EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 3
645-649
Year: 2018

INVITRO PHARMACOGNOSTIC EVALUATION & ANTICANCER ACTIVITY OF *ANDROGRAPHIS PANICULATA* EXTRACT IN HEP G2 CELL LINES

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Article Received on 10/01/2018 **Article Revised on 30/01/2018** **Article Accepted on 20/02/2018**

ABSTRACT
Cancer (medically termed as malignant neoplasm) is a class of disease which affects any parts of the body, with a characteristic feature in common, meant the uncontrollable cell growth, which divide and grow uncontrollably forming tumor. Human liver cancer is the fifth most common, which is a serious pop of most developing countries. It is a sad fact that, majority of hepatocellular carcinoma dies within one year after diagnosis. At present the treatment of hepatocellular carcinoma includes surgery and chemotherapy, but the curative effect of chemotherapy is not good enough since with major side effects. Therefore peeping for high efficient antitumor drugs remains a hotspot in life science research. Herbs have been a richest source of therapeutic agent and still an important source of new drugs for diseases such as cancer. Certain plant components capable of killing cancer cells are known to suppress tumor promoting action of Immune and other tumor stromal cells. Cancer is a group of characteristic disorder which shows "out-of -control "growth of cells and division. In contrast to a normal cell, a cancerous cell follow damaged or altered pattern that make a cell tumorous. When tumor spreads all over the body, it grows, invade other healthy tissues, difficult to treat. The present investigation proved that the ethanol extract of *Andrographis paniculata* significantly suppresses the growth and induces apoptosis in Liver cancer Cell lines (Hep G2).

KEYWORDS: Andrographis Paniculata; Hep G2 Cell Lines; Apoptosis.

INTRODUCTION
Cancer (medically termed as malignant neoplasm) is a class of disease which affects any parts of the body, with a characteristic feature in common, meant the uncontrollable cell growth, which divide and grow uncontrollably forming tumor. Human liver cancer is the fifth most common, which is a serious pop of most developing countries. It is a sad fact that, majority of hepatocellular carcinoma dies within one year after diagnosis. At present the treatment of hepatocellular carcinoma includes surgery and chemotherapy, but the curative effect of chemotherapy is not good enough since with major side effects. Therefore peeping for high efficient antitumor drugs remains a hotspot in life science research. Herbs have been a richest source of therapeutic agent and still an important source of new drugs for diseases such as cancer. Certain plant components capable of killing cancer cells are known to suppress tumor promoting action of Immune and other tumor stromal cells.

Andrographis paniculata (Burm.f.) Wall.ex Nees., (FamilyAcanthaceae) is a most annual herbaceous plant native of Southern Asia, has been reported to exhibit various mode of biological activities in vivo as well as in vitro viz., antibacterial, antiviral, anti-inflammatory, immunomodulating immunostimulatory and anticancer potential therapeutic action in curing liver disorders. *Andrographis paniculata* may have anti tumorous property which is evident from my GCMS report consisting several phytochemicalswhich is an anticancerous compound. Hence in this study I am thrived in finding the cytotoxicity and apototoxicity of *Andrographis paniculata* on Hep G2 Cell lines.

MATERIALS AND METHODS
Preparation of Media & Reagents required for Cell Culture
Preparation of DMEM for Cell Culturing
DMEM media was dissolved in 900 ml of autoclaved distilled water in a conical flask under sterile condition. To it about 3.7 g of Sodium bicarbonate was added and stirred until it is completely dissolved to form a uniform solution, then the pH of the medium was adjusted to 7.2 with 1N HCl. Following this antibiotics such as penicillin, streptomycin, gentamycin amphoterincin B was added. Finally, 10% FBS was added and the medium

was sterilized using 0.2 micron filter under pressure and stored at 4^0C.(Joshua Shemer et al).

Preparation of Saline: Trypsin Versene (STV)
10X Saline: 8 g NaCl, 0.4 g KCl, 1.0 g D-Glucose and 0.35 g $NaHCO_3$ were dissolved in 100 ml water. 10X saline was filter sterilized and stored at 4^0C.

