Pharmacogn J. 2023; 15(4): 612-621
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Research Article

The Effects of Andrographolide on Apoptosis in PC-3 Cell Line *Via* the Involvement of Caspases 3, 8 And 9

Janany Manimaran, Daruliza Kernain Mohd Azman*

ABSTRACT

Andrographolide is a labdane diterpenoid derived from the Andrographis paniculata species. This compound has multiple therapeutic applications, including anti-cancer properties. Previous research has demonstrated the effects of andrographolide on several cancer cells, including lung, brain, colon, and breast cancer. However, due to a lack of studies, the anti-cancer actions of andrographolide on prostate cancer cells are poorly understood. In this present work, the effects of andrographolide were evaluated on PC-3 cells, an aggressive androgen-independent prostate cancer cell line. In drug development research, the analysis of cytotoxicity is essential for determining the biocompatibility of the drug employed on cancer cells. This study utilises the WST-1 assay to measure cell viability when testing different concentrations of andrographolide (0-200 µM) on the PC-3 cancer cell line and the Hs27 normal cell line. The results indicate that andrographolide inhibits PC-3 cell growth in a dose-dependent manner but does not affect Hs27 cells. According to the National Cancer Institute, the LC 50 result of 26.42 µM (after 48 hours of incubation) is within an acceptable range. In this investigation, three concentrations of andrographolide were utilised for the first time: control, half the LC 50, and the full LC 50 (0, 13.21, and 26.42 µM) in all other analyses. Metastasis is crucial for disease progression. Therefore, the effects of andrographolide on PC-3 cells were tested using a scratch assay and transwell invasion assay. The results demonstrated that andrographolide reduces both migration and invasion compared to the control, indicating its role in metastasis inhibition. The existence of a comet tail has shown that 26.42 µM andrographolide treatment causes the greatest damage to single-cell DNA, followed by 13.21 µM and no treatment. Additionally, the apoptosis rate was found to be highest in the 26.42 µM andrographolide treatment and then at 13.21 µM followed by the control. The apoptosis-regulating caspase activity of caspases 3 (executor caspase), 8 (intrinsic pathway), and 9 (extrinsic pathway) increased significantly. According to the new findings associated with the caspase storm scenario, the half LC 50 (13.21 µM) exhibited higher activity in those caspases compared to the LC 50 (26.42 µM). This allowed us to determine that 13.21 µM is the optimal range for caspase activation. In summary, the potential of andrographolide has been demonstrated through the modulation of caspase-mediated apoptosis, inhibition of metastasis, and induction of DNA damage to combat the progression of cancer in PC-3 cancer cells.

Key words: Andrographolide, PC-3 cell line, Anti-cancer, Apoptosis, Caspase.

Janany Manimaran, Daruliza Kernain Mohd Azman*

Institute for Research in Molecular Medicine, University of Science Malaysia, 11800 Gelugor, Pulau Pinang, MALAYSIA.

Correspondence

Daruliza Kernain Mohd Azman

Institute for Research in Molecular Medicine, University of Science Malaysia, 11800 Gelugor, Pulau Pinang, MALAYSIA.

E-mail: daruliza@usm.my

History

- Submission Date: 21-05-2023;
- Review completed: 18-06-2023.
- Accepted Date: 06-07-2023.

DOI : 10.5530/pj.2023.15.128

Article Available online

http://www.phcogj.com/v15/i4

INTRODUCTION

Cancer disease has been a significant global health issue for humans over time. The expansion of research into the concept of expanding available treatment options is remarkable. Patients are frequently diagnosed with lung cancer, breast cancer, prostate cancer, and colon cancer, among others.[1] This study focuses on prostate cancer because of its severity and the significant rise in male cases diagnosed in recent years. Although there are treatment options for prostate cancer, more research is required to improve the treatment's efficacy and decrease its side effects. Chemotherapy, radiotherapy, surgery, monoclonal antibody therapy, and targeted therapy are the current treatment options for prostate cancer.[2] In the present study, a type of targeted therapy is evaluated in the PC-3 prostate cancer cell line. The PC-3 cancer cell line is an androgen-independent, aggressive form of the prostate cancer cell line.

Targeted therapy is a treatment that employs a compound or drug to target specific molecules that contribute to the progression of cancer, resulting in molecularly targeted therapies.[3] Andrographolide is the compound of interest in this investigation. Andrographolide is derived from Nees, also known as Andrographis paniculata. This substance has been used in traditional medicine due to its numerous medicinal properties. Andrographolide's pharmaceutical benefits include anti-inflammatory, anti-viral, anti-microbial, and significant anti-cancer effects.[4] Numerous studies on the activity of andrographolide on various cancers, including lung cancer, breast cancer, colon cancer, and brain cancer, have been conducted.[5] However, more research is needed to clarify the anticancer effects of andrographolide against prostate cancer.

Several *in vitro* studies on this compound have been conducted to test its anticancer effects. Previous studies have reported that andrographolide induces apoptosis *via* inhibition of breast cancer's growth as well as possessing higher nuclear DNA damage.[6] In another study, the cervical and neuroblastoma cells showed significant tumour inhibition during andrographolide treatment *via* apoptosis induced by the Bax gene. Colorectal cancer cells have also exhibited downregulation of glycolysis, which causes disruption in the PI3K-AKT-mTOR pathway when they are treated with andrographolide.[7] Besides, andrographolide has also displayed its ability to combat the progression of human osteosarcoma cells

Cite this article: Manimara J, Azman DKM. The Effects of Andrographolide on Apoptosis in PC-3 Cell Line *Via* the Involvement of Caspases 3, 8 And 9. Pharmacogn J. 2023;15(4): 612-621.

as well as cervical cancer cells *via* the inhibition of cell proliferation, migration, and aoptosis.[8,9]

In the present study, the andrographolide compound was evaluated against the PC-3 cancer cell line. This is to increase knowledge of andrographolide's effectiveness as a prostate cancer progression inhibitor. This study emphasised the use of three different concentrations of andrographolide, including the half-LC50 value, which has never been done with this compound. The purpose of this experiment is to determine whether a lower concentration is capable of exhibiting comparable inhibition activity on the PC-3 cancer cell line. In addition, metastasis is a significant factor in the progression of disease; therefore, it has been studied using scratch assays and transwell invasion assays. Besides, caspase-mediated apoptosis is an important pathway that could be utilised to combat the progression of this disease. According to previous research, the activity of several caspases, including caspases 3, 8, and 9, has been found to be downregulated in prostate cancer cells.[10] In addition, the involvement of caspases 8 and 9 indicates that both extrinsic and intrinsic pathways leading to apoptosis have been disrupted.[11] Thus, the caspase assay is used to determine the activity of caspase in PC-3 cancer cells following andrographolide treatment. Additionally, cytotoxicity analysis and the detection of DNA damage in single cells have been conducted.

