Pharmacogn J. 2021; 13(3): 660-668
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Original Article

Isolation of Andrographolide from *Andrographis lineata* Wall. ex Nees var. *lawii* C.B. Clarke and its Anticancer Activity against Human Ovarian Teratocarcinoma

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ABSTRACT

Introduction: Andrographolide is a well-known anticancer phytochemical often isolated from *Andrographis paniculata* (Burm. f.) Nees. (Acanthaceae). Though *Andrographis lineata* Wall. ex Nees var. *lawii* C.B. Clarke (ALw) which also belongs to the same family has an adequate amount of andrographolide; remained untouched for isolation of andrographolide and anticancer studies. Therefore, this study was targeted to isolate the andrographolide from the leaves of ALw and to assess its role inthe induction of apoptosis against the human ovarian teratocarcinoma (PA-1) cell line. **Methods:** Column chromatography, thin-layer chromatography (TLC), preparative TLC were used for the isolation and purification while melting point, ultraviolet (UV)-visible spectroscopy, Fourier transform infrared (FTIR), proton nuclear magnetic resonance (^{1}H NMR), carbon-13 (C13) nuclear magnetic resonance (^{13}C NMR) analysis were carried out for characterization of the compound. 3-(4,5-dimethylthiaxo-2yl) 2, 5-diphenyl tetrazolium bromide (MTT) assay was carried out for cytotoxicity test and further Annexin-V staining, caspase 3 activity, B-cell lymphoma-2 (Bcl-2) activity, cell cycle analysis, and DNA damage study by terminal deoxynucleotidyl transferase (dUTP) nick end labeling (TUNEL) assays were carried out for apoptosis study. **Results:** Andrographolide was isolated from the methanolic extract of leaves of ALw which had a melting point of 230 °C, λ_{max} at 223 nm. FTIR results proved the presence of hydroxyl group, alkanes, carbon-carbon double bond, and a characteristic gamma lactone carbonyl. NMR data confirmed the 20 carbon structure. In the MTT assay cytotoxicity against PA-1 was at 3.7 µg/ml with other apoptotic assays supporting the induction of apoptosis by the compound at that concentration. **Conclusion:** ALw is proved to be an alternate source of andrographolide with potential abilities to induce apoptosis in ovarian cancer cells.

Key words: *Andrographis*, Andrographolide, Anticancer activity, Apoptosis, Ovarian teratocarcinoma.

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History

- Submission Date: 09-01-2021;
- Review completed: 26-02-2021;
- Accepted Date: 05-03-2021.

DOI : 10.5530/pj.2021.13.84

Article Available online

http://www.phcogj.com/v13/i3

INTRODUCTION

Andrographolide, an *ent*-labdane diterpenoid compound that has been isolated from the stem and leaves of *Andrographis paniculata* (Burm.f.) Nees by Gorter.[1] It is proved to exhibit significant biological activities including antioxidant, anti-inflammatory, antimicrobial, antiseptic, hypolipidemic, cytotoxic, hepatoprotective, neuroprotective activities.[2] Andrographolide is also accepted as a potent anticancer drug, studied extensively for the treatment of various kinds of cancers like brain, heart, lung, pancreas, prostate, oral, liver, colon, kidney, breast, skin, colorectal, blood, and bone marrow cancers. Mechanism of anticancer activity of andrographolide includes suppression of cyclins and cyclin-dependent kinases, induction of tumour suppressor proteins p53 and p21, increased level of Bax protein and decreased level of Bcl-2 proteins in a leukaemia cell line (HL-60 cells), G0/G1 phase arrest in breast cancer cell line (MCF-7 cells), cell cycle arrest in G2/M phase in human glioblastoma cell lines (glioblastoma U251 and U87), breast cancer, human leukaemia K562 cells.[2]

Cancer has become the major cause of mortality in recent years. Nearly about 50% of currently used anticancer drugs are from natural sources.[3] Ovary cancer is the fifth common cause of death among women of the United States according to the study of the American Cancer Society. In India, the incidence of ovarian carcinoma is increasing over the years.[4] Studies have shown the cytotoxic activity of leaf extract *Pergularia daemia* against human ovarian teratocarcinoma (PA-1 cells).[5] Similarly studies have also shown that paclitaxel extracted from *Taxus* spp. inhibited the growth of PA-1 cells to 50% at a concentration of 30 nM.[6] However, PA-1 cells showed resistance against curcumin and thus it could not induce any apoptosis in the cells.[7] There is a lack of systematic work on the use of phytochemicals against PA-1 cells.

