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Antiproliferative activity of *Andrographis paniculata* seed extracts on hepatocellular carcinoma cells

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Abstract---Leading cause of death world side is because of severe health problems due to cancer. Compounds occurring naturally from plants known as phytochemical are rich sources of anticancer drug development, in depth screening assessments are necessary. The objective of the present study aimed to investigate the mechanistic pathway of antiproliferative activity of ethanolic *Andrographis paniculata* seed extract against Hepatocellular carcinoma cell (HepG2) cell line. Cell cycle arrest and apoptosis study was performed by Flow cytometry analysis. The expression of certain apoptosis gene associated with cell cycle regulation was conducted by QRT-PCR assay to assess the effect of ethanolic *Andrographis paniculata* seed extract on HepG2 cells. *Andrographis paniculata* seed extract inhibited cell proliferation and arrest proliferation on HepG2 cells at G2/M phase when compared to untreated cells. The ethanol extract of *Andrographis paniculata* seed found to be active cell inhibitor at the time and dose dependent manner. The QRT-PCR assay report reveals that pro-apoptotic genes BCL2 Associated X (BAX), Caspase-3, Caspase-8 genes on both treated and untreated cells. B-cell lymphoma 2 (Bcl-2) is down regulated in treated groups compared to untreated group and Beta actin was used as internal control. More over *Andrographis paniculata* seed extract triggered both extrinsic and intrinsic apoptosis by the activation of Bcl-2, BAX, Caspase-3 and Caspase-8 gene. The result indicate that *Andrographis paniculata* seed extract shows a promising potential to be used a nutraceutical for the development of anticancer agents to prevent and treat hepatocellular

cancer. However further detailed investigation required to make the *Andrographis paniculata* seeds applicable for therapeutic use.

Keywords---phytochemicals, cytotoxic, gene expression, caspase, pathway.

Introduction

In recent decades the use of plants as natural remedy has been used as traditional medicine for treatment of various diseases including cancer. For novel drug discovery and development natural products are considered as potent sources. Herbal medicine has increasingly grown in the last decade as an alternative medicine for cancer-related therapy, which increase efficacy, reduce toxicity when compared with chemotherapy treatment given for different malignancies. The natural compounds and its multiple therapeutic effects in traditional medicine motivated to evaluate the cytotoxic activity of *Andrographis Paniculata* seeds in HepG2 Cell lines *Andrographolide* involves a mechanisms like oxidative stress, cell cycle arrest, anti-inflammatory and immune system mediated effects, apoptosis, necrosis, autophagy, inhibition of cell adhesion, proliferation, migration, invasion, anti-angiogenic activity on different cell lines which proves that the it can be used as agents in the treatment of cancer. (Yunos *et al.*, 2013)

The study has reported that ethanolic extract of *Andrographis paniculata* leaf extract increased cell cycle arrest at G0/G1 and G2/M phases induced apoptosis in Hep2 and bile duct (HuCCA-1 and RMCCA-1) cells. The cyclin-D1, Bcl-2 and inactive proenzyme form of caspase-3 were reduced by *Andrographis paniculata* leaf ethanolic extract while proapoptotic protein Bax was increased. This suggests a promising herbal plant for treating intrahepatic cholangiocarcinoma. (Tawit *et al.*, 2014) The *Andrographolide* shows anticancer activity by inhibiting NF-kappa B, PI3K/AKT and other kinase pathways by inducing apoptosis. *Andrographolide* induces apoptosis pathway by both intrinsic and extrinsic in anti-apoptotic protein like Bax, p53 and activated caspases in different cancer cells. *Andrographolide* inhibited different cancers like human breast, prostate and hepatoma tumors. The antiproliferative and apoptotic effect of *andrographolide* along with combination of Cisplatin in Ovarian A2780 cancer cell lines are observed. The percentage of apoptotic cell death were found to be greater with combination of Andro and Cisplatin. (Rajagopal *et al.*, 2003)