Versene: 1 g of EDTA was weighed and added to 90 ml of distilled water. Then it was completely dissolved by adding 5N NaOH drop by drops then it is filtered, sterilized and stored at 4^0C.

STV Preparation: 100 mg of trypsin, 10 ml of 10X saline and 2.5 ml of versene was added and was made upto 100 ml using double distilled water. It was then filtered sterilized and stored at 4ºC.

Cell Culture
HepG2 cell lines were grown on polystyrene coated flasks with DMEM as the growth medium. The cell line was passaged after attainment of confluency, that is after every 2 days. Passaging was performed in a laminar flow hood as explained below.

The culture medium was removed completely and the traces of medium were removed by washing with STV then to it 2.5ml of STV was added to the flask and incubated at 37ºC for few minutes until the cells starts detaching from the surface. After complete detachment, STV action was neutralized using DMEM containing serum. The cells were pelleted by centrifugation at 1500 rpm for 3 min and the supernatant was discarded. The cell pellet was resuspended in fresh medium and seeded into flask or plates according to the requirement and incubated at 37ºC.

Preparation of Whole Plant Extract
The whole plants, free from soil matter was shade dried and powdered by using hand pulveriser then it is extracted with 70% Ethanol by soxhlet apparatus. The powdered plant sample was packed in a thimble, sealed properly with cotton. Ethanol in the round bottomed flask is boiled up to its boiling point, the vapours of it passes through the packed powder and collected as condensed extract in the RB flask. After repeated extraction of about 15-20 cycles the extract was evaporated to expel solvents, which was then used for further investigation.

Preparation of Drug
Andrographis paniculata whole plant extract was suspended in 1% dimethyl sulfoxide (DMSO) just before treatment and the final concentration of DMSO in the culture medium was 0.01% W/V and 0.01% DMSO was used as coolntr.

Cytotoxicity Assay Using MTT
MTT assay is a calorimetric method used to measure cell viability. Yellow MTT (3-(4,5-Dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. Different concentration of extracts was prepared as 100ng, 10ng, 1ng,1µg,10 µg, and 100µg respectively. Then the cells were grown in 96 well plates and treated with different concentration of the extracts. After 24hrs of incubation, cells are treated with MTT reagent and incubated for 2-4 hours. The reaction is terminated by aspiration of the media, the formed formazan crystals were dissolved in DMSO and the absorbance was read at 595nm which is directly proportional to cell viability. Based on cell viability, % cytotoxicity of the extracts was calculated as below.

$$\frac{\text{(Mean absorption of the control cells - Mean absorption of the test cells)} \times 100}{\text{Mean absorption of the control cells}}$$

Apototic Morphology Analysis
Dual Staining
Hep G2 cell lines were grown in 30mm petri dishes of concentration of 1.0×10^6 cells/plate. Then the plates were incubated for 48 hours supplemented with DMEM consisting 10% FBS to obtain monolayer of cells and then it is treated with 30µg of Drug prepared. After 24 hours the cell suspension is washed with cold PBS and then added to DMEM medium. 2 µl of combined dye consisting EQ & AR (each 100µg/ml) was added to 20µl of cell suspension. Then 5 µl of stained suspension was transferred to a glass slide which is analysed by fluorescence Microscope.

Intracellular ROS Assay
Intracellular ROS assay The effect of sample (MS1) on intracellular free radical production was assessed by 20, 70 –dichlorofluorescein diacetate (DCFDA) fluorescent dye. HepG2 were seeded in 96-well plates and up to 70 - 80% confluence, following which the cells were treated with MS1 and MS1 positive control drug. After the stipulated time (24h), cells were incubated with 20 mM of DCFDA for 30 min at 37 C. Finally, the supernatant was removed and relative fluorescence was measured using fluorimeter with excitation at 485 nm and emission at 530 nm.

