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Anti-proliferative activity and immunomodulator effects of *Andrographis paniculata* (Burm.f.) Wall. ex Nees against cancerous cell line

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ABSTRACT

Cancer not only affects the quality of lives of people but also it remains as a major economic burden. Hence there is a big avenue for research to develop new as well as cheap chemotherapeutics which will solve this mammoth task of eliminating cancer. In the ethnomedical approach, credence is given to oral or written information on medicinal use of the plant and based on this information this study was planned. Andrographis paniculata has been reported to have a broad range of pharmacological effects including anticancer. In this study the plant extracts were studied on human Peripheral Blood Mononuclear Cells (PBMC) and on HepG2 cell lines. Immunomodulatory and cytotoxic study was performed. The ability of the cell to proliferate and retains its normal morphological characteristic was determined by performing clonogenic assay. The content of Bcl-2 protein in HepG2 cells was analyzed using western blotting. For mitogen activation of mononuclear cells, the production of IL-2 was determined. The results of the study revealed, the extracts tested were not at all toxic at any of the concentrations. Protein and enzyme based assays SRB and MTT also revealed that the extracts were not toxic instead the results showed that the plant extracts proliferated the cells. By clonogenic assay the extracts showed antiproliferative activity on Hep G2 cell line. The results of this study clearly show that Andrographis paniculata used in this study can be effectively used to cure cancer.

Keywords: *Andrographis paniculata*, PBMC, HepG2 cell line, SRB and MTT.

INTRODUCTION

Cancer is one of the most dreaded diseases of the 20th century and spreading further continuously with increasing incidence in 21st century. As of 2004, worldwide cancer caused 13% of all deaths (7.4 million). The leading causes were: lung cancer (1.3 million deaths/year), stomach cancer (803,000 deaths), colorectal cancer (639,000 deaths), liver cancer (610,000 deaths), and breast cancer (519,000 deaths) [1]. It is hyper proliferative disorder that involves transformation, dysregulation of apoptosis, proliferation, invasion, angiogenesis and metastasis [2]. It is a major health problem both in developed and developing countries. Malignancies are one of the largest single-cause of death claiming over six million lives every year [3].

Immunomodulators are substances that have been shown to modify the immune systems response to a threat upon it. They modulate and potentiate the weapons of your immune system keeping them in a highly prepared state for any threat it may encounter. With this balancing effect, all subsequent immune responses improve. When your immune system is in this highly prepared state, the invading organisms do not have the time to build up force and strength before the immune system attacks destroys and/or weakens the invader. Immunomodulation is the process of modifying an immune response in a positive or negative manner by administration of a drug or compound [1, 4].

In spite of the recent domination of the synthetic chemistry as a method to discover and produce drugs, the potential of bioactive plants or their extracts to provide new and novel products for disease treatment and prevention is still enormous [5]. The antitumor area has the greatest impact of plant derived drugs, where drugs like vinblastine, vincristine, taxol, and campothecin have improved the chemotherapy of some cancers. Plants have an almost unlimited capacity to produce substances that attract researchers in the quest for new and novel chemotherapeutics [6]. The continuing search for new anticancer compounds in plant medicines and traditional foods is a realistic and promising strategy for its prevention [7]. Numerous groups with antitumor properties are plant derived natural products including alkaloids, phenylpropanoids, and terpenoids [8, 9] have been practiced these days.

Over the years plants have been a prime source of highly effective conventional drugs for the treatment of many diseases. There are different approaches for the selection of plants that may contain new biological agents. In the ethno medical approach, credence is given to oral or written information on medicinal use of the plant and based on this information the plant is collected and evaluated [10]. A retrospective analysis of the NCI program showed that the percentage of active leads based on ethnomedicine was substantially above that based on taxonomy, which itself was more than the active leads identified through random screening. The ethnomedicinal value of plants provides evidence of their biological activity that can be further utilized for the drug discovery process [11, 12]. In the present study the anti-proliferative activity and immunomodulatory effect of *Andrographis paniculata* against cancerous cell line HepG2 was studied.