Andrographolide induce apoptosis in TD-47 human breast cancer cell line by increasing the p53, bax, caspase-3 and decrease the expression of Bcl-2 which is determine by immunohistochemical analysis. Ethyl acetate fraction of *Andrographis paniculata* nees and 5-flourouracil combination against different human cancer cell line HeLa, Widr and T47D were evaluated. (sukardiman *et al.*, 2007) *Athyrium multidentatum* (Doll.) Ching strongly inhibited the cell growth and induced apoptosis and cell cycle arrest in HepG2 cells through both intrinsic and extrinsic pathways. The *Athyrium multidentatum* (Doll.) Ching upregulated the protein expression of Fas, Fas-L, Bax/Bcl-2, cyto-c, cleaved caspase-3 inducing apoptosis in HepG2 cells. (Guovauan *et al.*, 2017) Antiapoptotic BCl-2 protein over

expressed has implicated in different carcinomas. The mechanism of Bcl-2 inhibits apoptosis is considered to involved the inhibition of caspase protein.(Guo *et al.*, 2014) It has been reported that apoptosis was confirmed by caspases activity assay as well as gene expression *Bcl-2*. Caspase-8, -9, and -3 activity with downregulation of *Bcl-2* which illustrate intrinsic and extrinsic pathway in MCF7. The caspase-3 and 8 revealed extrinsic pathway of apoptosis and *Bcl-2* is down regulated. In Hela cells the *Bcl-2* is downregulated and activity of Caspase-9 and 2 shown intrinsic pathway and in HepG2 shows caspase independent apoptosis. (Luisa *et al.*, 2010) Yanmei *et al.*, in his study reported that *Andrographis paniculata* on HepG2 cells based on dose-dependent increase in proapoptotic Bax protein and no change in antiapoptotic *Bcl-2* protein in treated cells. Further expose to loss of mitochondrial membrane and the release of cytochrome c from the mitochondria to the cytosol result in the caspase-9 and caspase -3 were activated while caspase-8 was not affected in HepG2 cell lines. Result clearly point to the involvement of mitochondria-mediated signaling pathway in *Andrographis Paniculata* induced apoptosis.(Yanmei *et al.*, 2015)

Anti apoptotic gene *Bcl 2* gene prevents the initiation of apoptosis in programmed cell death. The *Annona* seed extract has higher potency against *Bcl-2* which is an anti apoptotic gene that prevents the initiation of apoptosis.(Droin *et al.*, 2004) Evidence suggest that *Bcl-2* acts as a downstream death, the caspase enzymes may able to inactivate the *Bcl-2* anti-apoptotic function further enhancing cell death.(Cheng *et al.*, 1997) *Andrographolide* induced apoptosis in breast cancer cells MDA-MB- 231 lacking functional p53 and estrogen receptor.(Raghavan *et al.*, 2018) The cytotoxicity of *andrographolide* on HepG2 human hepatoma cells was reported. Growth of HepG2 cells was affected in induced cell cycle arrest at G2/M phase and a late apoptosis of the cells. In the treated cells the BAX and pro-apoptotic gene P53 were upregulated but not other apoptotic proteins such as Bad, *Bcl-2* and *Bcl-X(L)*. The activity of Caspase-3 has direct effect on apoptosis which was enhanced by the presence of *andrographolide* cell death of HepG2 indicating caspase-independent cell death. (Jieliang *et al.*, 2007) The result of homogeneous polysaccharide (ATP-II), on glioma tumors induced apoptosis by increasing the ratio of Bax/*Bcl-2* and activation of caspase-3, caspase-8, and caspase-9 cascade.(13)(Lei *et al.*, 2014) *Azorella compacta Phil.* is an alpine medicinal plant the present study investigate action of *Azorella compacta Phil.* extract against human leukemia HL60 cells induced apoptosis was accompanied by activated/cleaved caspase-3, caspase-9 and poly(adenosine diphosphate-ribose) polymerase. The increase in apoptosis associate with decreases of apoptosis inhibitor B-cell lymphoma 2 (*Bcl-2*), upregulation of pro-apoptotic *Bcl-2*-associated X (Bax) protein and downregulation of anti-apoptotic *Bcl* extra large protein shows the anticancer activity of the extract. (Min *et al.*, 2015) *Chlorpyrifos* induces apoptosis of QSG7701 cells with a decrease in mitochondrial membrane releasing of cytochrome c into cytosol upregulating the Bax/*Bcl-2* and activation of caspase-9/-3. The result indicates the potential risk to induce apoptosis of human liver cells through caspase-dependent mitochondrial pathways.(Yang *et al.*, 2018) This study is the first one to investigate the cytotoxic effect, the expression of apoptotic-related genes, and the induction of apoptosis in HepG2 cell line after treated with ethanolic extract prepared from *Andrographis paniculata* seed extract.

