

**EVALUATION OF APOPTOTIC ACTIVITY OF ETHANOLIC EXTRACT OF
ANDROGRAPHIS PANICULATA LINN IN HUMAN HEPATOCELLULAR
CARCINOMA CELL LINES**

Running title: Evaluation of apoptotic activity by *Andrographis paniculata*

ABSTRACT

Introduction

Andrographis paniculata is one of the highly used potential medicinal plants in the world. This plant is traditionally used for the treatment of common cold, diarrhoea, fever due to several infective cause, jaundice, as a health tonic for the liver and cardiovascular health, and as an antioxidant. It is also used to improve sexual dysfunctions and serve as a contraceptive. All parts of this plant are used to extract the active phytochemicals, but the compositions of phytoconstituents widely differ from one part to another and with place, season, and time of harvest.

Materials and method

All data obtained were analysed by Student t test using MS- Excel. The results were computed statistically using one way ANOVA. The reagents used were MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide]: 0.5 mg MTT/ml of serum-free DMEM, Solubilization solution: Dimethyl sulfoxide (DMSO) and Phosphate Buffered Saline (PBS; pH 7.4). The cytotoxic effects of lycopene Ag NP on 3T3 cells. Cells were treated with Lycopene Ag NP (10, 20, 30, 50, 100 and 200 μ M) for 24 h, and cell viability was evaluated by MTT assay. Data are shown as means \pm SD (n = 3). * compared with the control-blank group, p < 0.001.

Results

The results were evaluated using three tests namely, MTT assay, Dual staining (acridine orange/ethidium bromide) and morphological changes.

Conclusion

Different stages of apoptosis revealed the importance of *A. paniculata* in hepatocellular carcinoma cell lines. Andrographolide and analogues have been subjected to extensive chemico- biological investigations for anticancer drug development. Several andrographolide analogues have shown superior anticancer activities in both in vitro and in vivo models. Further clinical and biomedical studies are required to confirm the pharmacological, pharmaceutical and toxicological properties of andrographolide.

Keywords: *Andrographis paniculata*, Carcinoma, Apoptosis, Viability

INTRODUCTION

Medicinal plant is an integral part of human life to combat the sufferings from the dawn of civilization (Alamgir, 2017). It is estimated that more than 80,000 of total plant species have been identified and used as medicinal plants around the world (Rajeshkumar *et al.*, 2018). Liver cancer is that the third leading explanation for cancer death worldwide and regarding half the patients with liver disease need adjuvant medical care when surgical operation (Duke, 2007; Gheena and Ezhilarasan, 2019). Therefore, development of novel agents to eradicate cancer cells might represent a viable approach to treat patients with liver disease (Nandhini, Rajeshkumar and Mythili, 2019). Andrographolide, a diterpenoid lactone isolated from *Andrographis paniculata*, is understood to possess potent inhibitor, medication, antineoplastic and antiviral properties (Sen and Chakraborty, 2019). During this study, we tend to investigate the cytotoxic impact of andrographolide on human liver disease cells and explore the necrobiosis mechanism (Vairavel, Devaraj and Shanmugam, 2020). Andrographolide iatrogenic a necrobiosis distinct from cell death in multiple human liver disease cells (Tang and Eisenbrand, 2013). The death was characterised by autophagy as proven by the buildup of LC3 II and autophagosomes, and therefore the formation of puncta GFP-LC3 (Malik *et al.*, 2021) (Ezhilarasan, Apoorva and Ashok, 2019) (Danda, Krishna, *et al.*, 2010) (Ramadurai *et al.*, 2019) (Sathivel *et al.*, 2008) (Panda *et al.*, 2016) (Neelakantan *et al.*, 2012) (Govindaraju, Neelakantan and Gutmann, 2017) (Sekhar, Narayanan and Baig, 2001) (DeSouza *et al.*, 2014) (Nasim *et al.*, 2010) (Danda, Muthusekhar, *et al.*, 2010)

(‘Molecular structure and vibrational spectra of 2,6-bis(benzylidene)cyclohexanone: A density functional theoretical study’, 2011) (Putchala *et al.*, 2013) (Neelakantan, Grotra and Sharma, 2013) (Suresh *et al.*, 2014). This autophagy similarly as toxicity caused by andrographolide (Dua *et al.*, 2019) may well be effectively prevented by 3-methyladenine (a chemical substance of autophagy) (M. Gomathi *et al.*, 2020).