MATERIALS AND METHODS

Collection and processing of the plants

Andrographis paniculata was identified and authenticated by a Botanist. After that the healthy plants were collected, washed with running tap water and were shade dried. Care was taken to avoid fungal contamination while drying. The dried plant material was stored for further test.

Preparation of the extract-cold extraction

The desired plant leaves were powdered making use of mechanical blender (Sumeet). A known quantity of the plant powder was taken in a clean conical flask. Known volume of 100% distilled ethanol was added to that and was kept in a shaker for 3 days. After that it was subjected for centrifugation. The supernatant was collected. This was repeated for few more times till the supernatant becomes colorless. The supernatants were pooled and dried. The dried extract was weighed and stored which were further used for the analysis. Known quantity of the dried plant extract was taken and was dissolved in PBS in the ratio of 1:1. This was vortexed and filters sterilized. This acted as the stock. From the stock known concentration was taken for further assays.

Immunomodulatory and cytotoxic nature of the plant extracts on human PBMC

Immunomodulation denotes a change, a strengthening of suppression or potentiation, of the indicators of cellular and humoral immunity and nonspecific defense factors. Cytotoxicity is the quality of being toxic to normal cells. The plant extracts were tested for immunomodulatory and cytotoxic property against human PBMC by performing three assays Viz. Dye exclusion method, Sulforhodamine B assay and MTT assay.

Separation of human Peripheral Blood Mononuclear Cells-PBMC [13]

Human PBMC was separated from heparinized human blood based on density gradient centrifugation.

Tryphan blue dye exclusion method Principle

A known concentration of the extract was taken and reconstituted with known volume of PBS pH-7.2, which was the stock concentration of the extract. This was centrifuged and the supernatant was membrane filtered and used. From the stock various concentrations of the extracts were prepared (20 µg, 40 µg 80 µg and 160 µg) and subjected for the *in-vitro* analysis on human PBMC. Constant cell number was maintained. The assay was performed in a 96

well tissue culture plate (Greiner, U.S.A), using various negative controls like plain media, complete media, vehicle, cell and extract. Positive controls like a known immunomodulator-PHA and known cytotoxic compound-LPS were also maintained. After the addition of cell, media and extract, the cultures were incubated in an incubator (TC2323, Shel lab, U.S.A) with 95% air, 5% CO_2 and humidified atmosphere at 37˚C. After the incubation period a suitable volume of a cell suspension (20-200µl) was taken in an appropriate tube and an equal volume of 0.4% tryphan blue was added and gently mixed. It was allowed to stand for 5 minutes at room temperature. 10 µl of stained cells were placed in a haemocytometer and the number of viable (unstained) and dead (stained) cells were counted. The average number of unstained cells in each quadrant was calculated and multiplied by 2×10^4 to find cells/ml. The percentage of viable cells is the number of viable cells divided by the number of dead and viable cells.

Sulforhodamine B assay [14]

Sulforhodamine B or kiton red is a fluorescent dye which uses spanning from LIF (Laser Induced Fluorescence) to the quantification of cellular proteins of cultured cells. The red, solid, water soluble dye is primarily used as a polar tracer. The dye absorbs 515 nm light and emits 586 nm light. It does not exhibit pH dependent absorption or fluorescence over the range of 3 to 10.

After treatment with extract at different concentration and incubation, TCA was added to fix the cells with the substratum. The fixed cells were stained with SRB that stains only cellular proteins. The SRB stained proteins were solubilized using trizma base. The intensity of the colour developed was read using ELISA reader (E1x 800 ELISA reader, Biotech Instruments Inc., USA) at 515 nm. Intensity of the colour developed is proportional to the number of viable cells.

MTT Assay [15]

Yellow MTT (3 - (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. A solubilization solution (usually dimethyl sulfoxide or a solution of the detergent sodium dodecyl sulphate in Dil. Hydrochloric Acid) is added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this coloured solution can be quantified by measuring at wavelength 520 nm by a spectrophotometer.

