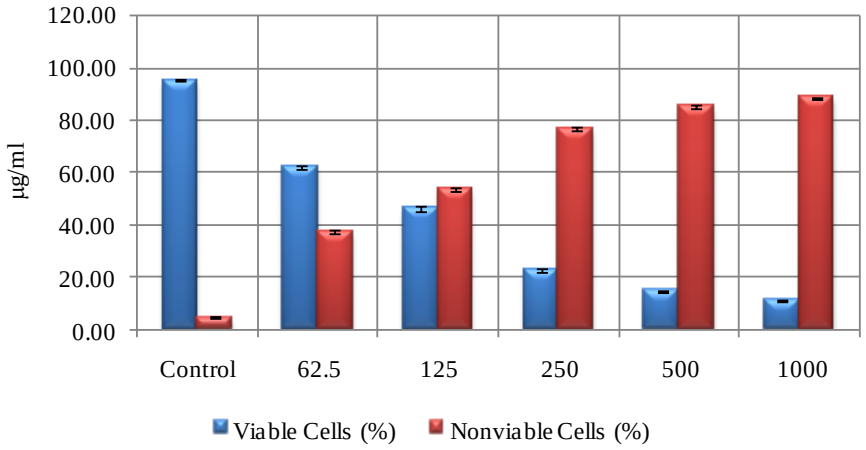


Figure 1: Effect of *Pp*-Aq on HT-29 (Tryphan Blue method)