To date, *Andrographis paniculata* (Acanthaceae) was being used as the only major source of andrographolide from which several researchers have isolated it using different techniques. *Andrographis lineata* Wall. ex Nees which belong to the same genus has potential medicinal values. It is being used as ethnobotanical medicine for skin diseases,

Cite this article: Bhat MA, Murthy HN. Isolation of Andrographolide from *Andrographis lineata* Wall. ex Nees var. *lawii* C.B. Clarke and its Anticancer Activity against Human Ovarian Teratocarcinoma. Pharmacog J. 2021;13(3): 660-8.

fever, constipation, bronchitis.[8] However, *Andrographis lineata* Wall. ex Nees var. *lawii* C.B. Clarke is an underexplored species, though it has a notable quantity of andrographolide.[9] Given the above, the present study was carried out with the objective isolation, characterization of andrographolide from *Andrographis lineata* Wall. ex Nees var. *lawii* C.B. Clarke and to study the anticancer activity of andrographolide on the human ovarian teratocarcinoma cell line.

MATERIALS AND METHODS

Extraction of phytochemicals

Plants of *Andrographis lineata* Wall. ex Nees var.*l awii* C.B. Clarke were collected from Jogimatti, Chitradurga district, Karnataka (14°09'31.9"N 76°23'43.8"E). The plants were maintained in the experimental garden and plants were identified using local flora and a voucher specimen (DSD-06A) was deposited at Herbarium, Shivaji University, Kolhapur (SUK), India. Leaves were collected from matured plants, washed thoroughly with distilled water, and then were dried to constant weight under shade and ground to powder. 30 g such powder was used for the extraction of phytochemicals with methanol (Analytical grade, HiMedia, Mumbai, India) using soxhlet apparatus at 70°C for 8 h. The solvent is evaporated after extraction to obtain a green extract.

Isolation of andrographolide

Four g of the extract was dissolved in 10 ml of methanol and loaded on analytical TLC (precoated sheets with silica, Merck, Mumbai, India) plates. Different solvent systems were tested for standardization viz. pure chloroform, ethyl acetate, ethanol, 9:1- chloroform: ethanol, 1:9 - chloroform: ethanol, 6:4- ethanol: ethyl acetate, 1:6:3- chloroform: methanol: ethyl acetate. Among the various solvents tested pure chloroform showed maximum bands. Methanol mixed with crude was evaporated and washed with chloroform to remove nonpolar and colored compounds. Crude left behind showed a brownish-red ring indicating the presence of terpenoids for the Salkowski test and a characteristic band for andrographolide.

The extract was saturated with 2 g of silica (mesh size 60- 120, HiMedia, Mumbai, India) and subjected to column chromatography where the column conditions were: internal diameter of the column – 4.5 cm; length of stationary phase – 20 cm; length of sample bind gel load – 1.5 cm; mobile phase –chloroform: methanol: ethyl acetate (1:6:3). The first fraction obtained had shown the band in TLC similar to the band of standard andrographolide (Sigma- Aldrich, USA).

Column chromatography was followed by preparative TLC where 5 g of silica (mesh size 60- 120, HiMedia, Mumbai, India) in 10 ml of distilled water was coated on a glass TLC plate. Silica was activated by keeping it in a hot air oven overnight at 80 °C. The fraction with andrographolide was streaked along the side and let run in ethanol. Band obtained was dissolved in ethanol to separate andrographolide from silica. The supernatant was dried to get the crystals of andrographolide at room temperature.

Spectral analysis

Maximum absorption in the UV-visible region of the electromagnetic spectrum was determined. A solution of 1 mg/ml isolated andrographolide was prepared using methanol and scanned for the range of UV-visible spectrum (190- 1000 nm) using a UV visible spectrophotometer (Jasco V 750, Japan). The melting point was determined using the melting point apparatus. FTIR analysis was done to determine the functional groups using IR transmission spectra. 5% of the sample was used to prepare the KBr disc and scanned at the wave length ranging from 400 to 4000 cm^{-1} (Thermo Fisher Scientific, Nicolet 6700, USA). ^{1}H NMR (399.8 MHz) and ^{13}C NMR (100.53 MHz) analyses were done for further confirmation using deuterated DMSO (Jeol, Japan).

Cell culture

PA-1 (human ovarian teratocarcinoma; ATCC CRL-1572) cells were obtained from The National centre for cell science, Pune, India. Cells were revived and were maintained in Dulbecco's Modified Eagles Medium (DMEM, HiMedia, Mumbai, India) supplemented with 10% fetal bovine serum (FBS) with 37°C in a humidified incubator (95% air and 5% CO_2).

Cytotoxicity test

Andrographolide isolated from ALw was tested for its *in vitro* cytotoxicity activity using the 3-(4,5-dimethylthiaxo-2yl) 2, 5-diphenyl tetrazolium bromide (MTT) assay.[10] The cell lines were maintained at 37°C in a humidified incubator (95% air and 5% CO_2) and plated into 96 well microplates (10,000 cells /200µl). The isolate was dissolved in dimethyl sulphoxide (DMSO) and further diluted with Dulbecco's modified eagle medium with high glucose (DMEM HG) medium to obtain a wide range of concentrations viz. 10, 20, 40, 80, and 160 µg/ml. After 24 h of incubation, the test sample was added to the wells containing the cells and incubated for 24 h. 200 µl of MTT solution was added while the DMSO solution was used as a negative control. After 3 h of incubation, 100 µl DMSO was used to dissolve formazan crystals formed. The optical density was measured at 570 nm using a microplate reader (Bio-Rad, model 3550, USA). Cell viability was calculated using the formula (mean optical density (OD) of test sample/ mean OD of untreated cells) x 100. IC_{50} was generated from the dose-response curve.