MATERIALS AND METHODS

Materials

List of reagents, list of equipment and list of cell lines are shown in tables 1 and 3.

Phosphate Buffer Saline (PBS): One-time PBS (Thermo Fisher Scientific, USA) was prepared by dissolving one tablet in 500mL of distilled water (dH2O) for cell culture work.

Complete media: Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, USA) was supplemented with 10 % of Fetal Bovine Serum (FBS; Thermo Fisher Scientific, USA) and 1% of Penicillin-Streptomycin (Nacalai Tesque, Japan).

Fixation solution: Fixation solution was prepared in the ratio of 1:7 (acetic acid: methanol) by combining 10 ml of acetic acid and 70 ml of methanol to obtain a total volume of 80 ml**.**

Lysis solution: The preparation of lysis solution was done by mingling the 30 mL lysis solution (TRIVEGEN, Bio-Techne: USA) together with 3 mL of DMSO.

Staining solution: Staining solution with the ratio of 0.5% of crystal violet to 25 % methanol was prepared by dissolving 0.25 g of crystal violet with 12.5 mL of DMSO.

Alkaline Unwinding Solution (AUS): Alkaline Unwinding Solution (AUS; 200 mM of NaOH, 1 Mm EDTA), pH > 13, was conditioned by mixing 0.4 g of NaOH pellets in 250 µl of 200 mM EDTA (TRIVEGEN, Bio-Techne: USA) 49.75 µl of dH2O.

Alkaline electrophoresis solution: Alkaline Electrophoresis Solution (AES; 200 mM NaOH, 1 mM EDTA), pH > 13 was prepared by dissolving 16 g of NaOH pellets with 4 mL of 500 mM EDTA, pH 8 and dH2O is added 1L is reached.

Ethidium bromide: Ethidium Bromide (EtBr; 20 µg/mL, stock solution) was prepared by mixing 28 µl of stock EtBr in 336 µl of DMSO.

Andrographolide: Andrographolide–98% (Sigma Aldrich (USA)) was prepared by dissolving 0.354 g of Andrographolide stock solution in DMSO to achieve a 100 mM concentration. Later, this solution was prepared by dissolving in the normal medium according to the concentration required.

Methods

Cell culture and treatment: PC-3 cells were cultured in DMEM medium (ATCC, Manassas, VA) supplemented with 10% US-qualified fetal bovine serum (FBS) (Invitrogen, Grand Island, NY). RWPE-

Table 1: List of reagents.

Reagents	Manufacturer	Catalog Number
Gibco™ Phosphate Buffer Saline (PBS)	Thermo Fisher Scientific (USA)	18912-014
Gibco™ Fetal Bovine Serum (FBS)	Thermo Fisher Scientific (USA)	A4766801
Gibco™ Dulbecco's Modified Eagle's Medium	Thermo Fisher Scientific (USA)	11885084
Dimethyl Sulfide (DMSO)	Sigma Aldrich (USA)	D8418
Andrographolide – 98 %	Sigma Aldrich (USA)	365645
Cell Proliferation Reagent WST-1	Roche Diagnostics	5015944001
EDTA (200 mM)	TREVIGEN, Bio-Techne (USA)	4250-050-K
Lysis Solution	TREVIGEN, Bio-Techne (USA)	4250-050-K
Comet Slide	TREVIGEN, Bio-Techne (USA)	4250-050-K
Penicillin-Streptomycin	Thermo Fisher Scientific (USA)	15140122
Trypsin	Thermo Fisher Scientific (USA)	25200056

Table 2: List of equipment.

Equipment	Brand	Models; Serial Numbers
Inverted Microscope	Meiji Techno. Binocular Inverted Brightfield Biological Microscope (Japan)	Meiji TC5100; 1035
Electrophoresis unit tank	Thermo Scientific; Owl ™ Easy Cast B1 Mini Gel Electrophoresis Systems (USA)	7309;170615-1781
High speed centrifuge	Sigma 3-16 KL (Germany)	3-16PK; 133579
Incubator	NuAire US Autoflow Automatic CO_2 Incubator (USA)	NU-4750E; 132274081109
Class II, Biological Safety Cabinet	NuAire LabGard ES Class II Biological Safety Cabinet	NU-425-400E; 125081011210
Micro-plate reader spectrophotometer	Thermo Scientific Multiskan Spectrum (USA)	NP-BP1KOE; 639B01894
Fluoroscence Microscope	ZEISS Axio Observer 1 Inverted Phase Contrast Fluorescence Microscope (Germany)	Axio Observer Z1; 3834002524

Table 3: **List of cell lines.**

Name of cell line	Origin / Cell Type	Supplier	Product ID	Media
PC-3	Prostate epithelial cancer cell	American Type Culture Collection (USA)	CRL-14345	DMEM
Hs27	Foreskin/Normal human fibroblast cell	American Type Culture Collection (USA)	CRL-1634	DMEM

Table 4: **The (A) half-maximal lethal concentration (LC 50) values for PC-3 and RWPE-1 cells after treated with andrographolide (B) the half maximal lethal concentration (LC 50) values for PC-3 cells after treated with docetaxel were estimated using nonlinear regression approximations in GraphPad Prism 8.0.1 (GraphPad software, Inc, San Diego, CA).**

Cell Line	[Andrographolide] LC 50 values		
	24 hours	**48 hours**	**72 hours**
PC-3	64.98 ± 9.51 µm	26.42 ± 1.52 µm	63.68 ± 2.16 µm
HS 27	77.20 ± 11.04 µm	85.63 ± 14.38 µm	83.10 ± 21.80 µm
	[Docataxel] LC 50 values		
PC-3	50.78 ± 4.99 µm	11.06 ± 0.087 µm	9.569 ± 0.521 µm