After treatment with extract at different concentration and incubation, MTT was added to the wells and incubated. During this, the MTT was reduced by mitochondrial dehydrogenase as a result of which the colour gets changed. Lysing solution was added to the wells to solubilize the formazan crystals. The absorbance was read by making use of an ELISA reader (E1x 800 ELISA reader, Biotech Instruments Inc., USA) at 570 nm. The data was analyzed by plotting cell number versus absorbance in a graph. The rate of tetrazolium reduction is directly proportional to the rate of cell proliferation.

Clonogenic assay

The effect of treatment on clonogenic survival of cancer cells was determined using colony formation assay. The cells were treated with increasing concentrations (20, 40 80 & 160µg/ml) of the plant extracts in RPMI-1640 complete medium. Following treatment, the cells were re-plated in triplicate on a 6-well tissue culture plate with 5000 cells/well and cultured in 5% CO2 at 37°C for 8 days with growth media being replaced with/without extracts every 2 days. The cells were then stained with 0.5% crystal violet (in methanol: H2O; 1:1) and the results were observed.

Effect of the extracts and the expression of Bcl-2 protein by Western blotting on HepG2 cells

Cell incubation and drug administration: HepG2 cells taken in the stage of logarithmic growth and were digested with pancreatin, and the cell concentrationwas adjusted by addingRPMI1640 culture medium with 10% fetal bovine serum. The sample was then put in Petri dishes at various concentration in dish and incubated in CO2 incubator for 24 h to make the cells adhere to the inner wall of the dishes. The experiment was performed on a negative control, a positive control, and with different extracts at various concentrations of 20, 40, 80 and 160 µg. The samples were then incubated in the CO2 incubator at 5% CO2 and 37 ◦C for 48 h.

Preparation of the samples: One ml of PBS was added to the culture flask. The cells were scraped using a cell scraper, added to a 1.5 ml centrifuge tube, and centrifuged at 1500 rpm for 5 min, after which the sample was rinsed twice with PBS. One hundred microlitre of cell lysate was added to the sample, which was then whirled, after which the sample was lysed from 30 min in an iced waterbath. It was then centrifuged for 10 min at 4 ◦C and 12,000×*g*.

The supernatant was collected and refrigerated at −20 ◦C for later use. Determination of the protein content was done by Bradford method (Kruger, 1994) to determine the protein content.

Electrophoresis: Twelve percent Resolving Gel and five percent Stacking Gel were prepared. Specimens were taken from the samples according to different protein contents, SDS buffer solution was added and mixed with the specimens, after which the specimens were boiled for 5 min in 100 ◦C water bath. The specimens were mounted after being cooled down. The gel was run at a voltage of 80V, which was then changed to 110V after the specimens had reached the separation gel.

Transfer: Following electrophoresis, the gel was peeled and cut off, and then transferred for 2 h at 50 mA. The membrane was taken out, sealed with TTBS containing 3% BSA, and then set under 4 ◦C for the night. The membrane was rinsed with TTBS three times for 5 min/time, after which murine anti-human Bcl-2 antibody I diluted 1:250 was added. Three hours later, the membrane was again rinsed with TTBS three times for 5 min/time, after which anti-murine IgG antibody II diluted 1:250 and labelled with alkaline phosphatase was added. Beta-actin was used as control. The membrane was taken out after 2 h and rinsed three times with TBS, 5 min each time. Film was then developed using western blot detection reagent.

Quantification of IL-2

IL-2 formerly called as growth factor, is a 14-16 kDA glycosylated polypeptide produced by activated $CD4^+$ Th cells which acts within an autocrine way to promote T cells and NK cells growth. T cells respond to IL-2 via binding to the high affinity IL-2 receptor made up of three subunits (α, β, γ). IL-2 can be found in culture supernatant after mitogen activation of mononuclear cells (PBMC) or T cell clones.