Detection of apoptosis by FITC annexin V assay

Fluorescein isothiocyanate Annexin-V (FITC Annexin V) staining was done by the flow cytometric method.[11] PA-1 cells were seeded on a 6-well plate at a density of 3 x 10^5 cells/ 2 ml and incubated in a CO_2 incubator at 37°C. Cells were washed with 1x phosphate-buffered saline (PBS) to remove the traces of medium after the incubation of 24 hours. Cells were treated with an IC_{50} value of isolated andrographolide (3.7 µg/ml) which was prepared in 2 ml of culture medium. Trypsinized cells were harvested by centrifugation at 300 x *g* at 25°C for further use. PBS washed cells were then treated with 1x binding buffer. Primary staining was done by adding 5 µl of FITCAnnexin V. After addition, gentle mixing was done and incubated at room temperature for 15 m in dark. Secondary staining was done with 5 µl of propidium iodide (PI) along with 400 µl of 1x binding buffer. Flow cytometric (Cytomics FC 500, Beckman Coulter, USA) analysis was carried out immediately using FL1 (525 nm bandpass filter) detector for FITC Annexin V fluorescence and FL3 (620 nm bandpass filter) detector for PI fluorescence.

Caspase 3 activity by flowcytometry[12]

Human ovarian teratocarcinoma cell cultures were treated with 3.7 µg/ml of isolated andrographolide and then harvested after 24 hours at 37°C incubation.0.5 ml 2% paraformaldehyde (PFA) solution was used to fix the cells followed by 20 m of incubation to facilitate further immune fluorescent staining. Cell lyses were done by the 0.1 % triton-x 100 detergent treatment. After washing with 0.5% bovine serum albumin (BSA), 20 µl FITC rabbit anti-active caspase-3 IgG antibodies were added and incubated in dark for 30 m. Unbound antibodies were removed by washing with 1x PBS. Green fluorescence was measured in the FL1 detector using a 525 nm band pass filter.

Determination of Bcl-2 activity by flow cytometry

PA-1 cells were treated with 3.7 µg/ml of isolated andrographolide then harvested after the incubation as explained previously. Fixation of the cells before the immunofluorescent staining was done by adding 0.5 ml

2% paraformaldehyde (PFA) solution followed by 20 m of incubation. After washing with 0.5% bovine serum albumin (BSA) in 1x phosphate-buffered saline (PBS), 0.1% of triton-x 100 (prepared in 0.5% BSA) was added and incubated for 10 m to remove the traces of triton-x 100 cells are washed with 0.5% bovine serum albumin (BSA) in 1x PBS 2 times. Along with 0.5% BSA, 20 µlPE mouse anti-human Bcl-2 antibodies were added, mixed well, and incubated for 30 m in dark at room temperature. After washing off the excess antibodies with 1x PBS, Bcl-2 activity was measured by measuring orange fluorescence in the FL2 detector using a 575 nm bandpass filter.

Cell cycle analysis by flowcytometry[13]

PA-1 cells pretreated with 3.7 µg/ml of andrographolide for 16 h were harvested and washed with 1x PBS. Fixation was done using chilled 70 % ethanol (-20 ºC) and 400 µl of PI-RNase solution was added to evaluate the DNA content. PI fluorescence was collected in an FL3 detector using a 620 nm bandpass filter.

Detection of DNA damage by TUNEL assay[14]

Andrographolide (3.7 µg/ml) treated cells were harvested, fixed with chilled 70 % ethanol (-20 ºC) and were suspended in 50 µl of DNA labeling solution (reaction buffer+ terminal deoxynucleotidyl transferase (tdt) enzyme + FITC deoxy-uridinetriphosphate (dUTP) + distilled water). After incubating it for 60 m at 37 ºC, 1 ml of rinse buffer (0.05 % sodium azide) was added and centrifuged at 300 x *g* for 5 minutes; repeat the same for one more time, and the supernatant was removed. 0.5 ml of PI-RNase was added and incubated for 30 m in dark at room temperature. The extent of DNA damage caused by andrographolide was analyzed by analyzing FITC (green) fluorescence in the FL1 detector using a 525 nm band pass filter.