Material and Methods

Preparation of ethanol *Andrographis paniculata* seed extract

The *Andrographis paniculata* seeds were collected from local market, Avadi, Tamil Nadu. The seed samples were wild varieties, which are not micro or macro propagated by human. The seeds were identified Reg no : of the certificate : PARC/2020/4258 and authenticated by Dr.P. Jayaraman, Director, Plant Anatomy research Centre, Tambaram. The seeds of *Andrographis paniculata* were shade dried , cleaned and powdered with blender. The 50 g of powder sample of *Andrographis paniculata* seed is dissolved in 250 mL of ethanol (V/V) in a Soxhlet apparatus. The extracts obtained were filtered and evaporated to dryness by using rota evaporator and stored in refrigerator at 2-8°C. The HepG2 is purchased from NCCS, Pune, India. The HepG2 cells were maintained in DMEM Low Glucose media supplemented with 10 % FBS along with the 1% antibiotic-antimycotic solution and 1% L-Glutamine (200mM) in atmosphere of 5% CO₂, 18-20% O₂ at 37°C temperature maintained in the CO₂ incubator and subcultured for every 2 days.

Cell cycle arrest study of *Andrographis paniculata* seed extract

Cell cycle study of the *Andrographis paniculata* seeds extract was determined in HepG2cells. HepG2 cells were cultured in a 6 well plate at a density of 2×10^5 cells/2 mL and incubated at 37°C for 24 h. After incubation, cells were treated with IC₅₀ concentration of 103uG/mL with an std control with the concentration of 5ug/mL in 2mL of DMEM Medium with low glucose and incubate the cells for 24 hours. After the incubation period, remove spent medium and washed cells with 2mL of 1X DPBS. Cells in each well were resuspended in 500 µl trypsin and incubate at 37°C for 3-4 minutes. Afterwards, 2 mL of culture medium was added and the cells were transferred directly into 12x75 mm tubes and centrifuged for 5 min at 300 x g at 25°C (REMI R- 8C, REMI, India). Pelleted Cells were washed within 1 mL of phosphate buffered saline. Cells were fixed and permeabilized with 70% of prechilled absolute ethanol for 30mins in -20°C deep freezer and again the cells were centrifuged to remove excess ethanol and washed with 1X DPBS and stain the cells with 400uL of Propidium iodide-RNase staining buffer Solution (BD Biosciences,CA, USA) and incubate the cells for 20-30 mins under dark at room temperature and analyze the cells immediately with a Flow cytometer (BD FACS Calibur, BD Biosciences, CA, USA).

Apoptosis study of *Andrographis paniculata* seed extract

Apoptosis/Necrosis analysis of the *Andrographis paniculata* seed extract was determined in HepG2 cells. HepG2 cells were cultured in a 6 well plate at a density of 2×10^5 cells/2 mL and incubated at 37°C for 24 h. After incubation, cells were treated with IC₅₀ concentration of 103uG/mL with an std control (Doxorubicin) with the concentration of 5ug/mL in 2mL of DMEM Medium with low glucose and Incubate the cells for 24 hours. After the incubation period, remove spent medium and washed cells with 2mL of 1X DPBS. Cells in each well were resuspended in 500µl trypsin and incubate at 37°C for 3-4 minutes.