Mechanistic study indicated that andrographolide induced autophagic necrobiosis by disruption of mitochondrial (Rajasekaran *et al.*, 2020) transmembrane potential and elevation of reactive element species (Malik *et al.*, 2021), that were related with mitochondrial porosity transition pore. Inhibition of cyclophilin D (a element of MPTP) by cyclosporin A or repeal of its expression by tiny meddlesome ribonucleic acid considerably suppressed the toxicity of andrographolide (Raj R, D and S, 2020), suggesting that cyclophilin D could play a very important role in mediating andrographolide-induced toxicity (Worakunphanich *et al.*, 2020) (Santhoshkumar *et al.*, 2019). Taken along, our findings unveil a unique mechanism of drug action by andrographolide in cancer of the liver cells (A. C. Gomathi *et al.*, 2020) and counsel that andrographolide could represent a promising novel agent within the treatment of cancer of the liver (Singh, 1999) (Saravanan *et al.*, 2018). The aim of this study is to evaluate apoptotic activity of *A. paniculata* in controlling hepatocellular carcinoma.

MATERIALS AND METHOD

Chemicals And Antibodies

DMEM medium, 0.25% Trypsin-EDTA solution, sodium bicarbonate solution, bovine serum albumin (BSA), low melting agarose, MTT from Sigma Chemicals Co., St. Louis, USA. fetal bovine serum (FBS) and antibiotic/antimycotic solution, DMSO were from Himedia, Sodium phosphate monobasic and dibasic, sodium chloride, sodium hydroxide, sodium carbonate, hydrochloric acid and methanol were purchased from Sisco Research Laboratories (SRL) India.

Cell Culture Reagents

DMEM

Commercially available DMEM contains 7.5% sodium bicarbonate solution. To 500ml of DMEM, 5ml of penicillin/streptomycin solution and 0.5ml of amphotericin B solution was added. Then the medium was sterile filtered (0.22 μ) inside the hood. The medium was then dispensed into sterile containers and stored at 4°C.

Growth Medium (DMEM with 10% FBS)

10ml of FBS was made up to 100ml using sterile DMEM. It was stored in a sterile container in cool and aseptic condition.

Phosphate Buffered Saline (PBS; pH 7.4)

0.63 g of sodium phosphate monobasic (NaH_2PO_4), 0.17 g of sodium phosphate dibasic (Na_2HPO_4) and 4.5 g of sodium chloride (NaCl) were dissolved in 500 ml of double autoclaved milliQ water. The pH was then adjusted to 7.4 using 1 N HCl and 1 N NaOH, sterile filtered (0.22 μ) and then stored in a sterile container.

Trypsin-EDTA

Trypsin was purchased as 1 x with EDTA (0.5% trypsin, 5.3 mM EDTA sodium salt). (Note: Freeze-thaw process does not affect the enzyme activity. Thawing is done at room temperature).

Cell Line Maintenance

Human breast cancer cell line was procured from the National Centre for Cell Science (NCCS, Pune), India. The cells were grown in T25 culture flasks containing DMEM medium supplemented with 10% FBS. Upon reaching confluence, the cells were detached using Trypsin-EDTA solution.

Passaging of Cells

Upon reaching the confluence cell line and were passed as follows.