RESULTS AND DISCUSSION

Spectral analysis of isolated andrographolide

Andrographolide which was isolated from ALw had a melting point of 230 ºC. Further, andrographolide was scanned for its absorbance in the range of 190-1000 nm it was found that at 223 nm it showed maximum absorption (Figure 1), these results were comparable to standard andrographolide (Sigma-Aldrich) and were similar to the earlier reported results. [15-17.] FTIR results of andrographolide isolated from ALw showed bond stretching at 3398 (hydroxyl group), 3099 to 2849 (alkanes), 1674, and 1647 (carbon-carbon double bond). A characteristic stretch of andrographolide corresponding to gamma lactone carbonyl stretching could be observed at 1727 cm^{-1} (Figure 2A). The above data were comparable to standard andrographolide (Figure 2B) as well as earlier reported results[18]. ^{1}H NMR (DMSO-d6, 399.87 MHz) was found

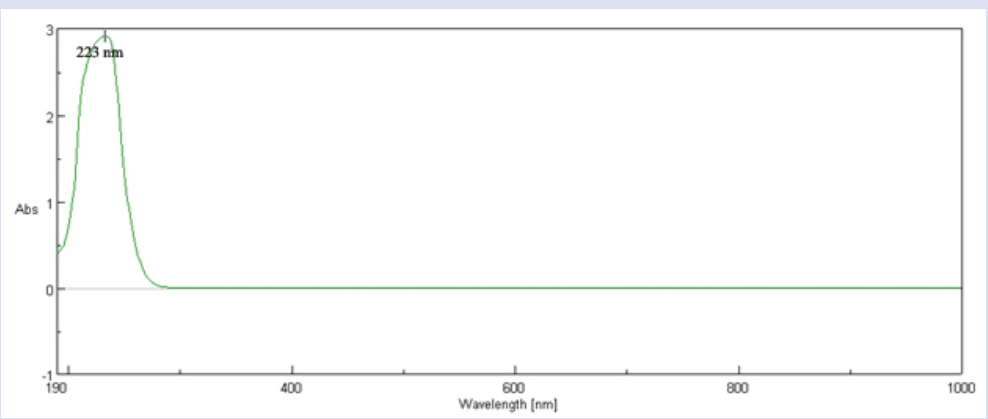

Figure 1: The UV-visible spectrum of isolated compound.

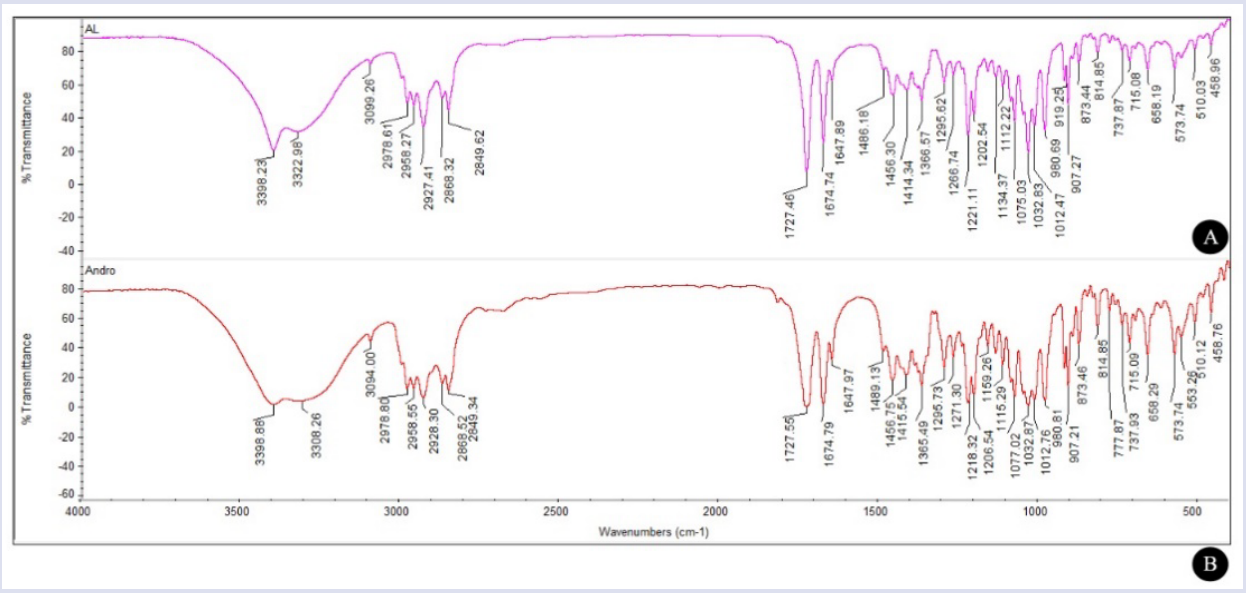

Figure 2: FTIR transmittance spectrum at 400-4000/cm of isolated compound (A) standard andrographolide (B).

to be δ ppm 6.59 (1H), 5.70 (1H), 5.05 (1H), 4.82 (2H), 4.59 (1H), 4.34 (1H), 4.04 (2H), 3.79 (1H), 3.20 (2H), 2.26 (1H), 1.79 (6H), 1.31 (1H), 1.18 (2H), 1.04 (3H), 0.62 (3H) and also a characteristic gamma lactone chiral carbon proton attached to –OH group had resonated at 6.579 ppm as triplet of doublet (Figure 3A). ^{13}C-NMR (101 MHz, DMSO-d6):δ ppm 170.49, 148.12, 146.84, 129.50, 108.80, 78.95, 74.87, 74.79, 65.03, 63.16, 56.00, 54.88, 42.80, 39.11, 38.03, 37.02, 28.41, 24.48, 23.61, 15.27. ^{13}C-NMR data presented of andrographolide isolated from ALw were correlating with earlier reports.[15]

Cytotoxicity test

Cell metabolic activity can be measured colorimetrically by MTT assay. NADPH-dependent cellular oxidoreductase enzymes can reduce the tetrazolium dye MTT to insoluble formazan crystals which is purple.