Afterwards, 2 mL of culture medium was added and the cells were transferred directly into 12x75 mm tubes and centrifuged for 5 min at 300 x g at 25°C (REMI R-8C, REMI, India). Cells were washed within 1 mL of phosphate buffered saline. Cells treated with Doxorubicin served as the positive control. Add 5 µl of Annexin V-FITC (Fluorescein isothiocyanate) and Propidium Iodide (PI) along with 100uL of 1X Annexin V Binding buffer and incubated the cells for 15-20mins in the absence of light. After the incubation period, 300uL of 1X Annexin V Binding Buffer and analyze the cells immediately with a Flow cytometer (BD FACS Calibur, BD Biosciences, CA, USA).

Gene expression study of *Andrographis paniculata* seed extract

Gene expression study of the *Andrographis paniculata* seed was determined in HepG2 cells. HepG2 cells were cultured in a 6 well plate at a density of 2×10^5 cells/2 mL and incubated at 37°C for 24 h. After incubation, cells were treated with IC50 concentration of 103uG/mL along with cell control an Std Control with the concentration of 5ug/mL in 2mL of DMEM Medium with low glucose and incubate the cells for 24 hours. After the incubation period, remove spent medium and washed cells with 2mL of 1X DPBS. Cells in each well were resuspended in 500 µl trypsin and incubate at 37°C for 3-4 minutes. Afterwards, 2 mL of culture medium was added and the cells were transferred directly into 12x75 mm tubes and centrifuged for 5 min at 300 x g at 25°C (REMI R-8C, REMI, India). Pelleted Cells were washed within 1 mL of phosphate buffered saline. Extraction of total cellular RNA from HepG2 cells was carried out using RNeasy Mini kit (Cat No: 74106, Qiagen) as per manufacturer instructions. Treated with DNase and purified. This is done to avoid genomic DNA contamination. RNA quantified by UV-Vis using Qiaexpert. Quality checked by 260/280. Quality is good (1.8 to 2). RNA was also run on agarose gel. Ladder used is Lambda HindIII/EcoRI.

Results

Cell cycle arrest study of *Andrographis paniculata* seed extract against the HepG2 cell line by Flow Cytometry

In this study, the experiments focused on investigating the response of *Andrographis paniculata* seed of IC50 concentration of 103.03µG/mL respectively against the HEPG2cell lines. The % of cells arrested at Sub g0/G1, g0/G1, S and G2/M phases of the cell cycle are summarized in Table 1. Doxorubicin with the concentration of 5ug/mL is used as a standard in these experiments. Cell Cycle study was evaluated by Flow Cytometry to check the stages of cell cycle arrest and the obtained results by Flow Cytometry as follows:

Table 1: Percentage of Cells in different cell cycle stages Vs HepG2 cell line

% of cells in different cell cycle stages vs HepG2					
Sl No	Cell stage	Cycle	Untreated	Standard	<i>Andrographis paniculata</i> seed extract

1	Sub G0/G1	0.93	7.6	0.59
2	G0/G1	70.24	51.04	59.68
3	S	2.97	4.64	6.72
4	G2/M	25.29	36.97	31.5

Total Events Selected per each group -10000

Table: 1 shows the % of cells get arrested in the different stages of HepG2 cell cycle. In Sub G0/G1 phase (Apoptotic phase), 0.93%, 7.6%, and 0.59% of cells get arrested in Untreated, Standard and *Andrographis paniculata* seed with IC50 concentration respectively. In G0/G1 phase (Growth Phase), 70.24%, 51.04%, and 59.68% of cells get arrested in Untreated, Standard and *Andrographis paniculata* seed with IC50 concentration respectively. In S phase (synthetic phase), 2.97%, 4.64%, and 6.72% of cells get arrested in Untreated, Standard and *Andrographis paniculata* seed extract with IC50 concentration respectively. On the other hand, in G2/M phase, 25.29%, 36.97%, and 31.5% of cells get arrested in Untreated, Standard and *Andrographis paniculata* seed extract with IC50 concentration respectively.

The cells treated with std control and *Andrographis paniculata* seed extract with IC50 concentration are showing high % of cells at G2/M stage arrest, when compared to untreated cells. Hence the cell cycle got arrested at G2/M stage. Extract exhibiting prominent Cell Cycle phase arrest similar to the std Control, doxorubicin on HepG2 cells.