Table 5: **Shows the cell migration of PC3 cells treated with different concentration of andrographolide: (a), (d) 0 µM (as the control), (b), (e) 13.2 µM (half LC 50 value) and (c), (f) 26.4 µM (LC 50 value). Analysis was done using image J software.**

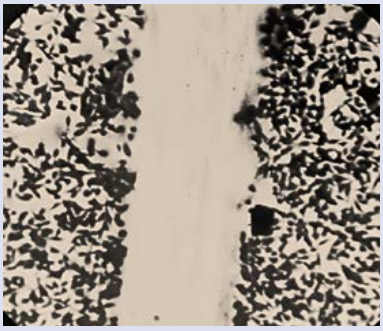
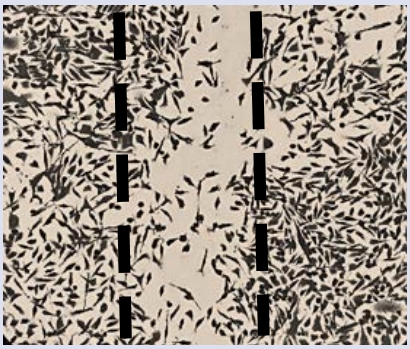
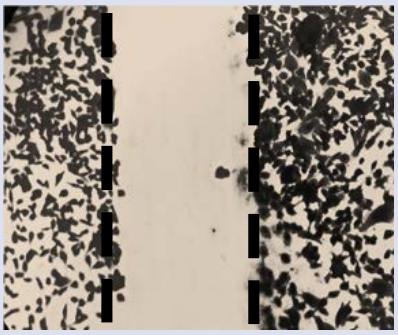
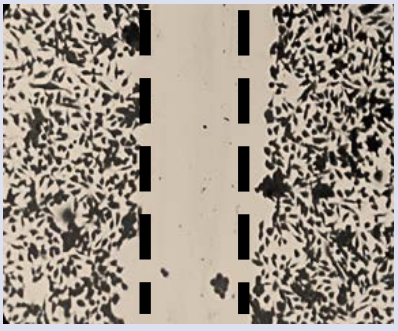
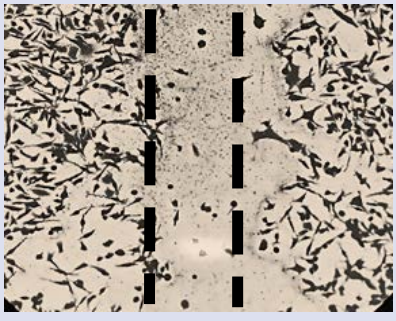
Before Incubation	After Incubation (48 hours)
 (a)	 (d)
 (b)	 (e)
 (c)	 (f)

Table 6: Shows the cell invasion of PC3 cells treated with different concentration of andrographolide: (a) 0 µM (as the control), (b) 13.2 µM (half LC 50 value) and (c) 26.4 µM (LC 50 value). Analysis was done using image J software.

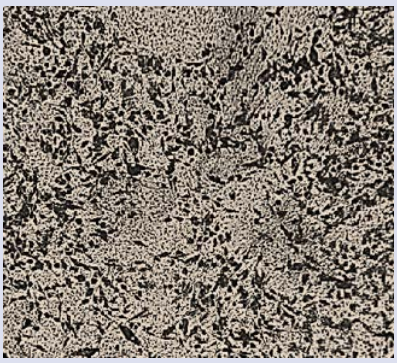
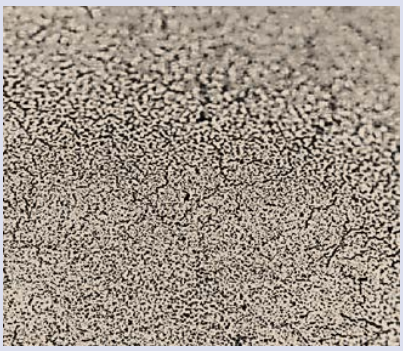
INVADED CELL UNDER PHASE CONTRAST MICROSCOPE
(a) 
(b) 
(c) 

Table 7: Shows the visualization of cells stained with Ethidium Bromide, after 48 hours of treatment with different concentration under fluorescent microscope.

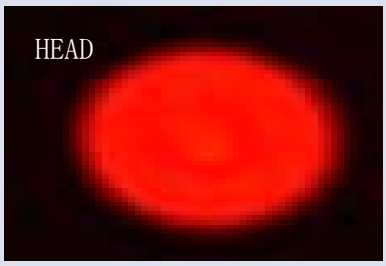
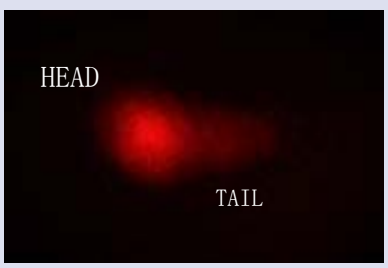
PC-3 cell without andrographolide treatment	
PC-3 cell after andrographolide treatment	

Table 8: Shows the details of alkaline comet assay analysis on prostatic cancer cell line PC3 with various concentrations of andrographolide which is Control (0 µM), Half LC 50, (13.21 µM) and LC 50 (26.42 µM) obtained from CaspLab software.

GROUP PARAMETER	n	0 µM	13.21 µM	26.42 µM
HeadArea	50	581.66	2046.31	1024.8
TailArea	50	22.66	390.82	588.08
HeadDNA	50	98.25	171.88	78.92
TailDNA	50	0.83	12.33	24.08
HeadDNA%	50	99.15	93.40	77.84
TailDNA%	50	0.71	6.60	22.16
HeadRadius	50	13.18	21.65	16.14
TailLength	50	3.16	8.47	16.16
CometLength	50	30.52	52.31	49.43
HeadMeanX	50	36.49	57.63	52.26
TailMeanX	50	50.67	77.01	70.23
TailMoment	50	0.02	0.67	5.52
OliveTailMoment	50	0.11	1.30	4.55