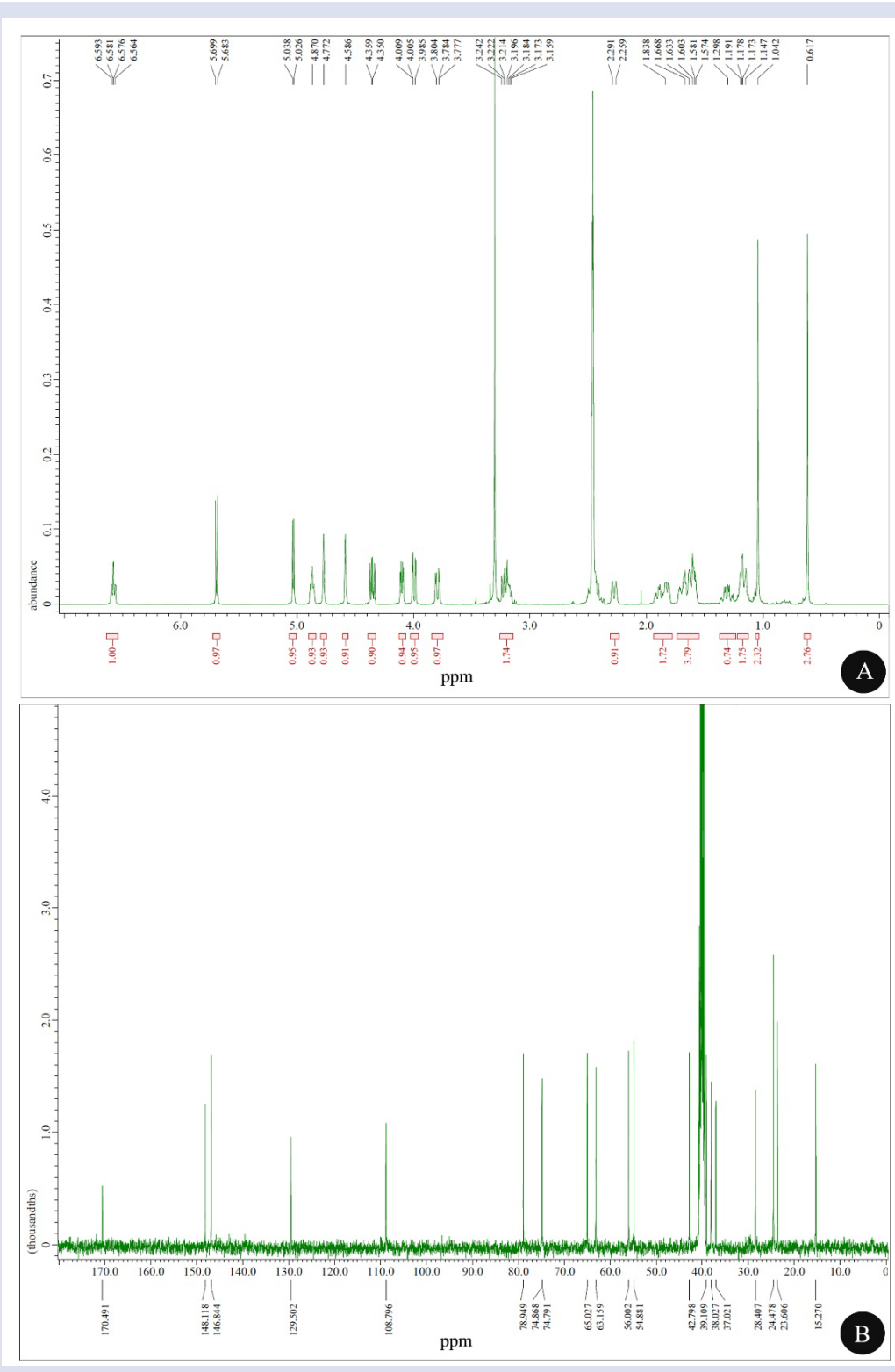

Figure 3: ^{1}H NMR spectra of isolated compound (A) and ^{13}C NMR spectra of isolated compound (B).