Various concentrations of the extracts were prepared and subjected for the *in-vitro* analysis on human PBMC alone treated with mitogen to quantify IL-2. Constant cell number was maintained. The assay was performed in a 96 well tissue culture plate (Greiner, U.S.A), using various negative controls like plain media, complete media, vehicle, cell and extract. Positive controls like a known immunomodulator (PHA) and a known cytotoxic compound (LPS) were also maintained. To quantify IL-2 secreted mitogen activated human PBMC were treated with the extract. After the addition of cell, media, mitogen and extract, the cultures were incubated in an incubator (TC2323, Shel lab, U.S.A) with 95% air, 5% CO2 and humidified atmosphere at 37˚C for 72 hrs. After the incubation period, the culture supernatant was taken and used for quantification of IL-2 secreted. IL-2 was quantified by making use of IL-2 ELISA kit manufactured by M/s. BioSource, Belgium.

Statistical analysis

Statistical analysis was done using EXCEL (DATA ANALYSIS) for determining ANOVA. A p value of < 0.01 was considered statistically highly significant, $p < 0.05$ significant and $p > 0.05$ in significant.

RESULTS AND DISCUSSION

Immunomodulatory and cytotoxic nature of the extracts

When extracts were treated on PBMC and tested by DEM the results revealed that 20 µg concentration was found to be non toxic up to 72 hours exposure. At 80 µg concentration it was found be an immunmodulator when exposed to 72 hours. Similar results were found at 160 µg concentrations (Figure 1). When extracts were treated on HepG2 and tested by DEM the results revealed that 100 µg concentrations was found to be cytotoxic up to 72 hours of exposure. Similar results were found at 160 µg concentrations (Figure 4). Agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy [17] Immunomodulatory agents of plant and animal origin enhance the immune response of an organism against a pathogen by activating the immune system. Many Indian herbs are being used in traditional practices to cure various human ailments. Immunomodulators are used as an adjuvant in conditions of immunodeficiency in cancer and to a limited extent in acquired immunodeficiency syndrome [18]. However these agents should be subjected to systematic studies to substantiate the therapeutic claims made with regard to their clinical utility Thus in this study the plants chosen were studied for their antiproliferative and immunomodulatory properties. Results of this study showed that extracts increased the proliferation of cells by SRB assay, similarly on another study *Solanum nigrum* shows considerable activity on HeLa cell and little beat effect on Vero cell, and these activities was checked by using second cytotoxicity assay, MTT assay [19]. MTT assay also shows significant effect on HeLa cell and had little beat significant value on Vero cell. The extract reduced cell viability in a concentration-dependent fashion as

shown in the MTT studies. Similar results were observed in a study conducted earlier, where proliferation of T47D cells exposed to the leaf extract was potently inhibited compared to the unexposed cells, based on microscopic studies and no cell apoptosis [18].