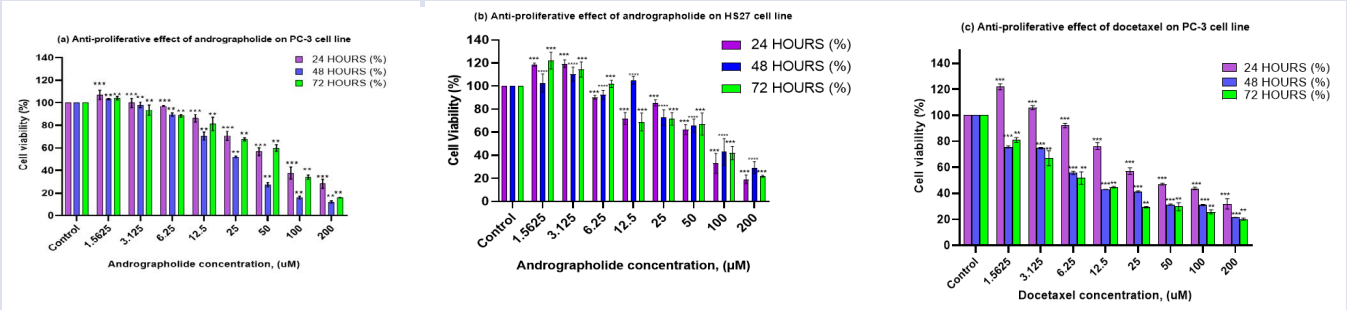


Figure 1: The bar graph above illustrates the percentage of cell viability of PC-3 cells treated with different concentration of andrographolide (0-200 µM) on (a) PC-3 cell line, (b) HS 27 cell line, for three different timelines which are 24 hours, 48 hours and 72 hours and (c) illustrates the percentage of cell viability of PC-3 cells treated with different concentration of docetaxel (0-200 µM) on PC-3 cell line, for three different timelines which are 24 hours, 48 hours and 72 hours . The results were analyzed using the GraphPad Prism 8.0.1 (GraphPad software, Inc, San Diego, CA). Data above was expressed as mean ± SEM from 3 independent experiments. ****P < 0.00001 as compared to control, ***P < 0.0001 as compared to control. **P < 0.001 as compared to control.

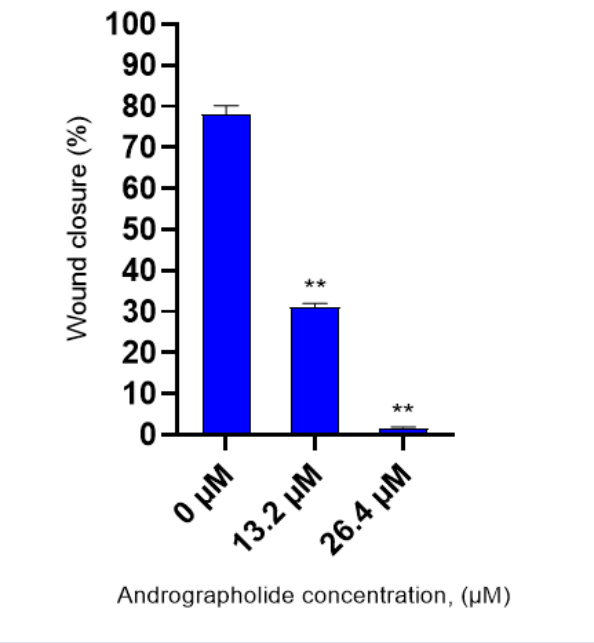


Figure 2: Shows the bar chart for migration rate of PC3 cells treated with different concentration of andrographolide, 0 µM (as the control), 13.2 µM (half LC 50 value) and 26.4 µM (LC 50 value). The results were analyzed using the GraphPad Prism 8.0.1 (GraphPad software, Inc, San Diego, CA). Data above was expressed as mean ± SEM from 3 independent experiments. **P < 0.01 as compared to control.

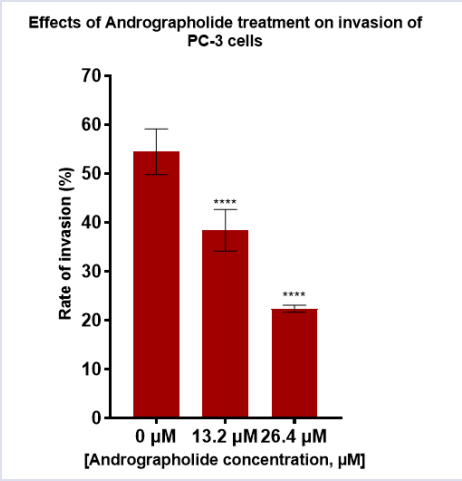


Figure 3: Shows the bar chart for invasion rate of PC3 cells treated with different concentration of andrographolide, 0 µM (as the control), 13.2 µM (half LC 50 value) and 26.4 µM (LC 50 value). The results were analyzed using the GraphPad Prism 8.0.1 (GraphPad software, Inc, San Diego, CA). Data above was expressed as mean ± SEM from 3 independent experiments. ****P < 0.0001 as compared to control.

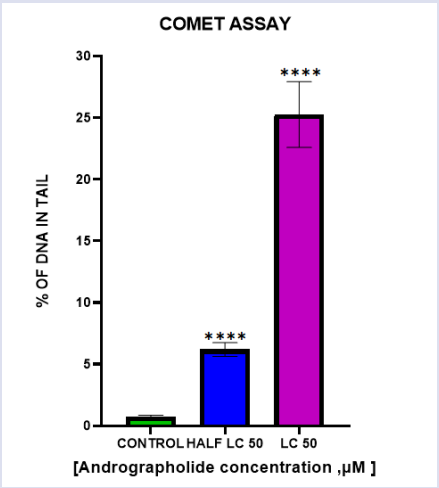


Figure 4: Shows the bar chart for percentage of DNA in tails of PC3 cells treated with different concentration of andrographolide, 0 µM (as the control), 13.2 µM (half LC 50 value) and 26.4 µM (LC 50 value). The results were analyzed using the GraphPad Prism 8.0.1 (GraphPad software, Inc, San Diego, CA). Data above was expressed as mean ± SEM from 3 independent experiments. ****P < 0.0001 as compared to control.