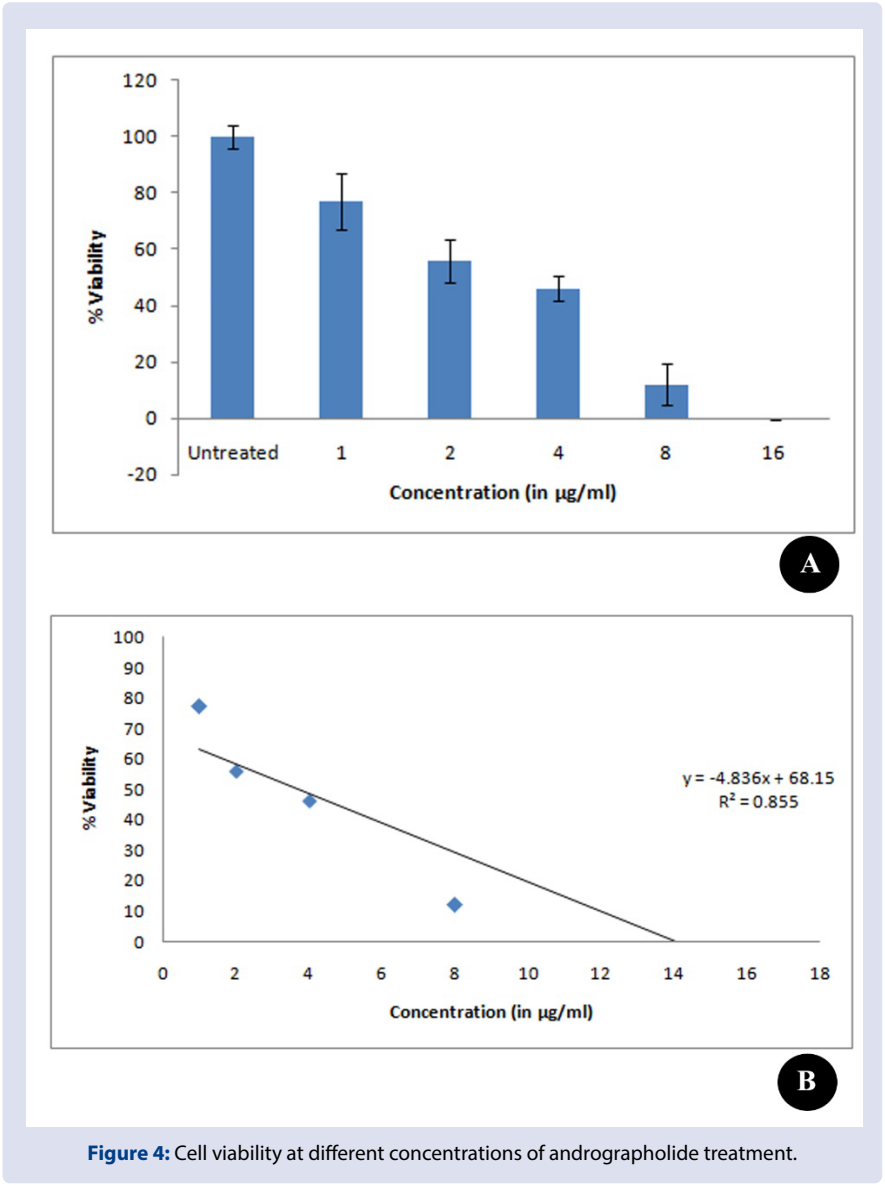

Figure 4: Cell viability at different concentrations of andrographolide treatment.

Thus, the intensity of the colour measures the cell viability in terms of enzymatic conversion of MTT into formazan. IC_{50} value calculated by the dose-response curve was found to be 3.7 µg/ml (Figure 4). Such a lower IC_{50} value proved the good cytotoxicity of andrographolide against human ovarian teratocarcinoma cells.

Detection of apoptosis by FITC Annexin V assay

Differential staining with annexin V fluorescein isothiocyanate and propidium iodide showed that the cells were shifted to the second quadrant representing early apoptosis (from 6.25% to 21.8%) (Figure 5A and 5B) in the andrographolide treated cells when compared to untreated cells indicating that andrographolide is inducing the apoptosis in PA-1 cells.

Caspase 3 activity by flowcytometry

Cysteinyl aspartate proteinases 3 (Caspase 3) are effecter caspases that execute apoptosis. These are the important caspases that can be activated by both intrinsic and extrinsic pathways responsible for the cleavage of multiple regulatory and structural proteins that are necessary for the survival of the cell.[19] Flowcytometric estimation of caspase 3 using FITC rabbit anti-active caspase-3 IgG antibodies had shownan increased number of cells with activated caspase 3 after the andrographolide treatment (15%; Figure 5D) when compared to control (8.4%) (Figure 5C). Increased caspase 3 positive cells indicated an increased level of apoptosis.

Determination of Bcl-2 activity by flowcytometry

Bcl-2 are anti-apoptotic proteins that protect the cells from apoptosis[20]. Estimation of Bcl-2 activity by PE mouse anti-human Bcl-2 antibodies had shown a considerable increase in the number of cells with a low level of Bcl-2 (16.9%, Figure 5F) when compared to untreated cells (4.19%, Figure 5E).

Cell cycle analysis by flowcytometry

The results of cell cycle studies reveal that a higher level of cells was existing at the G2/M phase with the andrographolide treatment (55.6%, Figure 5H) when compared to control (33.7%, Figure 5G), this evidence suggests that cell cycle arrest occurs at G2/M phase. Similar to the present studies Liu and Chu[21] have reported that andrographolide was arresting the human melanoma cells at the G2/M phase. Sub G1 cells had increased from 1.21 to 5.42% indicating DNA damage after the treatment of andrographolide.