The medium from the culture flask was aspirated. The flask was rinsed with 2ml of PBS and aspirated again quickly. 1.5-ml of trypsin-EDTA solution was added and incubated at 37°C for about 2 minutes until cells started detaching. As soon as the cells were detached, transfer the trypsinized medium containing cells using a transfer pipette into a 15 ml falcon tube and it was centrifuged at 1000 rpm for 5 min. The medium was carefully aspirated off and care was taken not to put the pipette tip in the bottom of the tube, where the cells were pelleted. The cells were gently resuspended in the DMEM medium with 10% FBS by pipetting up and down 5-8 times gently. From the cell suspension, a drop was placed on the edge of the coverslip of Neubauer hemocytometer. The drop was led to run under the cover slip by capillary action. Care was taken not to force the liquid and the entry of air bubble was avoided. Then the cells from the E1, E2, E3, E4 and E5 squares were counted under microscope. The cells were then gently resuspended and transferred to sterile culture flasks and the volume of medium was made up to required volume with growth medium per flask.

Cell Proliferation (MTT) Assay

The proliferation of MCF-7 cells was assessed by MTT assay Safadi et al., (2003).

Principle

The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. Only live cells are able to take up the tetrazolium salt (Ezhilarasan, Sokal and Najimi, 2018). The enzyme (mitochondrial dehydrogenase) present in the mitochondria of the live cells is able to convert internalized tetrazolium salt to formazan crystals, which are purple in colour. Then the cells are lysed using a 20% SDS solution, which releases the formazan crystal (Ezhilarasan, 2018). These crystals are solubilized by DMF present in the solubilizer. The colour developed is then determined in an ELISA reader at 620 nm.

Reagents

1. MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide]: 0.5 mg MTT/ml of serum-free DMEM.
2. Solubilization solution: Dimethyl sulfoxide (DMSO)
3. Phosphate Buffered Saline (PBS; pH 7.4).

Procedure

A549 and MCF-7 cells were plated in 48 well plates at a concentration of 2×10^4 cells/well 24 hours after plating, cells were washed twice with 500 μ l of serum-free medium and starved by incubating the cells in serum-free medium for 3 hours at 37°C (Raghunandhakumar *et al.*, 2013). After starvation, cells were treated with Compound Name different concentrations for 24 hours. At the end of treatment, the medium from control and Compound Name treated cells were discarded and 200 μ l of MTT containing DMEM (0.5 mg/ml) was added to each well. The cells were then incubated for 4h at 37°C in the CO₂ incubator (Asokkumar *et al.*, 2012).

The MTT containing medium was then discarded and the cells were washed with 1x PBS. The crystals were then dissolved by adding 200 μ l of solubilization solution and this was mixed properly by pipetting up and down. Then the formazan formed crystals were dissolved in dimethyl sulfoxide (200 μ l) and incubated in dark for an hour. Then the intensity of the color developed was assayed using a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed as the percentage of control cells cultured in serum-free medium. Cell viability in control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability = [A_{570 nm} of treated cells/A_{570 nm} of control cells] \times 100.

Morphology Study

Based on MTT assay we selected the optimal doses (---) for further studies. Analysis of cell morphology changes by a phase contrast microscope. 3×10^4 cells were seeded in 6 well plates and treated with Compound Name (concentration for MCF-7 cells) for 24h (Anandakumar *et al.*, 2012) (Ramesh *et al.*, 2018). At the end of the incubation

period, the medium was removed and cells were washed once with a phosphate buffer saline (PBS pH 7.4). The plates were observed under a phase contrast microscope.

ETHIDIUM BROMIDE/ACRIDINE ORANGE STAINING (AO/EB STAINING OR DUAL STAINING)

A549/HepG2 cells were plated at a density of 5×10^4 in 6 well plates containing sterile coverslips. They were allowed to grow at 37°C in a humidified CO₂ incubator until they are 70 - 80% confluent (Paramasivam *et al.*, 2015).