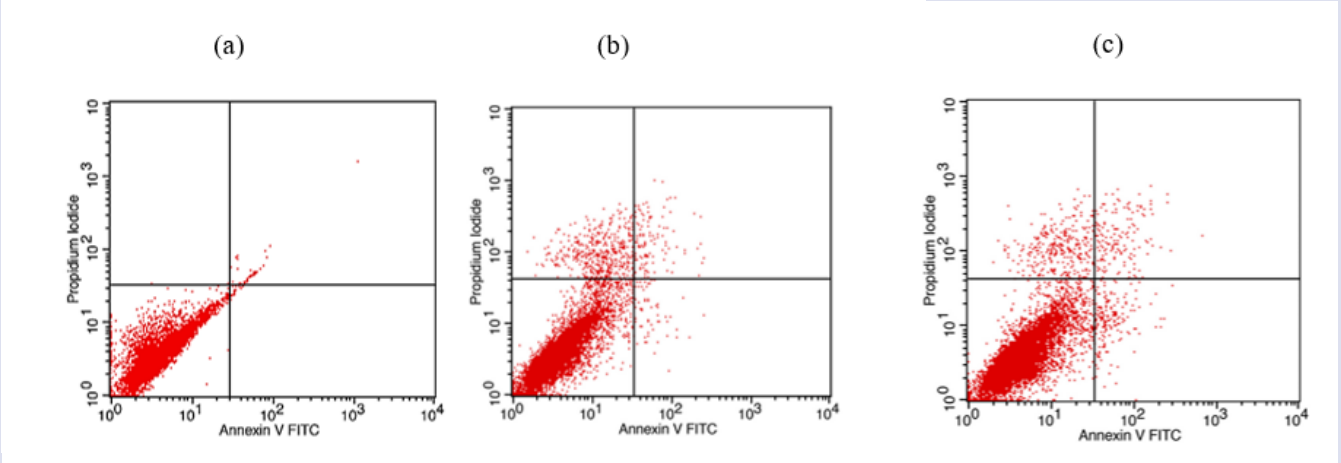


Figure 5: Shows scatter plot obtained from apoptosis analysis in PC3 cells treated with different concentration of andrographolide, (a) 0 µM (as the control), (b) 13.2 µM (half LC 50 value) and (c) 26.4 µM (LC 50 value).

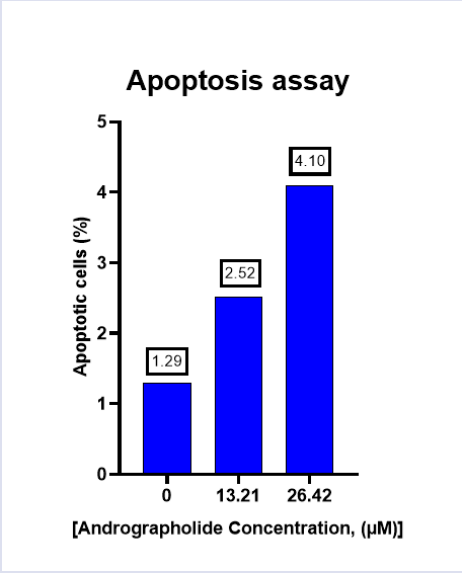


Figure 6: Shows the bar chart of the rate of apoptosis in PC3 cells treated with different concentration of andrographolide, 0 µM (as the control), 13.2 µM (half LC 50 value) and 26.4 µM (LC 50 value).

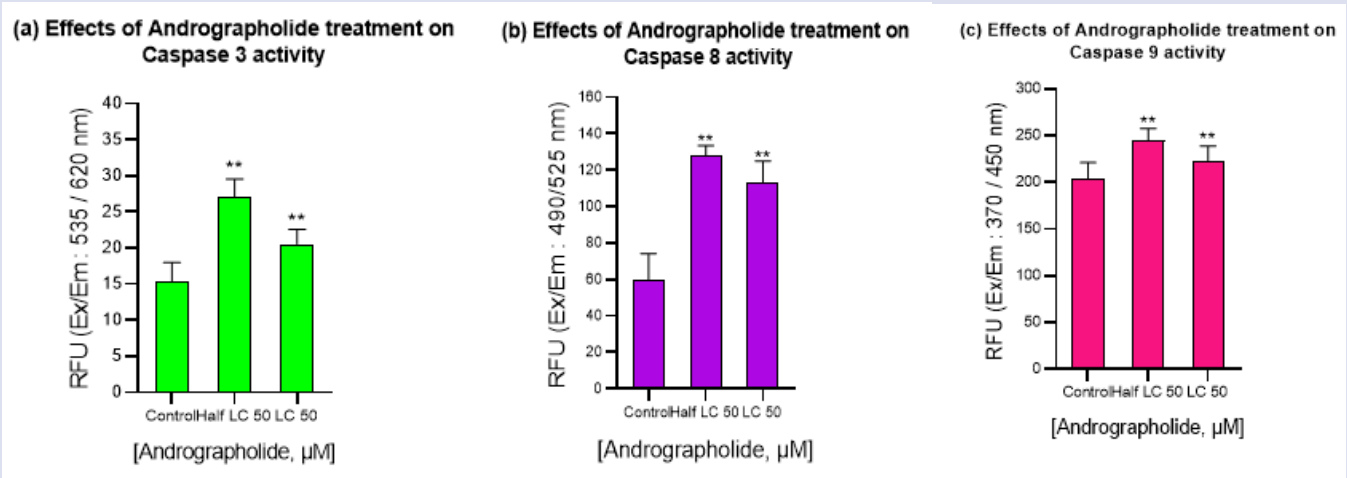


Figure 7: Shows the bar chart of (a) caspase 3 (b) caspase 8 and (c) caspase 9 activity in PC-3 cells treated with different concentration of andrographolide, 0 µM (as the control), 13.2 µM (half LC 50 value) and 26.4 µM (LC 50 value). The results were analyzed using the GraphPad Prism 8.0.1 (GraphPad software, Inc, San Diego, CA). Data above was expressed as mean ± SEM from 3 independent experiments.

1 cells were cultured in Eagle's minimum essential medium (ATCC, Manassas, VA) supplemented with 10% US-qualified fetal bovine serum. All cells were cultured in a cell culture incubator with 5% CO2 at 37°C. Andrographolide was dissolved in dimethyl sulfoxide (DMSO) to make stock solutions of 100 mM.