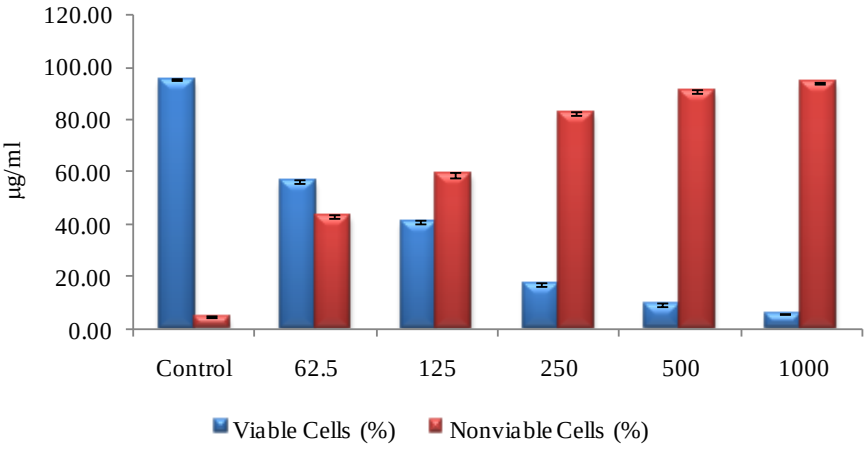
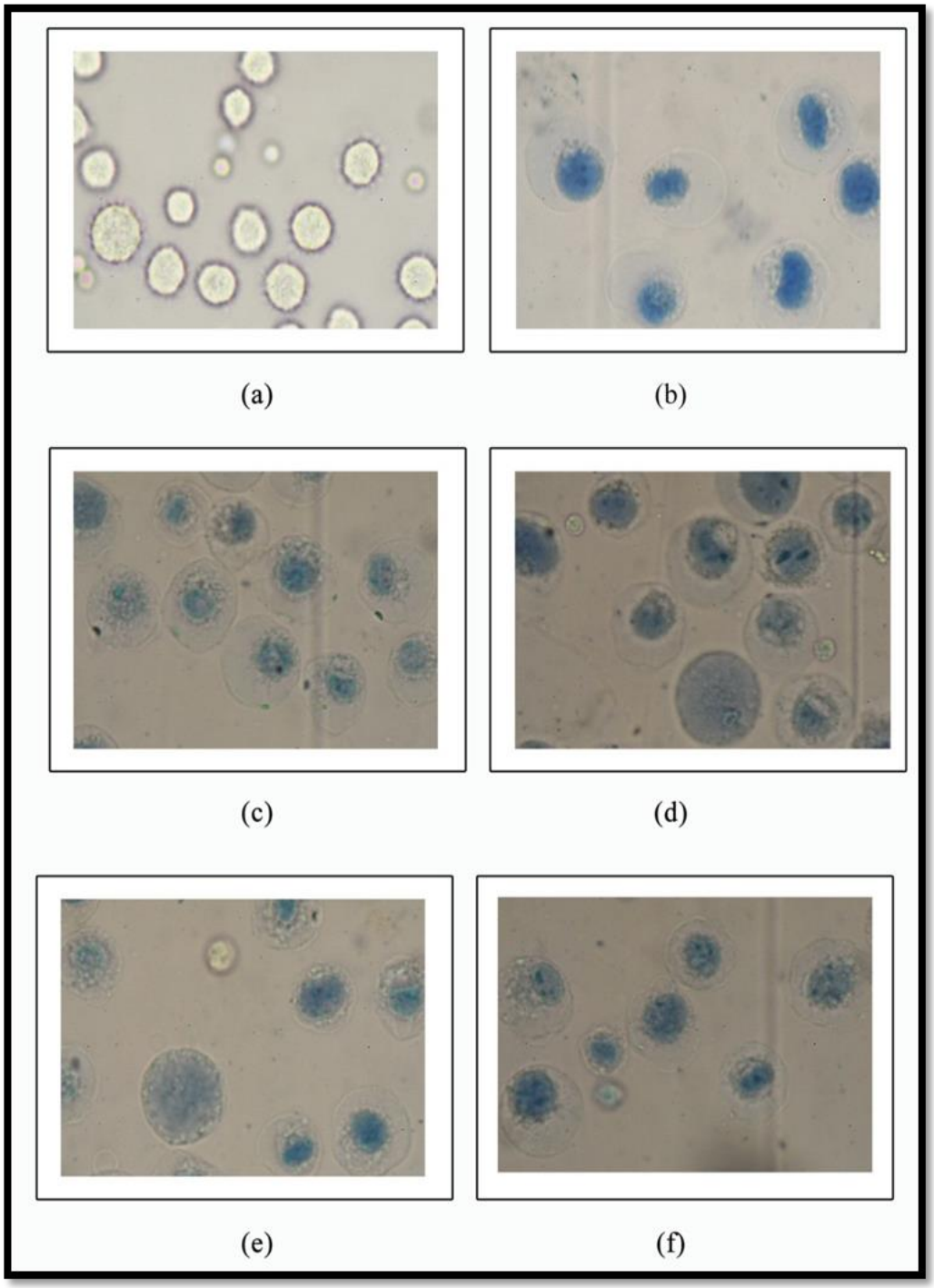