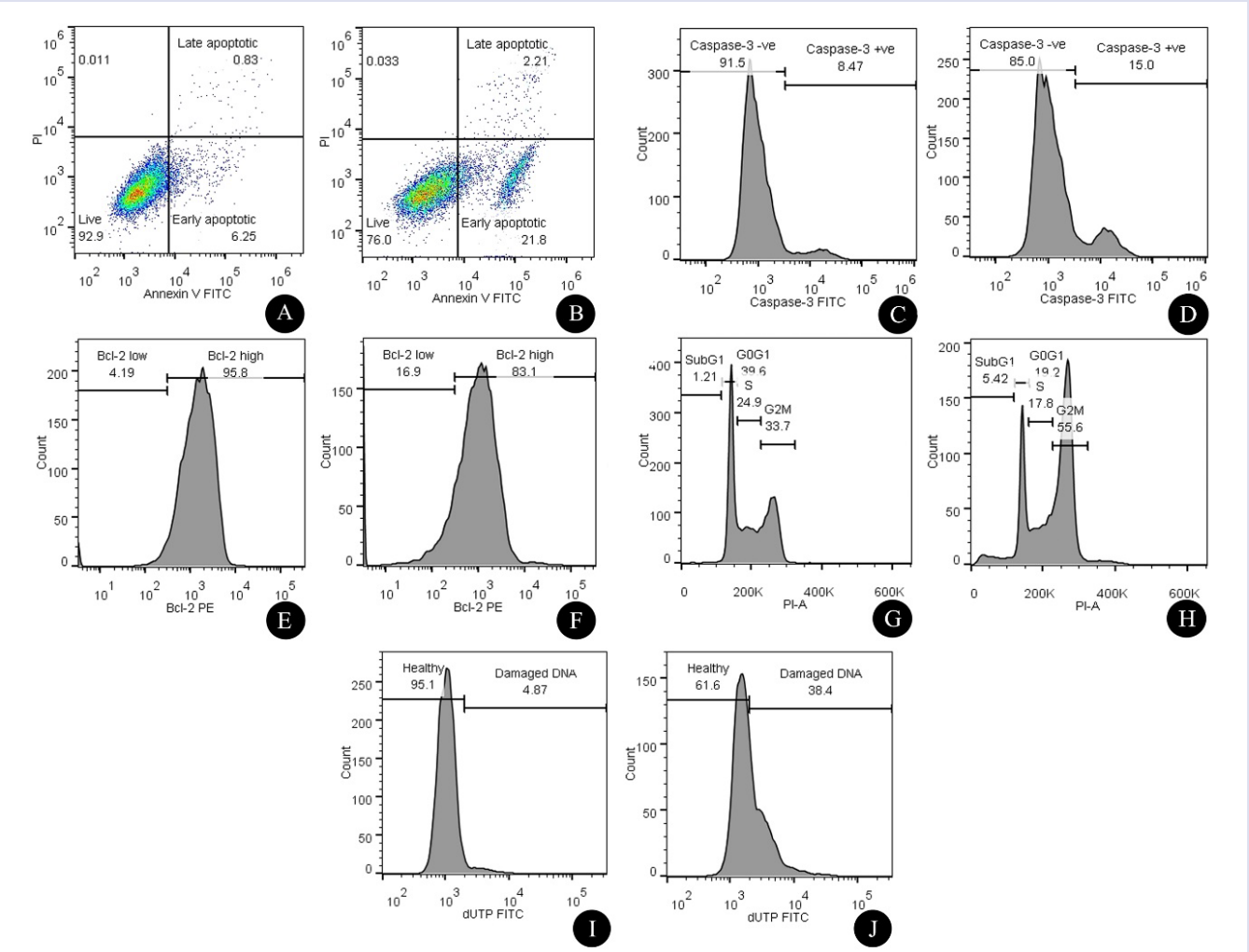

Figure 5: Flow cytometry analysis of andrographolide induced apoptosis in PA1 cells, Annexin V FITC staining of untreated cells (A) and treated PA1 cells (B); caspase-3 activity in untreated cells (C) and treated PA1 cells (D); Bcl-2 activity of untreated cells (E) and treated PA1 cell (F); cell cycle analysis of untreated cells (G) and treated PA1 cells (H); TUNEL assay showing damaged DNA of untreated cells (I) and treated PA1 cells (J).

Detection of DNA damage by TUNEL assay

The extent of DNA fragmentation was determined by terminal deoxynucleotidyl transferase end labeling (TUNEL) assay. In untreated cells, 95.1% cells were healthy (Figure 5I), whereas, in treated cells, damaged cells were increased to 38.4% and there was a decline in the healthy cells to 61.6% (Figure 5J).