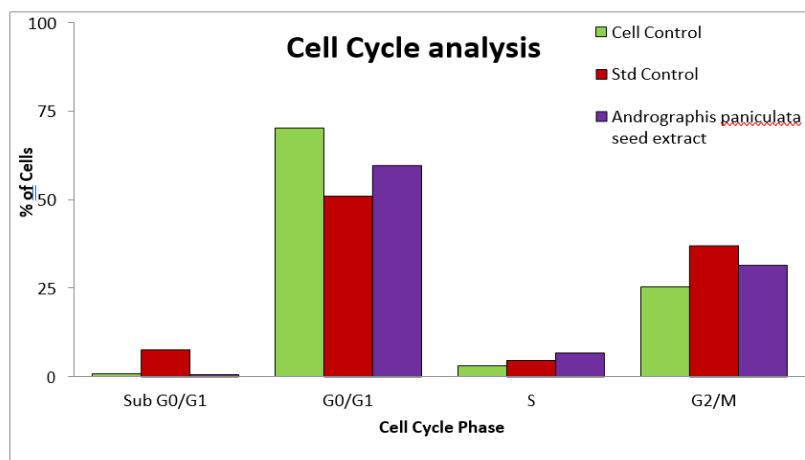


Figure 1 : Overlay showing the % of cells get arrested in the different stages of HepG2 cell cycle.

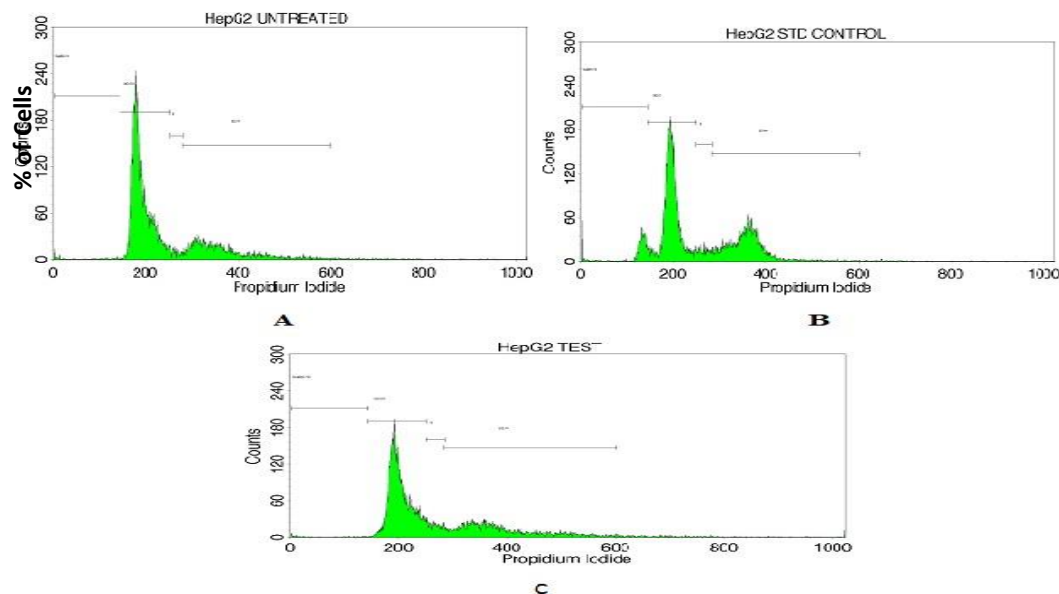


Figure 2: Flow cytometric histograms showing the phases of cell cycle distribution in the HepG2 cell line treated with *Andrographis paniculata* seed extract, extract with IC₅₀ concentration and standard drug, doxorubicin at 10 µg/mL concentration compared to the Untreated control. (A-Cell Control, B -Std control, C- *Andrographis paniculata* seed extract)

Apoptosis study of *Andrographis paniculata* seed extract against the HepG2 cell Line by Flow Cytometry

The anticancer activity of the *Andrographis paniculata* seed is evaluated to analyse the apoptosis effect on HepG2 cell lines. The concentrations of the *Andrographis paniculata* seed used to treat the cells are given in Table 2. Annexin V and PI staining are used to examine possible induction of cell death (necrosis and/or apoptosis). PI is membrane impermeant it does not pass through intact cell membranes and are excluded from viable cells. Annexin-V is used as a probe to detect cells that have expressed phosphatidylserine on the cell surface, an event found in apoptosis as well as other forms of cell death. After staining the green stain represents Annexin V and Pink stain represents Propidium iodide.