Figure 1: DEM on PBMC using *Andrographis paniculata* extracts

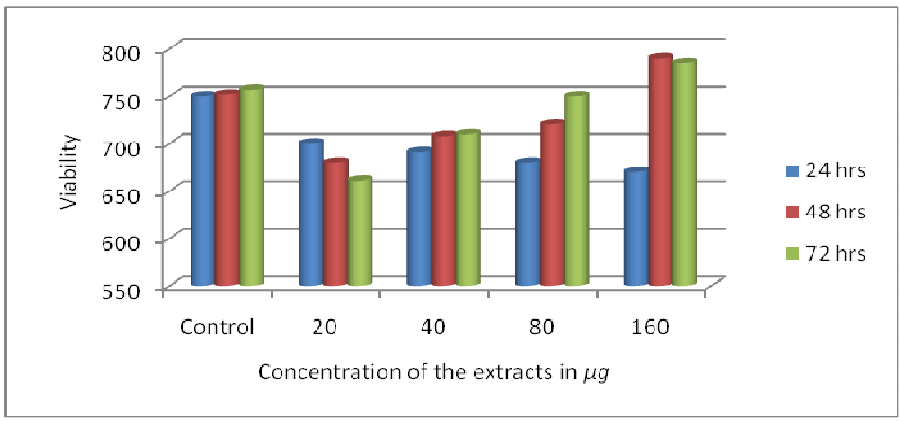


Figure 2: SRB assay on PBMC using *Andrographis paniculata* extracts

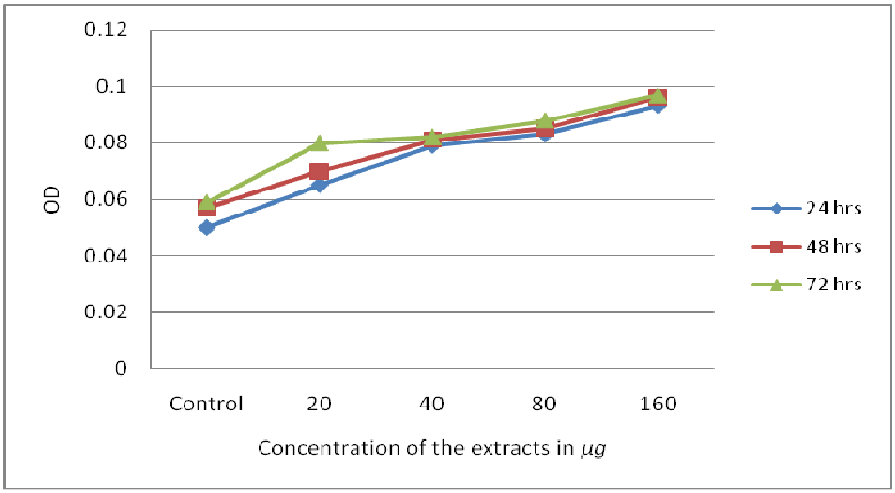
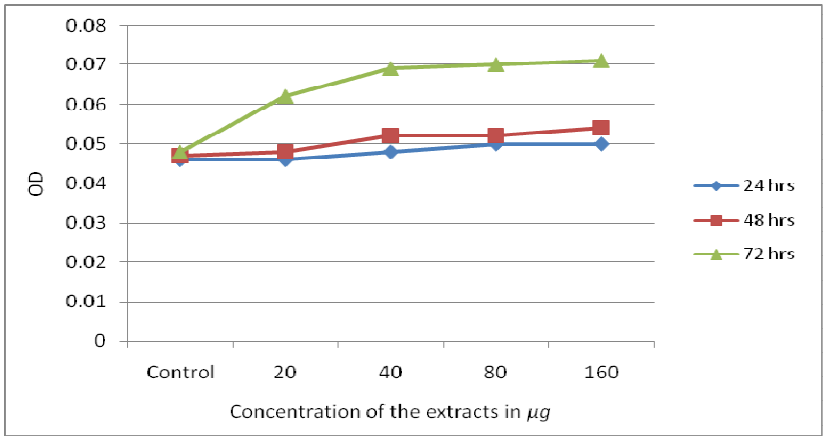


Figure 3: MTT assay on PBMC using *Andrographis paniculata* extracts



Results based on SRB on PBMC showed that *Andrographis paniculata* extracts increased the proliferation of cells evident by increased in the OD values (Figure 2). On HepG2 cell line *Andrographis paniculata* extracts increased the proliferation of cells when time exposure was increased. On PBMC the extracts of *Andrographis paniculata* increased the proliferation of cells and on HepG2 cell line too proliferation of cells were found (Figure 1-8).