Anti-cell proliferation assay: Effects of andrographolide on prostate cancer cells (PC-3) viability were determined with a "(WST-1 Assay, Roche). Cells (1 x 104 cells / well) were seeded into 96-well plates and incubated overnight before being treated with 0 to 200 µM andrographolide in triplicates for 24 h, 48 hr and 72 hr with DMSO as solvent control. After removing the medium, cells were washed with phosphate buffered saline (PBS), and then 10µl of WST-1 was added into the wells that contain treated cells. Cells were incubated at 37°C for 4 h according to manufacturer's instructions and measured for optical density (OD) values at 540 nm. Cell viability was expressed as a percentage of control from three independent experiments.

Scratch assay: Cells were plated in 6-well plate and allowed to form a confluent monolayer. The cell-surface was scratched using 1000 µl pipette tip. Cells were allowed to fill the scratched area and monitored over the course of 48 hours. Images were taken using a microscope. Cell migration was quantified by using the wound closure formula measuring the distance between the migrating cells.

Transwell invasion assay: Invasion assay has been carried out using transwell assay method. Transwell chambers with polycarbonate membranes (8-µm pores, Costar, CA) in 24-well culture plates were coated with Matrigel diluted in serum-free Dulbecco's modified Eagle's medium (DMEM) and incubated at 37 °C overnight. A 300-µL suspension of 60000 cells with andrographolide treatment layered to the upper compartment of the transwell chambers and cultured for a further 48 h at 37 °C with serum-free DMEM. Then, the cells were motile and invaded through the Matrigel membrane into the lower chamber. The medium from the lower chambers are removed and the

cells are fixed and permeabilized using cold methanol. The methanol is allowed to incubate for 10 minutes before removing by manual aspiration. Later, 500 µl of diluted crystal violet is added for staining purpose and incubated for 30 minutes. The inserts are then washed and allowed to dry before quantification.

Quantification of invaded cells: The 400 µl of acetic acid was diluted and added into the insert. The eluent from the lower chamber was transferred to 96-well clear microplate and the absorbance at 590 nm was measured.

Comet assay: The neutral comet assay was performed according to manufacturer protocol (Trevigen #4250-050-K) followed by the staining using ethidium bromide staining. In detail, after the treatment of andrographolide on PC-3 cells, the cells are placed one agarose gels on comet slides and used for single-cell electrophoresis and DNA precipitation. Later, the gel slides were stained using diluted ethidium bromide for better observation under fluorescence microscope. The slides were observed immediately after staining for efficient results. The comet tail and head percentage of the cells are analysed using CasP analyser.

Apoptosis assay: PC-3 cells are treated with andrographolide concentration before the apoptosis assay is carried out. The cells are then centrifuged to obtain cell pellet which is resuspended with 1 X Annexin Binding Buffer (AVBB). The cell suspension is transferred to round bottom culture tube followed by the addition of 5 ul of Annexin V-Fitc and 5 ul of PI staining in the tube. The tubes are gently mixed followed by the incubation in the dark at room temperature for 15 minutes. Then, the AVBB is added into the tubes to stop the reaction and kept on ice followed by the apoptosis analysis using flow cytometer.

Caspase assay: The cells are seeded in 96-well plate at the confluency of 5000 cells/well. The PC-3 cells are allowed to incubate overnight before treated with andrographolide concentration. After 48 hours of incubation of andrographolide treated cells, the activity of caspases 3,8 and 9, is measured using the Caspase 3, 8 and 9 Multiplex Activity Assay Kit. Add 100 µL/well of Caspase assay loading solution without removing media containing andrographolide treatment. The plate is incubated at room temperature 30-60 minutes, protected from light. The fluorescence is identified using the fluorescence microplate reader at the specific wavelengths; Caspase 3: Ex/Em = 535/620 nm (red), Caspase 8: Ex/Em = 490/525 nm (green) and Caspase 9: Ex/Em = 370/450 nm (blue). The bar graph of fold change between the treated and control cells are plotted.

RESULTS

Andrographolide reduces the viability of cells in PC-3 cells

The anti-proliferative ability of the prostate cancer cells has been evaluated in this study using the PC-3, which was then treated with various concentrations of andrographolide treatment ranging from 200 uM to 3.125 uM. The effects of treatment have been evaluated within three different timelines, which include 24 hours, 48 hours, and 72 hours. The PC-3 cells showed a positive concentration-dependent activity upon the treatment of andrographolide for all three timelines. The highest anti-proliferative activity was observed at the highest treatment concentration, which is 200 uM. However, the effect of andrographolide was not time-dependent on PC-3 cells, in which higher anti-proliferative activity has been recorded at 48 hours compared to 72 hours of treatment incubation. Hence, the viability of the cells was lowest during the 48-hour incubation of PC-3 cells with andrographolide treatment. The LC50 value calculated in this study indicates 26.42 uM at 48 hours of andrograpolide treatment. The cytotoxicity of andrographolide was tested on the HS 27 cell line in order to determine the effect of andrographolide on normal cells. The results showed that the andrographolide has a very low effect on HS 27 cells, indicating that the andrographolide compound is not toxic to normal cells.

Andrographolide suppresses the PC-3 cells to undergo angiogenesis

The Scratch assay was used to determine the migration ability of PC-3 cells, and the results showed that andrographolide is capable of inhibiting the migration of PC-3 cells. Andrographolide showed a higher inhibitoron rate in the migration of PC-3 when the cells are treated for 48 hours with the LC50 concentration. The PC-3 cells treated with 26.42 Um (LC50 concentration) showed a higher inhibition rate compared to 13.21 uM (half LC50 concentration) followed by 0 Um (served as control). The rate was determined according to the wound closure rate in which it was the highest for the LC 50 concentration of andrographolide, which is approximately 1.56 % compared to cells not treated with andrographolide, which is about 76.6%. In addition, similar results were obtained when the invasion activity of andrographolide was measured using the Transwell assay. The rate of invasion has been reduced by up to 50% after the PC-3 cells were treated with andrographolide.