Table 2: Concentration of the *Andrographis paniculata* seed extract

S.No	Test Compounds	Cell line	Concentration treated to cells
1	Untreated	HepG2	No treatment
2	Standard (Doxorubicin)	HepG2	10 µg/mL
3	<i>Andrographis paniculata</i> seed extract	HepG2	IC ₅₀ -103.03 µg/mL

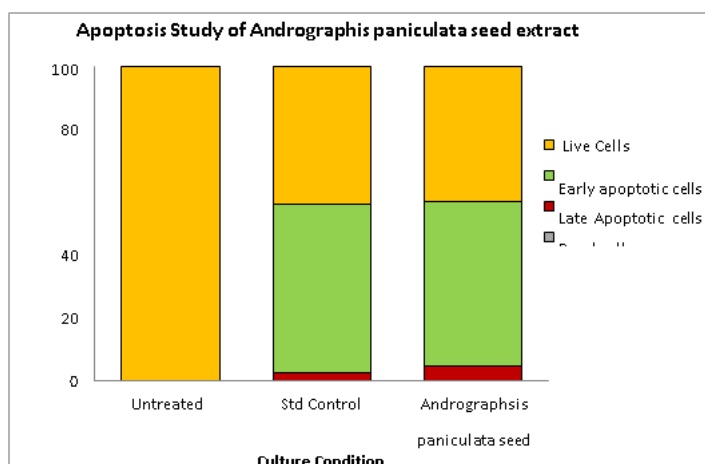


Figure 3: % of live, apoptosis and necrotic HepG2 cells treated with culture medium alone (Untreated), Doxorubicin and *Andrographis paniculata* seed extract.

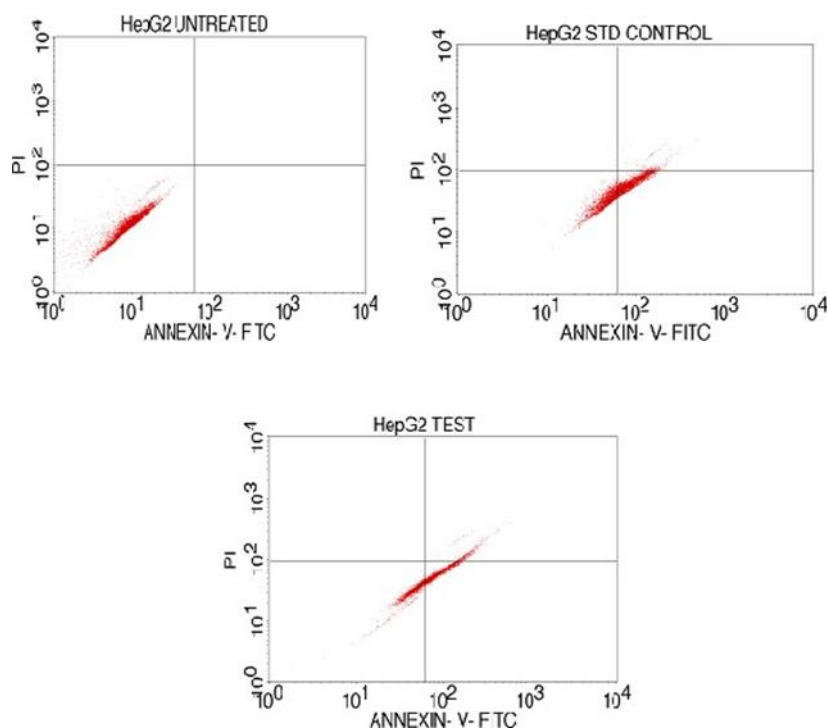


Figure 4: Flow cytometric histograms apoptosis distribution in the HepG2 cell line treated with *Andrographis paniculata* seed extract