Figure 2: Effect of *Pp*-Et on HT-29 (Tryphan Blue method)

Plate I Cytotoxic effects of *Pp*-Aq on HT-29 cell lines

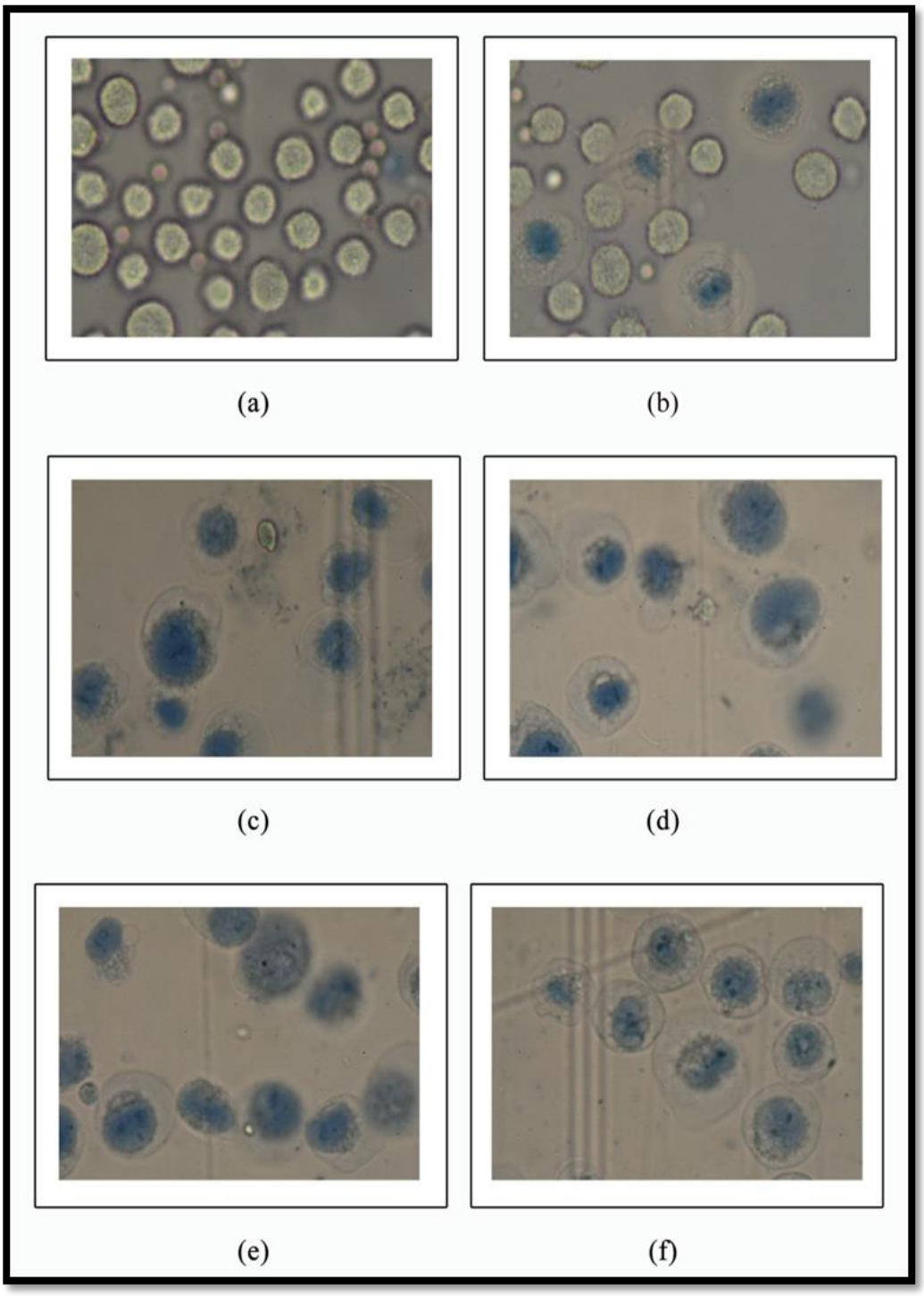
(Trypan Blue Method)



(a) Control **(b) 62.5 µg/ml of Pp-Aq** **(c) 125 µg/ml of Pp-Aq**

(d) 250 µg/ml of Pp-Aq **(e) 500 µg/ml of Pp-Aq** **(f) 1000 µg/ml of Pp-Aq**

Plate II Cytotoxic effects of *Pp*-Et on HT-29 cell lines (Trypan Blue Method)



(g) Control **(b) 62.5 µg/ml of *Pp*-Et** **(c) 125 µg/ml of *Pp*-Et**

(d) 250 µg/ml of *Pp*-Et **(e) 500 µg/ml of *Pp*-Et** **(f) 1000 µg/ml of *Pp*-Et**

From Figure 1 and 2, it was observed that HT-29 cell lines treated with various concentrations of *Pp*-Aq (62.5, 125, 250, 500 and 1000µg/ml) and *Pp*-Et (62.5, 125, 250, 500 and 1000µg/ml) showed progressive reduction in cell viability over a period of 3h. In fact, at 3h there was a complete loss of cell viability in all the cancer cell lines. With treatment of *Pp*-Aq against HT-29, the percentage of nonviability cells at 0, 62.5, 125, 250, 500 and 1000µg/ml were found to be 4.64±0.42, 37.83±0.75, 53.53±0.87, 77.02±0.67, 85.26±0.56 and 88.63±0.19 (%) respectively (Figure 1). IC_{50} of *Pp*-Aq dose was analyzed and noted as 169.97µg/ml. Similarly the cytotoxicity effects of *Pp*-Et against HT-29 was analyzed and graphically represented in Figure 2. The percentage of non viable cells was evaluated for control and extracts (0, 62.5, 125, 250, 500 and 1000µg/ml) and results were noted as 4.64±0.42, 43.33±0.75, 59.03±0.87, 82.52±0.67, 90.76±0.56 and 94.13±0.19%. IC_{50} of *Pp*-Et was marked as 129.16µg/ml.