CONCLUSION

The results obtained at the end of the study conveyed that *Andrographis lineata* Wall. ex Nees var. *lawii* C.B. Clarke can be used as an alternative source for andrographolide. The andrographolide thus isolated was a potent anticancer agent against ovarian teratocarcinoma leading to further studies in this regard.

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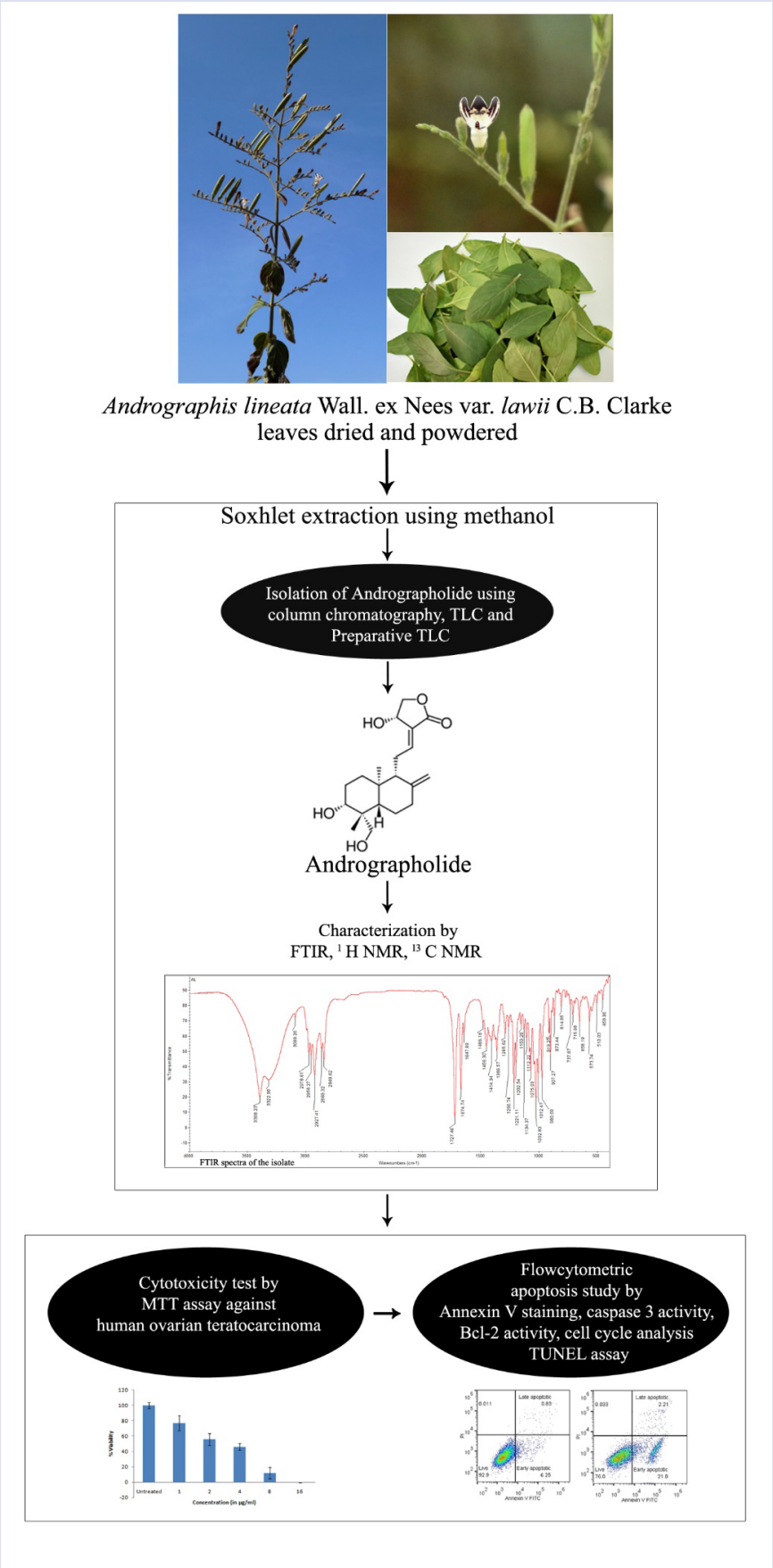
GRAPHICAL ABSTRACT



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Hosakatte Niranjana Murthy professor in Department of Botany, Karnatak University, Dharwad, India, has obtained Ph.D. degree from the same university. He has tremendous passion for research and academics. Since 1986, Apart from his teaching experience of 34 years, he possesses extensive research experience in the area of plant biotechnology. Prof. Murthy has post-doctoral and collaborative research experience in many foreign research institutes. He has successfully completed more than 15 research projects funded by various agencies and guided several Ph.D. students. Prof. Murthy has published more than 200 research articles in international peer reviewed journals with high impact factor.

Cite this article: Bhat MA, Murthy HN. Isolation of Andrographolide from *Andrographis lineata* Wall. ex Nees var. *lawii* C.B. Clarke and its Anticancer Activity against Human Ovarian Teratocarcinoma. Pharmacog J. 2021;13(3): 660-8.