Gene expression Study of *Andrographis paniculata* seed extract against the HepG2 cell Line

To accurately and reliably determine gene expression values, raw fluorescence data (Ct values) generated by the real-time PCR instrument (Qiagen). The

following formula could be applied to generate relative quantities: $\Delta Ct = \text{Average Ct of extract sample} - \text{Average Ct of calibrator}$. Relative expression is a variation of the expression of a gene between two samples. The Relative quantification (RQ) value is the fold change compared to the calibrator (untreated sample, time zero, etc.). The calibrator has a RQ value of 1. If any values exceed RQ 1 the gene is said to be up regulated otherwise down regulated. All samples are compared to the calibrator.

Table 3 : Gene expression study of *Andrographis paniculata* seed extract against the HepG2 cell Line

	Bcl-2	Bax	Caspase-3	Caspase-8
Untreated	0	0	0	0
Standard control	-0.7206 + 0.10	0.8790 + 0.14	2.1492 + 0.17	1.8679 + 0.26
<i>Andrographis paniculata</i> seed extract	-0.4947 + 0.13	0.1850 + 0.11	0.8921 + 0.09	1.0994 + 0.25

Values were expressed as Mean $\pm$ Standard deviation for triplicates

Table 3 shows the normalized fold change (mRNA expression) values of Bcl-2, Bax, Caspase-3 and Caspase-8 in Untreated, Std and *Andrographis paniculata* seed extract treated in HepG2 cells. Beta actin was used as a internal control for the study.

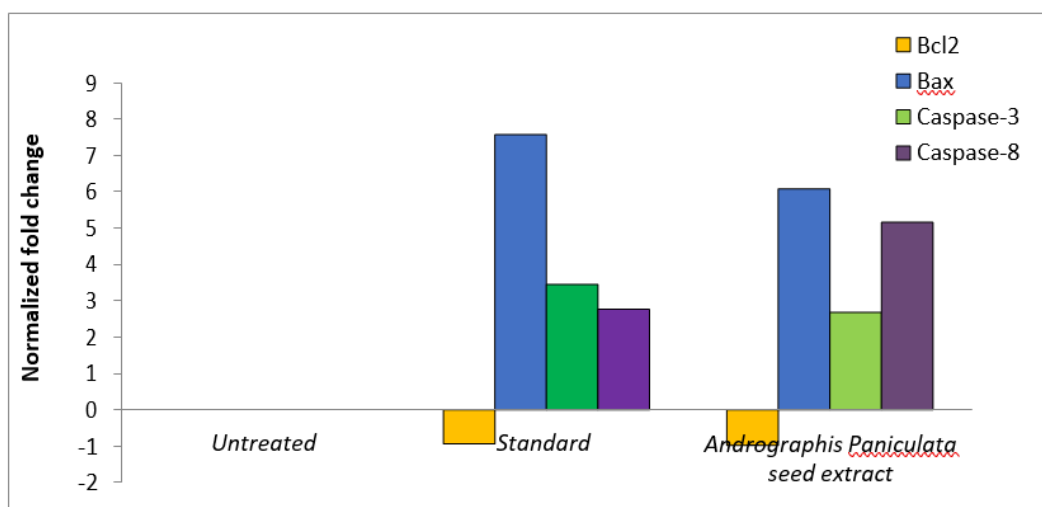


Figure 5 : Relative mRNA expression of Bcl-2, Bax, caspase-3, Caspase-8 in Untreated, Standard and *Andrographis paniculata* seed extract treated HepG2 cells

Discussion

In previous study by Tawit Suriya *et al.*, 2014 reported that ethanolic extract of *Andrographis paniculata* leaf extract showed increased cell arrest at G0/G1 and

G2/M phases in HepG2 and bile duct (HuCCA-1 and RMCCA-1) cells. Thus concluding that *Andrographis paniculata* to be a promising plant for treatment of Cholangiocarcinoma. The present anticancer study illustrated that the ethanol extract of *Andrographis paniculata* seeds showed significant cell inhibition after the treatment period of 24 hrs at IC₅₀ concentration of 103 µg/mL against HepG2 cell lines showing high % of cells at G2/M stage arrest, compared to untreated cells.