Plate I and II shows the morphological changes occurred during cytotoxic effect of *Pp*-Aq and *Pp*-Et. Tryphan blue, a diazo dye, is a vital stain used to selectively colour dead tissues or cells blue. Live tissues or cells with unharmed cell membranes are not coloured. Since cells are very selective in the compounds that pass through the membrane, in a viable cell, tryphan blue is not absorbed; however, it navigates the membrane of dead cells. Hence, dead cells are shown as a distinctive blue colour under a light microscope. Since live cells are excluded from this staining, this aglycones could provide the chemical basis of its staining method described as a dye exclusion cytotoxic effect against HT-29 cells (Shapiro, 1988; Haldar, *et al*., 2010).

Similar to this study, the ethanolic extract from leaves of *Holarrhena antidysenterica* has significant *in vitro* cytotoxic activity. It was found that it inhibited the growth in the range of 71-99% against seven human cancer cell lines from five different tissues, OVCAR-5 (ovary), HT-29 (colon), SK-N-MC (neuroblastoma), HEP-2 (liver), COLO-205 (colon), NIH-OVCAR-3 (ovary) and A-549 (lung) (Sharma *et al*., 2014). An another study also reported that the ethanolic extract from the leaves of *Nardostachys jatamansi* exhibited *in vitro* anticancer effects against five human cancer lines such as COLO-205, SW-620, HeLa, SK-N-MC and NCI-H23 and in the range of 70-93% (Sharma and Hussain, 2013).

From the results of Tryphan blue dye exclusion method, it was observed that the cytotoxic effect of *Peperomia pellucida* was increased with increase in the concentration of extract. It was found that both the *Pp*-Aq and *Pp*-Et have good cytotoxic effect against HT-29 cell lines.