Then cells were treated with synthesized (50µM) for 24hrs. The culture medium was aspirated from each well and the cells were gently rinsed twice with PBS at room temperature (Anandakumar *et al.*, 2012). Then the coverslips were taken and kept on glass slides and stained with 100 µl of dye mixture (1:1 of EB and AO), immediately viewed under the fluorescence microscope.

Viable cells had green fluorescent nuclei with an organized structure. The early apoptotic cells had yellow chromatin in nuclei that were highly condensed or fragmented. Apoptotic cells also exhibited membrane blebbing (Ezhilarasan, Apoorva and Vardhan, 2018; Arumugam, George and Jayaseelan, 2021). The late apoptotic cells had orange chromatin with nuclei that were highly condensed and fragmented. The necrotic cells had bright orange chromatin in round nuclei. Only cells with yellow, condensed, or fragmented nuclei were counted as apoptotic cells in a blinded, non biased manner. (Duraismy *et al.*, 2019) For each sample, random 300 cells were observed in a fluorescent microscope (EVOS FL Cell Imaging System, Thermo Fisher Scientific, USA) and observed at x400 magnification. at least 500 cells/well and 4 wells/condition were counted, and the percentage of apoptotic cells was determined [% of apoptotic cells = (total number of apoptotic cells/total number of cells counted) x 100]. Dual acridine orange/ethidium bromide (AO/EB) staining method was repeated 3 times at least.

RESULTS

MTT assay : Figure 1

The cytotoxic effects of lycopene Ag NP on 3T3 cells. Cells were treated with Lycopene Ag NP (10, 20, 30, 50, 100 and 200 μ M) for 24 h, and cell viability was evaluated by MTT assay (Koka *et al.*, 2018). Data are shown as means \pm SD (n = 3). * compared with the control-blank group, p < 0.001.

AO/EB: Figure 2

Acridine orange/Ethidium bromide (AO/EtBr) staining of MDA-MB-231 cells after treatment of IC50 concentration of andrographolide for 24h and compared with untreated control cells using fluorescence microscopy (Gnanavel, Roopan and Rajeshkumar, 2019). Cells showing bright orange fluorescence indicate apoptosis in comparison to control cells showing green fluorescence.

Morphology: Figure 3

Assessment of cell morphology of MDA-MB-231 treated without or with andrographolide. Cells were treated with Lycopene Ag NP (100 μ M) for 24 h along with the control group. (Gnanavel, Roopan and Rajeshkumar, 2019; Markov *et al.*, 2021) Images were obtained using an inverted Phase contrast microscope..