In existing study, (Nida Nayyar *et al.*, 2014) the *Portulaca oleracea* (PO) and *Petroselinum sativum* (PS) extracts showed anticancer activity on HepG2 cell when exposed with 5-500 µg/mL, result showed significant reduced in cell viability in both extract. HepG2 cell exposed with 50 µg/mL and higher concentration of PO and PS lost their typical morphology. The result revealed that the extract showed cytotoxic and HepG2 activities showed high due the presence of secondary metabolites such as phenols, flavonoids, alkaloids and glycosides. The present study illustrates that the ethanol extract of *Andrographis paniculata* seeds showed the significant cytotoxicity activity against HepG2 cells, which results in decrease in viability of cells. The findings showed that the ethanol extract was found to poses high flavonoid content which can also be one of the reason for the cytotoxic activity on the hepG-2 liver cancer cells. (Sahabjada Siddiqui *et al.*, 2019) evaluated that the ethanol extract of extract of *Ajwa date pulp* induced apoptosis on HepG2 cells showed significant dose and time dependent inhibition of HCC cell growth. *Ajwa date pulp* extract induced elevation of S and G2/M phases of cell cycle and proportionally decreases in the percentage of cells in G0/G1 phase inducing apoptosis in HepG2. In the present study, the ethanol extract of *Andrographis paniculata* seed showed significant cytotoxicity activity against HepG2 cells and IC₅₀ concentration show high % of cell arrest at G2/M stage arrest which similar to the std control doxorubicin on HepG2 cells.

Kim in his study reported that *Andrographis* found to be activate proapoptotic Bcl-2 family in human leukemia HL-60 cells the same has been reported in our study that *Andrographis paniculata* seed extract exhibits apoptosis by down regulation Bcl-2. (Kim *et al.*, 2005) In Yanmei *et al.*, in his study reported that *Andrographis paniculata* extract on HepG2 increases Bax protein and no change in antiapoptotic Bcl-2 protein in treated cells. Loss of mitochondrial membrane result in caspase-9 and caspase -3 were activated while caspase-8 was not affected in HepG2 cell lines compared to our present study Bax , caspase-3 and caspase-8 act as proapoptotic gene and the BCL-2 protein activate as antiapoptotic gene.(Yanmei *et al.*, 2015) Another study Luisa (20) has reported that Apoptosis was confirmed by caspases activity assay as well as gene expression *Bcl-2*. Caspase-8, 9, and 3 activity with downregulation of *Bcl-2* which illustrate intrinsic and extrinsic pathway in MCF-7, same has been reported in reverse way in our present study that *Andrographis paniculata* seed extract obtained result suggested the Bax, Caspase-3, Caspase-8 genes was up regulated in treated groups and Bcl-2 is down regulated in treated groups.(Luisa *et al*, 2010)

Conclusion

The present study is the first to demonstrate the anti-cancer properties of *Andrographis paniculata* seed extract to have potent cytotoxic activity against HepG2 cells. A significant increase in cytotoxicity were recorded with increase concentration of the *Andrographis paniculata* seed extract. The cells treated with standard and *Andrographis paniculata* seed extract with IC50 concentration are showing high % of cells at G2/M stage arrest, when compared to untreated cells. Hence the cell cycle got arrested at G2/M stage accompanied by induction of apoptosis through both extrinsic and intrinsic apoptosis pathways. The obtained results suggest that relative gene expression level of Bax, Caspase-3, Caspase-8 genes was up regulated in treated groups compared to untreated group and other gene, Bcl-2 is down regulated in treated groups compared to untreated group. Beta actin was used as internal control in the current study. The result of our study suggest that *Andrographis paniculata* seed extract suppress HepG2 cell proliferation, induces cancer cell apoptosis and inhibits cancer cell growth.

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