Measurement of Lipid peroxidation byproducts & antioxidants
Hep G2 cells were seeded in a T75 flask at a density of 1×10^6 cells/ flask for a day. The cells were then harvested by trypinisation and washed with PBS. Then the washed out cells are suspended in 130mM KCl, 50mM PBS and 10µM dithiothreitol, then it is centrifuged at 20,000 RPM for 15min at 4˚C, then the supernatant was collected and subjected to biochemical estimations includes lipid peroxidation by product thiobarbituric acid (TBARS) & enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutatathione peroxidase (GP_x).

RESULTS AND DISCUSSION

Cytotoxicity Assay Using MTT

Naturals are renowned to be with excellent therapeutic properties with tolerability and reliability for the development of newer drugs. Cytotoxicity tests generally posses a broad spectrum of sensitivity in developing novel anticancer drugs, which inhibits the metabolism of cancer cells of both animal & human origin. Supplementation of the extract with the culture medium inhibits growth of Hep G2 Cell lines in Dose – Time Dependent manner, reveal the Cytotoxicity of the extract. The cells were treated with gdifferent concentration of the extract and the data are expressed as means ± SD of six independent experiments. Hep G2 Cell lines requires a concentration of 100µg of the extract to induce 72% of cancer cell death, whereas the positive control (cisplatin) of concentration 10ng induces only 31% of cancer cell death. (Table 1 & Figure 1).

Table 1: Time dependent Effect of *Andrographis paniculata* whole plant extract on Hep G2 Cancer Cell Line Cytotoxicity.

Concentration	Inhibition of Cell Viability / 24 Hours	SD
SC	4.122246	0.41855
MS1ng	23.45416	0.767725
MS10ng	25.01777	1.178925
MS100ng	36.6027	0.835723
MS1ug	53.87349	0.211343
MS10ug	69.36745	1.242328
MS100ug	72.1393	1.15825
P.Control	30.7747	0.987338

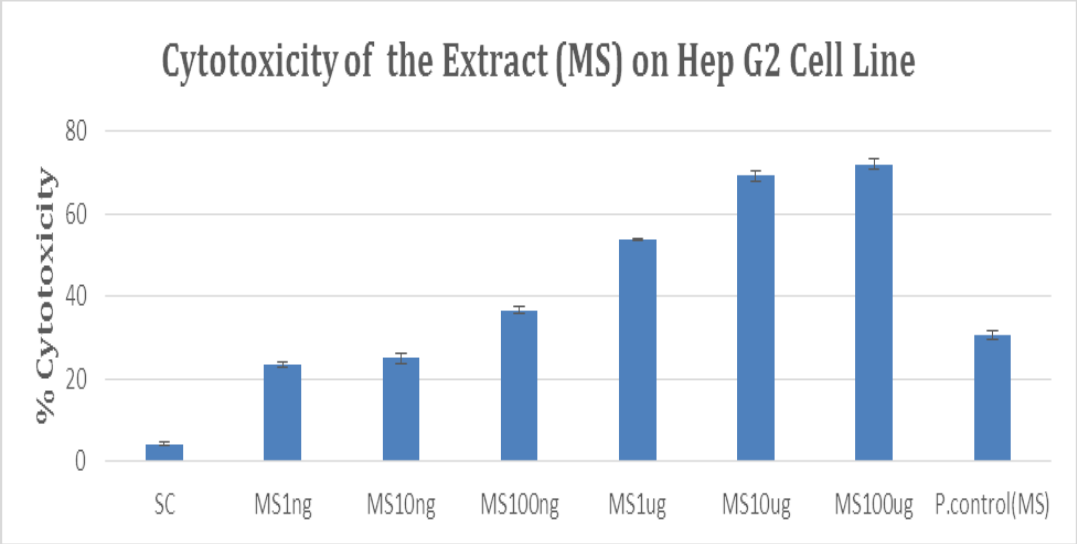
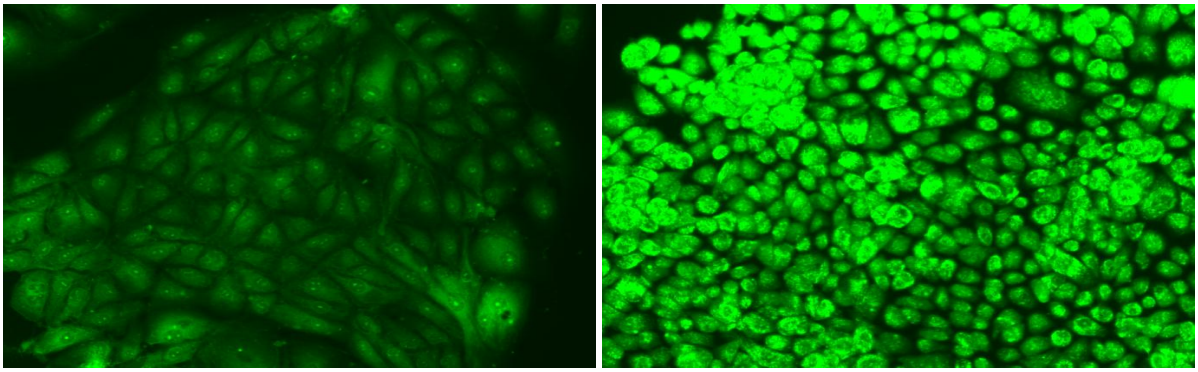