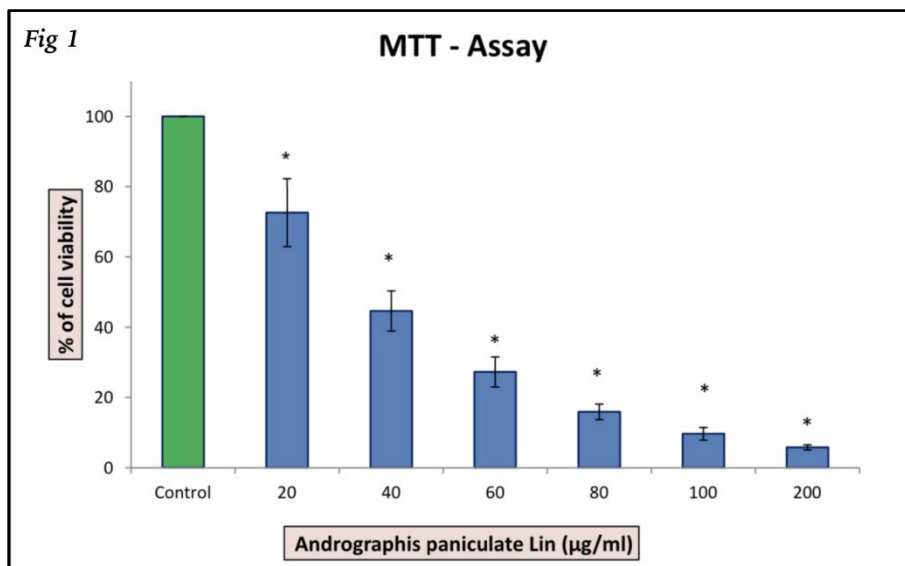


Figure 1: Bar graph represents the percentage of cell viability of HepG2 cells against *A. paniculata*, where x axis represents *Andrographis paniculata* and y axis represents percentage of cell viability. The control cell against *A.paniculata* showed 100% cell viability. MTT assay shows that when there is an increase in *A. paniculata* Lin, there is subsequent decrease in cell viability.

Fig 2

Control

Andrographis paniculata Lin (30µg/ml)

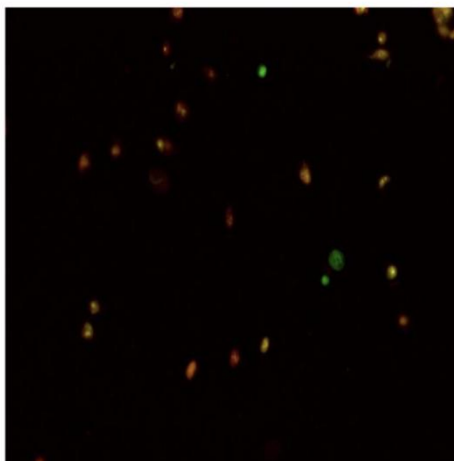
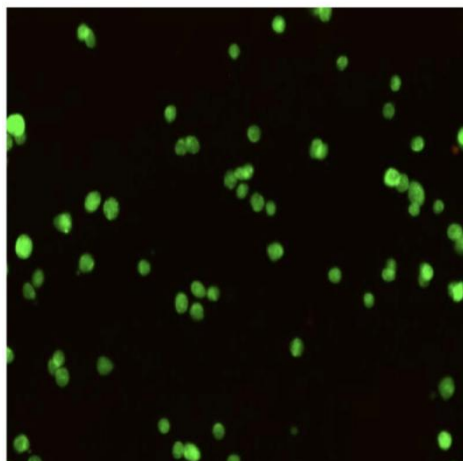


Figure 2: Acridine orange/Ethidium bromide (AO/EtBr) staining of HepG2 cells after treatment of IC₅₀ concentration of andrographolide for 24h and compared with untreated control cells using fluorescent microscopy. Cells showing bright orange fluorescence indicate apoptosis in comparison to control cells showing green fluorescence

Fig 3

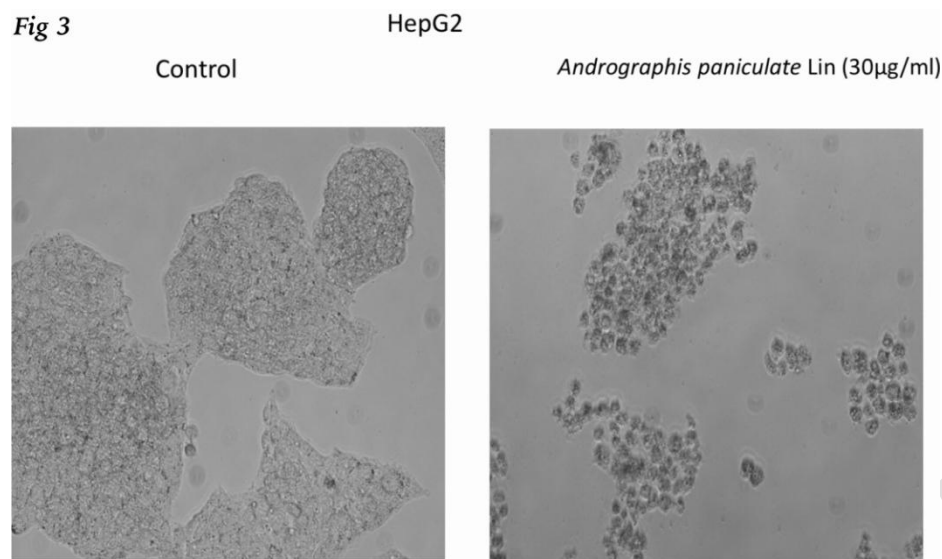


Figure 3: Morphological changes are visible between control and *Andrographis paniculata* Lin. It shows shrunken nucleus, chromatin and membrane blebbing seen in *Andrographis paniculata* Lin compared to that with viable cells.