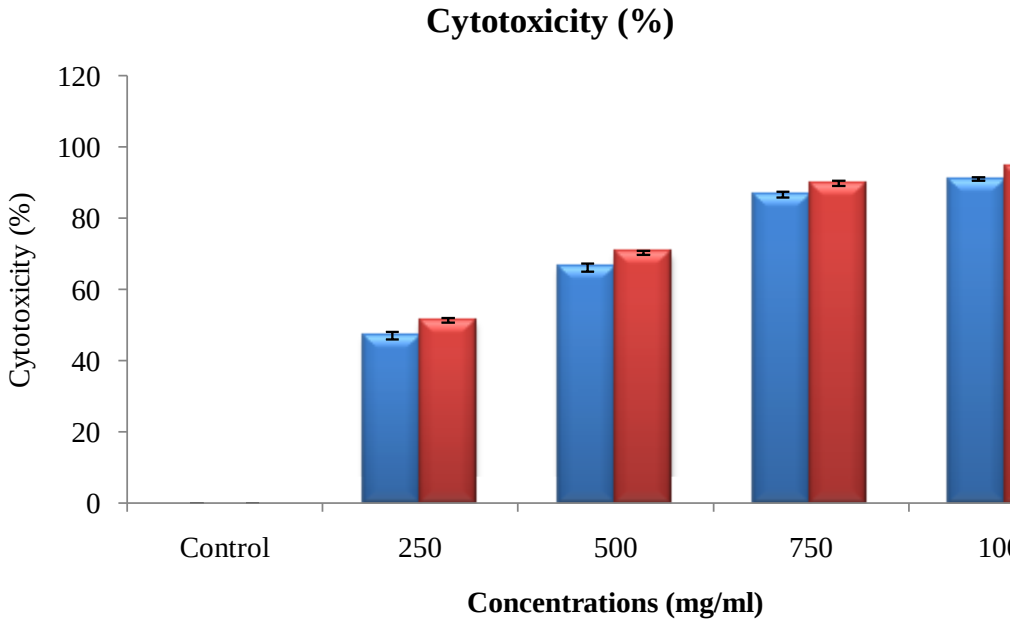
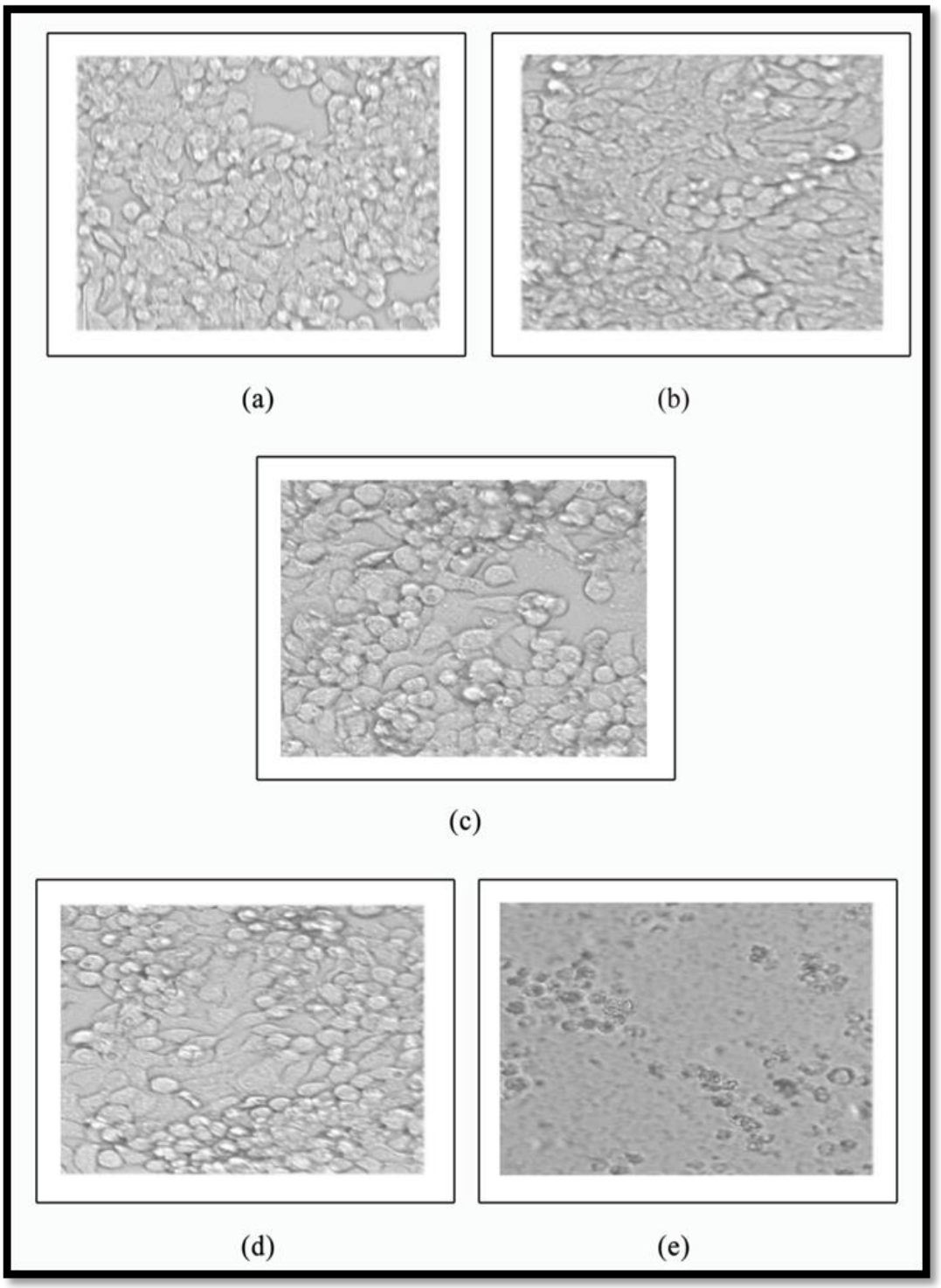