Andrographolide induces DNA damage in PC-3 cells

The prostate cancer cells were treated with andrographolide, which was followed by an evaluation of the DNA damage in the cells after the treatment. The Comet assay was used to determine the percentage of DNA damage through the calculation of the tail percentage present in the cells after andrographolide treatment. The comet assay results indicate that the highest percentage of tail observed was in PC-3 cells treated with 26.42 Um of andrographolide compared to 13.21 Um. The DNA damage observed in andrographolide treated cells was more than 100% in both the concentrations compared to control. Hereby, it is deduced that andrographolide is a strong inducer of DNA damage in prostate cancer cells.

Andrographolide increases the rate of apoptosis in PC-3 cells

The ability of andrographolide to escalate the apoptosis rate has been tested using the flow cytometry method. The cells were stained with Annexin V-FITC and Propidium Iodide after the treatment of andrographolide. It is observed that the andrographolide treated cells have shown an increase of about 50% more than cells without treatment of andrographolide. The Q2 & Q4 quadrants have a higher apoptotic rate in andrographolide treated cells, indicating the rise in early and late apoptosis of the cells.

Activity of caspases on PC-3 cells after andrographolide treatment

The activities of three main caspases have been determined after the andrographolide treatment. The caspase activity for all three caspases is higher in andrographolide-treated cells. However, the caspase activity is observed to be higher in the lower concentration of andrographolide, which is 13.21 compared to the LC 50 concentration of 26.42. These results prove that apoptosis is induced through an increase in caspase 3, 8, and 9 activity. Caspase 8 and 9 function as initiator caspases, and caspase 9's role is in the execution of the apoptosis phenomenon. The increase in caspase activity is correlated to the ability of andrographolide to induce a higher rate of apoptosis in prostate cancer cells.

DISCUSSION

Numerous natural compounds are tested against various cancer cells to determine their potential for suppressing and rectifying this

disease. Natural compounds are used in regards to widening the therapeutic window as well as providing a better treatment option through an herbal approach. Cytotoxicity studies have been done *via* the WST-1 assay. The WST-1 assay is a type of cell proliferation assay that is used for drug screening to determine if the test molecules (Andrographolide) have effects on cell proliferation or exhibit direct cytotoxic effects on the cell lines under analysis.[12] Andrographolide clearly inhibits PC-3 cell line proliferation. This rate of inhibition of PC-3 cell lines increases as the andrographolide concentration rises. This emphasises the dose-dependent role of andrographolide in PC-3 cell proliferation. The ability of andrographolide to inhibit cell proliferation in a dose-dependent manner has been examined in a variety of cancer cell lines, including HCT116 colorectal cancer cells, with similar results.[13] In addition to that, the normal cells, the Hs27 cell line, exhibit no significant effect in cell viability when tested with the desired concentration of andrographolide.

Cancer metastasis to bone remains one of the crucial contributors to the mortality rate in prostate cancer. It is important for the treatment to significantly reduce the metastasis in order to be helpful in the inhibition of cancer progression.[14] The Scratch Assay is used to identify the ability of PC-3 cells upon andrographolide treatment to migrate as determined by their ability to fill in a scratched or wounded area.[15] The phase contrast microscope images demonstrate that as the concentration of andro-grapholide rises, the number of cells in the injured area decreases dramatically. In addition, the image of the LC 50 concentration of andrographolide treatment reveals a significant reduction in the non-wounded area's cell confluence. In a previous study, andrographolide was shown to inhibit the migration of human glioblastoma cells.[16] Similar results were obtained when the rate of invasion of PC-3 cells was analysed after treatment with andrographolide. The Transwell Invasion Assay is utilised to determine the invading capacity of cells by depicting their ability to invade through a Matrigel-formed polycarbonate membrane.[17] The phase contrast microscope images demonstrate that as the concentration of andro-grapholide rises, the number of cells that have invaded the membrane falls. The population of invaded cells visible in the image is much smaller than the population of cells that manage to invade without treatment. Additionally, a previous study found that andrographolide inhibited the migration and invasion of colorectal cancer cells by reducing MMP-7 gene expression.[18]

The injury or damage that occurs in DNA is critical for the prevention of cancer development. The DNA damage attracts the immune cells *via* the signals emitted, which identify the cancer present.[19] The current study, which used a comet assay to test for DNA damage, found that andrographolide has a positive effect on DNA damage. The comet tails are observed to be higher in andrographolide treated PC-3 cells. This serves as evidence to support previous studies on the ability of andrographolide to induce DNA damage in the PC-3 cell line. Wang has reported that andrographolide induces DNA damage in human esophageal cancer.[20] DNA damage is related to the apoptosis incidence in a cell. The suppression of programmed cell death, also known as apoptosis, is essential for the progress of cancer. The apoptotic activity of PC-3 cells treated with andrographolide is increased. According to the findings, andrographolide increases the rate of apoptosis in PC-3 cells by 50%. Previous research indicates that andrographolide increases the rate of apoptosis in prostate cells by about 50%.[21] The higher apoptotic rates of Q2 and Q4 in andrographolide-treated cells indicate an increase in early and late apoptosis. This result suggests that the rate of apoptosis of PC-3 cells increases proportionally to their concentration. Apoptosis is mediated in cells through various pathways, which include caspases. This study emphasised caspase activity due to the nature of caspase that has been downregulated in prostate cancer and has served as a hall maker.[10]

To better comprehend the role of caspases in PC-3 cells, a caspase assay focusing on caspases 3, 8, and 9 was conducted. Caspase 3 is an executor caspase, whereas caspases 8 and 9 are initiator caspases in the extrinsic and intrinsic pathways, respectively.[22] The results confirmed the upregulation of all three caspases following andrographolide treatment of cells, as well as the role of andrographolide in inducing apoptosis *via* both the intrinsic and extrinsic pathways. In this study, the treatment of andrographolide on the PC-3 cell line has given insight into the ability of andrographolide to specifically target the hallmakers of prostate cancer by upregulating caspases. The higher activity of caspases in the presence of a lower concentration of andrographolide is supported by the "storm-like effect" observed in a previous study. As lower concentrations are closer to the optimal range, they exhibit greater caspase activity. Since higher concentrations exceed the optimal range, it can be concluded that they exhibit less caspase activity.[23]

CONCLUSION

The emerging studies on natural compounds and their pharmaceutical benefits have brought significant advancements in the drug discovery field. Andrographolide is one of the compounds that has exhibited its medicinal benefits in combating various disease progression. Hence, in this study, the anti-cancer properties of andrographolide are tested on an aggressive prostate cancer cell line, which is the PC-3 cell line. The ability of andrographolide to inhibit the progression of the PC-3 cell line is clearly demonstrated *via* several pathways such as migration, invasion, DNA damage, and also apoptosis. In addition, the involvement of caspase 3, 8, and 9 in aiding apoptosis has also shown significant elevation after the andrographolide treatment. This finding suggests that andrographolide has the potential to be developed into an anti-cancer drug.