Figure 4: DEM on HepG2 cell line using *Andrographis paniculata* extracts

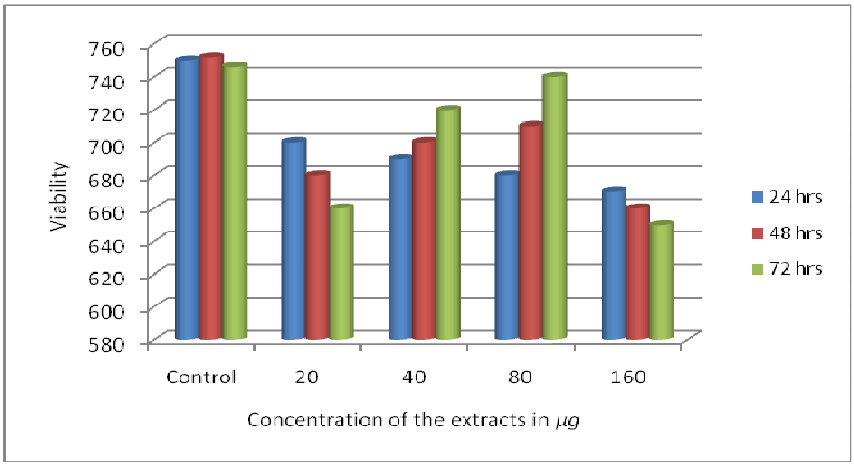


Figure 5: SRB assay on HepG2 cell line using *Andrographis paniculata* extracts

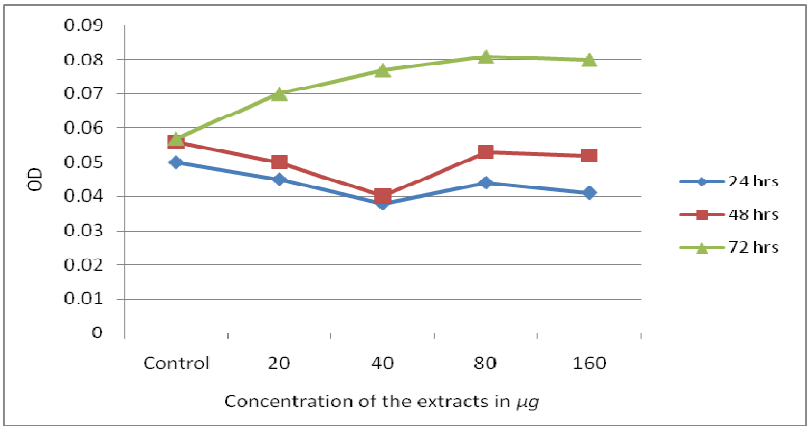


Figure 6: MTT assay on HepG2 cell line using *Andrographis paniculata* extracts

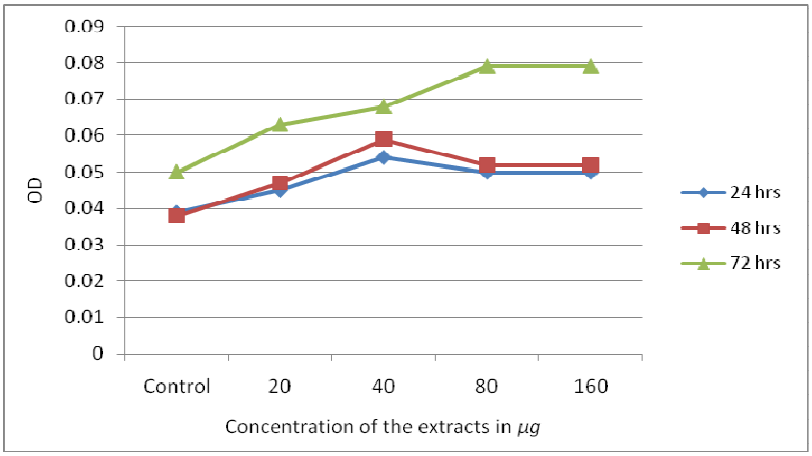
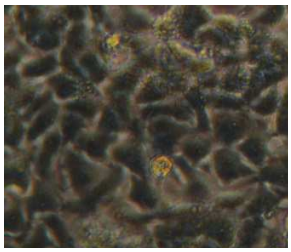
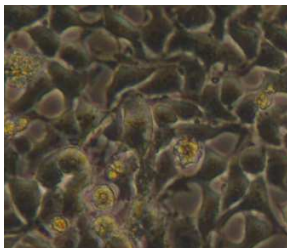