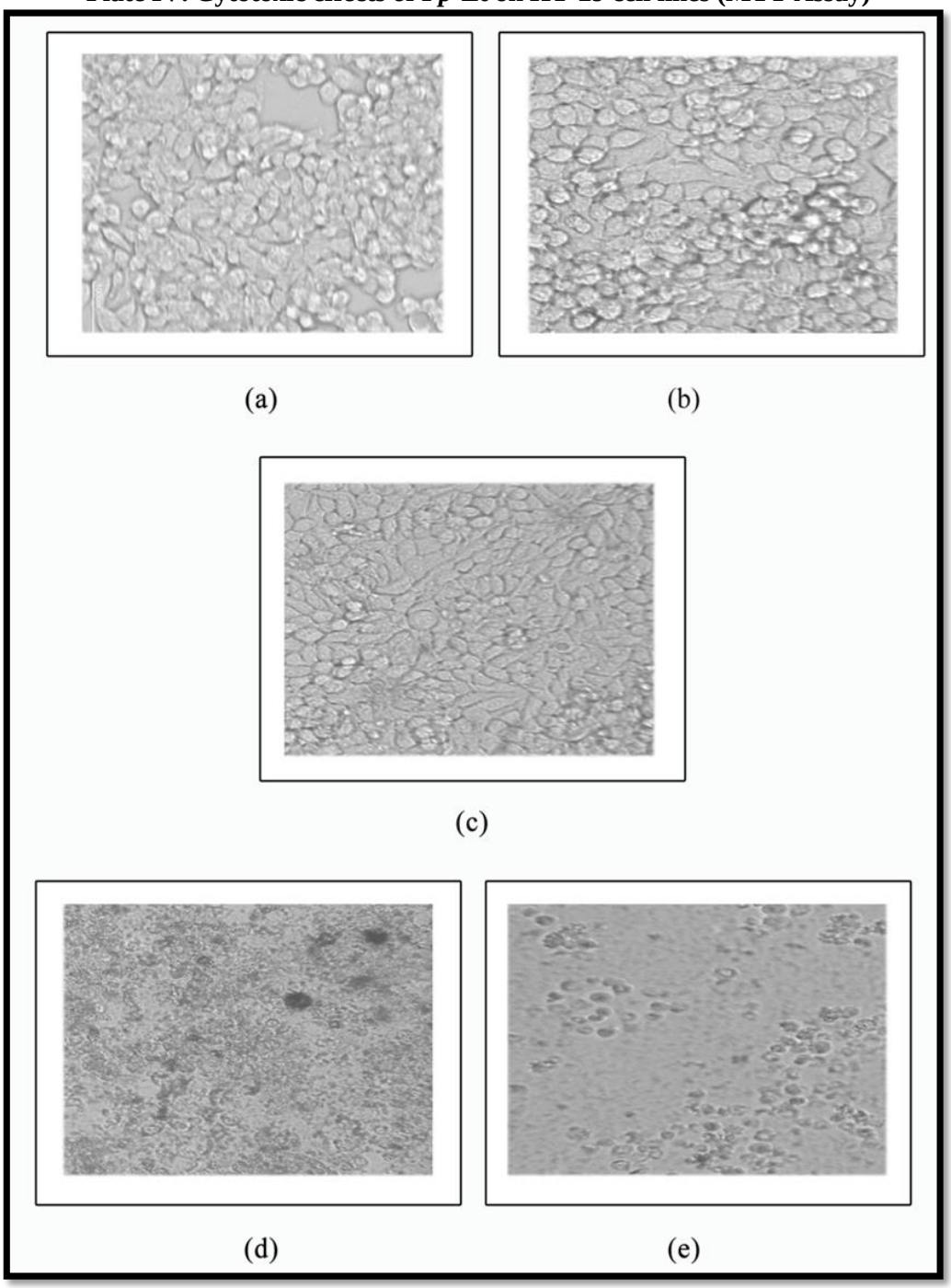
Figure 3: Effect of *Pp*-Aq and *Pp*-Et on HT-29 cell lines (MTT Assay)

Plate III. Cytotoxic effects of *Pp*-Aq on HT-29 cell lines (MTT Assay)



(a) Control **(b) 250 µg/ml of *Pp*-Aq** **(c) 500 µg/ml of *Pp*-Aq**
(d) 750 µg/ml of *Pp*-Aq **(e) 1000 µg/ml of *Pp*-Aq**

Plate IV. Cytotoxic effects of *Pp*-Et on HT-29 cell lines (MTT Assay)



(a) Control (b) 250 µg/ml of *Pp*-Et (c) 500 µg/ml of *Pp*-Et
(d) 750 µg/ml of *Pp*-Et (e) 1000 µg/ml of *Pp*-Et

MTT Assay

In this study, HT-29 cell lines were treated with *Pp*-Aq and *Pp*-Et at different concentrations for 48h. A mitochondrial enzyme in living cells, succinate dehydrogenase cleaves the tetrazolium ring of MTT and converting it to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. Viability of the HT-29 cancer cells was decreased with increasing concentration of *Pp*-Aq (250 to 1000µg/ml) and of *Pp*-Et (250 to 1000µg/ml) (Figure 10).