Figure 1: Effect of *Andrographis paniculata* whole plant extract on Hep G2 Cancer Cell Line Cytotoxicity.

Apoptotic Morphological Analysis

Apoptosis refers Programmed Cell Death and literally it is defined as a physiological process leads to cell death far distinct from necrosis. Thus apoptosis can be considered as a therapeutic target for cancerous cells, while apoptosis induced by harmful stimuli should be prevented in normal cells. Biochemically apoptosis involves chromatin condensation, cell shrinkage, DNA fragmentation, Plasma membrane blebbling and formation of membrane enclosed apoptotic bodies. In this study Hep G2 cell lines treated with *Andrographis paniculata* whole plant ethanolic extract was stained with AO/EtBr staining, it exhibits many characteristic properties of apoptosis such as Cell Shrinkage, Loss of cell membrane integrity, impactation of Nuclei & most cells appears as granules in size and shape, while no such changes quoted above are seen in control cells.



A B

Figure 2: Effect of *Andrographis paniculata* whole plant extract on cell morphology with Acridine Orange /Ethidium Bromide Staining

A- Control cells showing well defined cellular morphology

B- Test cells characteristic properties of Apoptosis

Intracellular ROS Assay

ROS involves in triggering apoptotic signals by inducing depolarization of mitochondrial membrane which lead to the increased level of pro-apoptotic molecules intracellularly. Treatment of *Andrographis paniculata* extract on Hep G2 cell lines shows significant increase in the intracellular ROS production compared with the control cell lines. However more amount of ROS was observed in 100ng / 24 Hours.

Table 2: Effect of *Andrographis paniculata* whole plant extract on the Production of ROS.

Concentration	Production of ROS / 24 Hours	SD
SC	20.74283	4.645795
MS 1ng	28.19599	3.672866
MS 10ng	36.68546	6.563833
MS 100ng	38.17775	8.888237
MS 1ug	33.78378	5.275939
MS 10ug	25.50158	7.007719
MS 100ug	20.65992	6.778126
P.Control	49.46112	6.675961