Figure 8: HepG2 Cell line



(Without treatment with the extracts)



Treated with *Andrographis paniculata* (160 µg)

Clonogenic assay and Western Blotting

S. No	Plant Extracts	Concentration of the extract in µg	Time Exposure		
			24 hours	48 hours	72 hours
01	*Andrographis paniculata*	20 µg	NT	NT	NT
		40 µg	NT	NT	AP
		80 µg	NT	AP	AP
		160 µg	NT	AP	AP

Clonogenic assay refers to the ability of the cell to proliferate and retains its normal morphological characteristic when the plant extracts were added to the cancer cells. The loss of reproducible ability and inability to proliferate is the common cause of the cell death, which was evident following a period of extract exposure. Results revealed that the extract has the antiproliferative activity on Hep G2 cell line. Western blot analysis shows, that with the increase in the dosage of the plant extract, the content of Bcl-2 protein in HepG2 cells gradually decreased (Table 1).

RESULTS

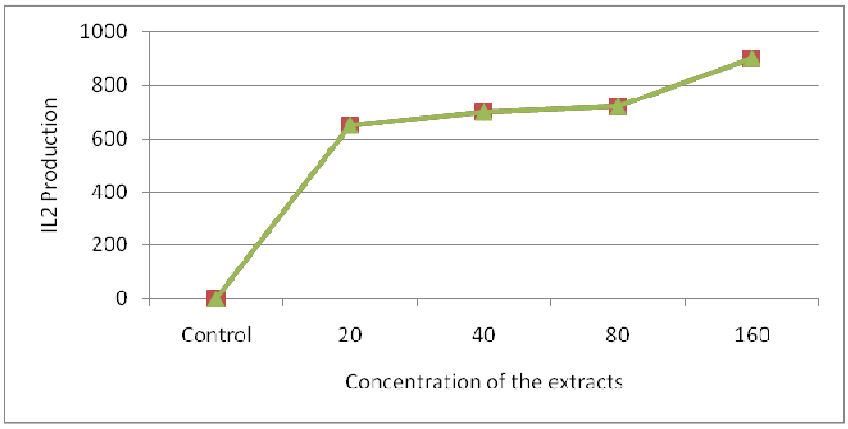
Results of Clonogenic assay on HepG2 Cell lines

NT – Non Toxic / Normal, AP-Antiproliferative

Interleukin 2 (IL_2)

Human PBMC alone when treated with the plant extract which produced good quantity of IL_2 at increased concentration. There was a direct correlation between concentration of the extract and the quantity of IL_2 produced (Figure 7).

Figure 7: IL2 production on PBMC using *Andrographis paniculata* extracts



Cell culture systems have been a useful tool to study the effects and associated mechanisms of individual phytochemicals on cancer. Carotenoids, phenolics and organosulfur compounds are only some of the phytochemicals that have been studied with this approach. Nevertheless, it was proposed that the health benefits of vegetables and fruits might result from multiple combined effects of their phytochemicals rather than from the action of a single active ingredient. In this context, different combinations of phytochemicals have been tested in order to explore synergistic and additive effects on cancer cells proliferation. It has been shown that several whole fruit and vegetable extracts have antiproliferative effects on the hepatocarcinoma cell line HepG2. [20, 21] Showed that aqueous extract of mango inhibited cell proliferation in leukemia cell line HL-60 as well as the neoplastic transformation of BALB/3T3 cells initiated by benzo(a)pyrene. All these studies strongly suggest that mixtures of phytochemicals and extracts of whole foods could be a good strategy to screen cancer chemopreventive activities of phytochemicals present in fruit and vegetables using cell culture models.

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