Statistical Analysis

All data obtained were analyzed by Student's-t-test using MS-Excel, represented as mean \pm SD for triplicates. The results were computed statistically (SPSS/10 Software Package; SPSS Inc., Chicago, IL, USA) using one-way ANOVA. The level of statistical significance was set at $p < 0.05$.

DISCUSSION

Kumar et al. fractionated the methanol extract of *A. paniculata* into dichloromethane, petroleum ether and aqueous extracts and found that only the dichloromethane fraction significantly inhibited the proliferation of HT-29 colon cancer cells (Vairavel, Devaraj and Shanmugam, 2020). They further fractionated the dichloromethane extract and yielded three diterpene compounds, namely andrographolide, 14-deoxyandrographolide and 14-deoxy-11,12-didehydro andrographolide (Menon, S., Agarwal, H., Rajeshkumar, S., 2020). Andrographolide showed the greatest anti-cancer activity on a range of cancer cells. The *A. paniculata* ethanol extract showed cytotoxic activities against human

cancer cell lines(Raj R, D and S, 2020), such as Jurkat (lymphocytic), PC-3 (prostate), HepG2 (hepatoma) and Colon 205 (colonic) cells (Mohapatra *et al.*, 2020). An in vivo study demonstrated that *A. paniculata* 70% ethanol extract and andrographolide increased the life spans of mice injected with thymoma cells. Isolated from 85% ethanol extract of *A. paniculata*, andrographolide and isoandrographolide exhibited higher antiproliferative activities in human leukaemia HL-60 cells than other 16 ent-labdan diterpenoids with IC₅₀'s of 9.33 and 6.30 μ M(Barma, 2020).

In study conducted by Wei, we investigated the effects of andrographolide on human liver cancer cells and further examined the cell death mechanism(Barma, 2020; Vikneshan *et al.*, 2020). Our observations demonstrated that andrographolide-induced cytotoxicity was attributed to autophagy but not apoptosis in human liver cancer cells and that this autophagy-inducing activity was closely associated with the cyclophilin D-mediated mitochondrial permeability transition pore (MPTP)(Nandhini, Ezhilarasan and Rajeshkumar, 2020). The preclinical studies conducted by Karl suggest that andrographolide could be useful for the treatment of liver cancer(Vikneshan *et al.*, 2020). Andrographolide suppresses apoptosis of human umbilical vein endothelial cells (HUVECs) induced by growth factor deprivation via the activation of PI3/Akt pathway.

CONCLUSION

The three findings confirm that andrographolide induces apoptosis effectively in HepG2 human hepato cellular carcinoma cells in vitro(Rajendran *et al.*, 2019)(Ashok, Ajith and Sivanesan, 2017)(Malli *et al.*, 2019)(Mohan and Jagannathan, 2014)(Menon *et al.*, 2018)(Samuel, Acharya and Rao, 2020)(Praveen *et al.*, 2001)(Neelakantan *et al.*, 2011)(‘Oligonucleotide therapy: An emerging focus area for drug delivery in chronic inflammatory respiratory diseases’, 2019)(Kumar *et al.*, 2006). Taken together, our findings unveil a novel mechanism of drug action by andrographolide in liver cancer cells and suggest that andrographolide may represent a promising novel agent in the treatment of liver cancer. Further investigations revealed needed at pre-clinical and clinical levels for establishing it as a potential agent for cancer therapy.

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