FUNDING

This research and the APC was funded by FRGS, grant number "203/CIPPM/6711721".

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Ferlay J, Colombet M, Soerjomataram I, Parkin DM, Piñeros M, Znaor A, *et al.* Cancer statistics for the year 2020: An overview. Int J Cancer. 2021;149(4):778-89.
2. Culp MB, Soerjomataram I, Efstathiou JA, Bray F, Jemal A. Recent global patterns in prostate cancer incidence and mortality rates. Europ Urol. 2020;77(1):38-52.
3. Tsimberidou AM. Targeted therapy in cancer. Cancer Chemother Pharmacol. 2015;76(6):1113-32.
4. Mussard E, Cesaro A, Lespessailles E, Legrain B, Berteina-Raboin S, Toumi H. Andrographolide, a natural antioxidant: an update. Antioxidants. 2019;8(12):571.
5. Parveen R, Ahmad FJ, Iqbal Z, Samim M, Ahmad S. Solid lipid nanoparticles of anticancer drug andrographolide: formulation, in vitro and in vivo studies. Drug Dev Ind Pharm. 2014;40(9):1206-12.
6. Banerjee M, Chattopadhyay S, Choudhuri T, Bera R, Kumar S, Chakraborty B, *et al.* Cytotoxicity and cell cycle arrest induced by andrographolide lead to programmed cell death of MDA-MB-231 breast cancer cell line. J Biomed Sci. 2016;23(1):1-17.
7. Loh SH, Tsai YT, Huang SF, Yu TC, Kuo PC, Chao SC, *et al.* Effects of Andrographolide on Intracellular pH Regulation, Cellular Migration, and Apoptosis in Human Cervical Cancer Cells (Running Tittle: Effects of Andrographolide on pH Regulators and Apoptosis in Cervical Cancer). Cancers. 2020;12(2):387.

8. Sanati P, Chua LS, Nasiri R, Hashemi SS. Nanoencapsulation of andrographolide rich extract for the inhibition of cervical and neuroblastoma cancer cells. J Biomed Nanotechnol. 2020;16(9):1370-80.
9. Li Y, Zhang P, Qiu F, Chen L, Miao C, Li J, *et al*. Inactivation of PI3K/Akt signaling mediates proliferation inhibition and G2/M phase arrest induced by andrographolide in human glioblastoma cells. Life Sci. 2012;90(25-26):962-7.
10. Winter RN, Kramer A, Borkowski A, Kyprianou N. Loss of caspase-1 and caspase-3 protein expression in human prostate cancer. Cancer Res. 2001;61(3):1227-32.
11. Hongmei Z. Extrinsic and intrinsic apoptosis signal pathway review. In Apoptosis and medicine. InTechOpen. 2012.
12. Hodroj MH, Jardaly A, Abi Raad S, Zouein A, Rizk S. Andrographolide potentiates the antitumor effect of topotecan in acute myeloid leukemia cells through an intrinsic apoptotic pathway. Cancer Manage Res. 2018;10(1):1079.
13. Chowdhury KD, Sarkar A, Chatterjee S, Patra D, Sengupta D, Banerjee S, *et al*. Cathepsin B mediated scramblase activation triggers cytotoxicity and cell cycle arrest by andrographolide to overcome cellular resistance in cisplatin resistant human hepatocellular carcinoma HepG2 cells. Environ Toxicol Pharmacol. 2019;68:120-32.
14. Yücel B, Bahar S, Kaçan T, Şeker M, Celasun M, Bahçeci A. Importance of metastasis site in survival of patients with breast cancer. Austin J Med Oncol. 2014;1(2):7.
15. Dai L, Wang G, Pan W. Andrographolide inhibits proliferation and metastasis of SGC7901 gastric cancer cells. BioMed Res Int. 2017;2017:6242103.
16. Chiu SP, Batsaikhan B, Huang HM, Wang JY. Application of electric cell-substrate impedance sensing to investigate the cytotoxic effects of andrographolide on U-87 MG glioblastoma cell migration and apoptosis. Sensors. 2019;19(10):2275.
17. Yi-Chieh Lee. Inhibitory effects of andrographolide on migration and invasion in human non-small cell lung cancer A549 cells via down-regulation of PI3K/Akt signaling pathway. Eur J Pharmacol. 2010;632(1-3):23-32.
18. Wang W, Guo W, Li L, Fu Z, Liu W, Gao J, *et al*. Andrographolide reversed 5-FU resistance in human colorectal cancer by elevating BAX expression. Biochem Pharmacol. 2016;121:8-17.
19. Reisländer T, Groelly FJ, Tarsounas M. DNA damage and cancer immunotherapy: a STING in the tale. Molecular cell. 2020;80(1):21-8.
20. Wang ZM, Kang YH, Yang X, Wang JF, Zhang Q, Yang BX, *et al*. Andrographolide radiosensitizes human esophageal cancer cell line ECA109 to radiation in vitro. Dis Esophagus. 2016;29(1):54-61.
21. Ingrid S, Forestier-Román AL. Andrographolide induces DNA damage in prostate cancer cells. Oncotarget. 2019;10(10):1085-101.
22. Lu Yang DW. Andrographolide enhances 5-fluorouracil-induced apoptosis via caspase-8-dependent mitochondrial pathway involving p53 participation in hepatocellular carcinoma (SMMC-7721) cells. Cancer Lett. 2019;276(2):180-8.
23. Mark B, Hampton SO. Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis. FEBS Lett. 1997;414(3):552-6.

Cite this article: Manimara J, Azman DKM. The Effects of Andrographolide on Apoptosis in PC-3 Cell Line *Via* the Involvement of Caspases 3, 8 And 9. Pharmacogn J. 2023;15(4): 612-621.