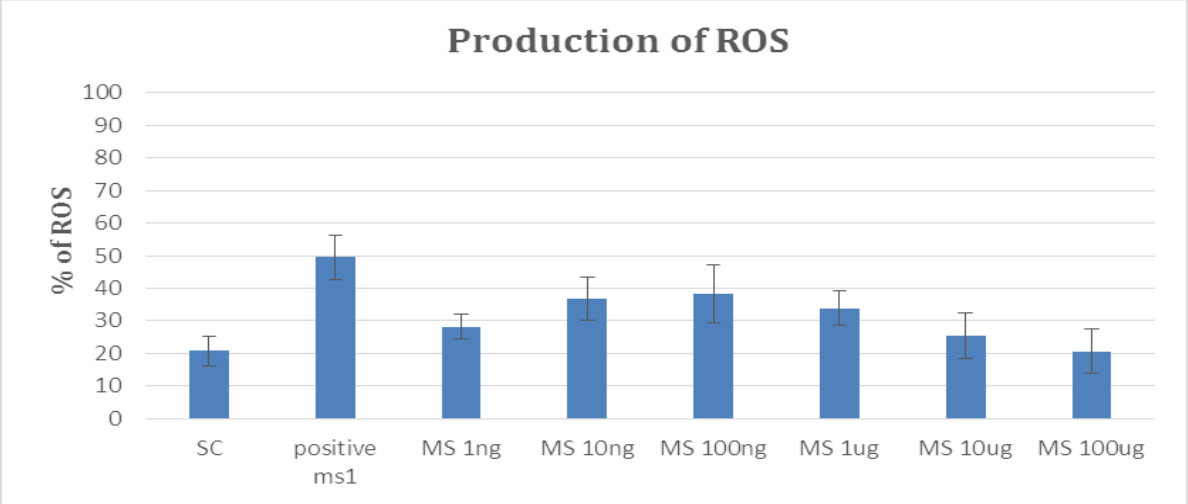


Figure 3: Effect of *Andrographis paniculata* whole plant extract on the Production of ROS.

Measurement of Lipid peroxidation byproducts & antioxidants

Enhanced lipid Peroxidation and decreased activityof antioxidant enzymes (SOD, CAT & GPx) conclude with *Andrographis paniculata* whole plant extract ROS Production. Hence our Extract excerts beneficiary in cancer cells in the presence of low antioxidant defense.

Table 3: Effect of *Andrographis paniculata* whole plant extract on Antioxidant Enzymes.

Test Parameter	Hep G2 Cell lines			
	Control	Positive control	MS1 Drug 100ng	MS1 Drug 1000ng
GPx	60mU/ml	25 mU/ml	39 mU/ml	45mU/ml
CAT	35 mU/ml	21 mU/ml	23 mU/ml	29 mU/ml
SOD	29 mU/ml	15 mU/ml	22 mU/ml	28 mU/ml

To add, the decreased activity of antioxidant enzymes may also be summated with cell proliferation, induction of tumor cell death & alterations in the levels of oxidative stress markers.

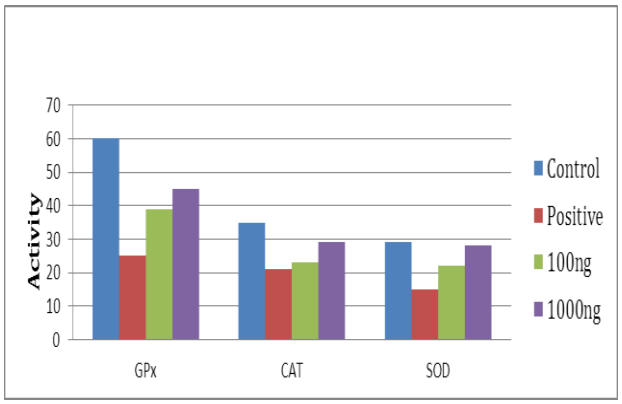


Figure 4: Effect of *Andrographis paniculata* whole plant extract on Antioxidant Enzymes.

CONCLUSION

Apoptosis is an ideal cancer therapy strategy. To conclude my study pin pointedly reveal the selective induction of cell death through ROS dependent apoptotic pathway and thus I strongly suggest my study that the whole plant ethanolic extract selectively posses potent anti cancer properties and hence *Andrographis paniculata* ethanolic extract can be used as the potential candidature for the development of anticancer drug for treating liver cancer.

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