Aqueous extract of *P. pellucida* exhibited a high cell inhibition rate against HT-29 as 47.03±0.98, 66.15±1.20 and 86.66±0.86 and the ethanol extracts showed inhibition activity as 51.33±0.60, 70.35±0.55, 89.81±0.76 and 94.67±0.62 for HT-29at 250, 500, 750 and 1000 (µg/ml) respectively (Figure 3; Plate III & IV). IC_{50} value for *Pp*-Aq was found to be 128.37 µg/ml and for HT-29 was 218.58µg/ml.

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Similar to present study, *in vitro* cytotoxicity of ethanolic extract of *Dendrobium formosum* on Dalton's lymphoma (Ritika and Biplob, 2014), *Mikania laevigata* against tumor (Hep-2, HeLa) and non tumor (MRC-5) cell lines (Rufatto *et al*., 2013), seed ethanolic extract of *Hibiscus cannabinus* L. (Yu Hua *et al*., 2014) was evaluated using MTT assay. Three new compounds from the flower of *Lawsonia inermis* L. showed cytotoxic activity on MCF-7, Hela, HCT-116 and HT-29 cell lines (Qian *et al*., 2014). Four new compounds isolated from the leaves of *Prunus tomentosa* as a valuable source of new potent anticancer drug (Wei *et al*., 2014) and the compounds isolated from *Combretum paniculatum* against HT-29, MCF-7 and HeLa using the methyl thiazolyl tetrazolium (MTT) assay and reported that cytotoxic effect through the induction of cell cycle arrest in the G0/G1 phase of the cell cycle (Sowemimo *et al*., 2012).

Earlier studies carried out on cell cultures and animal models indicated that polyphenols were the main phytochemicals with antioxidant and anti-proliferative properties from higher plants (Carvalho *et al*., 2010). These molecules may act as cancer-blocking agents, preventing the initiation of the carcinogenic process as cancer-suppressing agents and inhibiting cancer promotion and progression (Russo, 2007). In our preliminary study, *Pp*-Aq contained a higher phenolic content than other extracts, which coincides with its cytotoxic activity. This finding showed that the phenolic compounds in *Peperomia pellucida*whole plant are responsible for cytotoxic properties.

From figure 3, it was observed that *Pp*-Et exhibited a maximum rate of cell inhibition against HT-29. Results from Plate III and III also confirmed the cytotoxicity through with morphological changes of HT-29 cancer cells treated with *Pp*-Aq and *Pp*-Et at various concentrations. Similar study has been carried out in *Morinda pubescens* which can be potent natural antioxidants and can be essential for health preservation against oxidative stress related degenerative diseases, such as cancer (Inbathamizh *et al*., 2013).

The results of tryphan Blue exclusion method are in agreement with the IC_{50} values calculated in the MTT assay. Both cytotoxic assays presented have comparable results and the small differences was observed in the cytotoxic values might be due to their assay principles, MTT reduction assessing the functional metabolic activity of mitochondria based on the enzymatic reduction of a tetrazolium salt by mitochondrial dehydrogenases of viable cells, while trypan blue is based on cell membrane integrity. The use of different test confirms the cytotoxicity of the *Pp*-Aq and *Pp*-Et.

CONCLUSION

In conclusion, *P. pellucida* is one of the effective medicinal plants used in traditional medicinal systems of India. All the parts of the plants have the efficient medicinal property and used to heal many diseases. The current exploration also added one more potent activity of the plant. This study provides only the baseline data and thus calls for further studies on the active components for proper assessment of their chemotherapeutic properties as well as their possible development as promising anticancer drugs.

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How to cite this article

Narayana moorthi, V., K. Vasantha and A. Maruthasalam, 2018. *In vitro* Evaluation of Cytotoxic Properties of *Peperomia pellucida* (L.) H.B.K. Against Human Cancer Cell Lines. *Bioscience Discovery*, **9**(3):344-355.