AN INVITRO CELL LINE STUDY TO EVALUATE THE EFFECT OF *ARKA LAVANA* IN HEPATOCELLULAR CARCINOMA

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***Abstract*- Hepatocellular carcinoma is the fifth common malignancy worldwide with a continuously increasing incidence. Orthotopic liver transplantation, surgical resection and local destruction are the treatment options available for this condition. Further it depends on the extent as well as location of the tumour. The overall disappointing results as well as the expense of the procedure support the research for other more active and specific treatments to be administered alone or with the combination of those therapies. Hepatocellular carcinoma can be understood as an *Avasthabheda* of *Udara, Gulma, Kamala* and *Arbuda*. *Arka lavana* is a unique preparation indicated for *Yakrit Pleeha Rogas*. *Arka (Calotropis procera)* is a herb of choice with promising anti cancerous activity. Hence an attempt has been made to see the anticancerous effect of *Arka lavana* in Hepatocellular carcinoma cell lines.**

Keywords: **Cell lines, HepG2, *Arka lavana*, Cancer.**

INTRODUCTION

The liver is the largest and complex internal organ of the body maintaining the body's internal environment[1]. It is the primary site of biotransformation and detoxification of xenobiotics. The liver is expected not only to perform a physiological function but it has to protect itself against the hazards of harmful toxins, chemicals and medicines. Thus, to maintain a healthy liver is a crucial factor for overall health and wellbeing. But it is continuously and variedly exposed to environmental toxins, and abused by poor drug habits, alcohol which can eventually lead to various liver ailments like hepatitis, cirrhosis, alcoholic liver disease, Hepatocellular carcinoma etc[2].

Cell lines have revolutionized scientific research because of their similarity to primary tissues, low cost, and ease of use and culture. In addition, such cells provide an unlimited supply of biomaterials, and their use in research avoids ethical problems associated with the utilization of animal and human tissues[3]. The use of immortalized hepatic tumor cell lines has become a widespread practice not only for the cancer research, but also for the study of hepatitis B (HBV) and hepatitis D (HDV) viral infections. Currently, there are about 40 various hepatic tumor cell lines, but the most commonly used are HepaRG, Huh7, SK-Hep-1, Hep3B, and HepG2, obtained from various tumors[4]. Among the cell culture mentioned above, the HepG2 cell line has gained popularity due to its wide range of applications in scientific research.

Arka lavana is well-recognized and highly effective *Lavana Kalpana*. In *Lavana Kalpana,* particular heating pattern is followed for drug along with *Lavana* in *Sharava* (earthen crucible) by subjecting it to *Putapaka* [5]. *Arka lavana* is one of the commonly used preparations containing very safe and easily available herbo-mineral drugs. It is mentioned in various classical texts like *Rasa Tarangini, Bhaishajya Ratnavali* and *Chakradatta.* A scrutiny of classical literature revealed use of *Arka Lavana* in *Yakrit-Pleeha roga, Gulma, Ajirna, Agnimandya* and *Udara*[6].

AIM OF THE STUDY

The present study was designed to assess and establish the role of an aqueous extract of *Arka lavana* as an anticancer agent using the HepG2 cell line.

MATERIAL AND METHOD

Drug source

Arka lavana was prepared according to the classical textual reference from Rasa tarangini and standardization was done in SDM centre for Research, Udupi.

CELL LINE CULTURING

HepG2 cell line was used for study which was purchased from the National Centre for Cell Science (NCCS) Pune. HepG2 cell line was human liver cancer cell line. It was cultured in medium (MEM)E, (Eagle's Minimum Essential Media) containing 10% FBS (Foetal Bovine Serum).

Experimental Study

Aims and Objectives:

- The screening for anticancer activity of aqueous extract of *Arka lavana* against HepG2 cell lines by MTT assay.

Principle

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay is a simple colorimetric assay for screening cell viability, depends on cellular NAD(P)H oxidoreductase enzymes of live cells. The mitochondrial succinate dehydrogenase from live cells which reduces yellow 3-(4, 5-dimethythiazol2-yl)-2, 5- diphenyl tetrazolium bromide (MTT) to an insoluble, dark purple coloured formazan crystals. Further these formazan crystals are solubilised with suitable organic solvent and measured between 500 and 600 nm by a spectrophotometer. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells (Mosmann 1983).

Materials and Methods

- HepG2 cell lines were procured from NCCS Pune (CSIR lab) and grown and sub cultured at Sri Dharmasthala Manjunatheshwara Centre for Research in Ayurveda and Allied Sciences, Udupi. In vitro anticancer activities of *Arka lavana* was carried out by MTT assay.

Preparation of Extract

- 10 g of *Arka lavana* was taken and 100 ml of double distilled water was added and mixed properly.
- The aqueous extract *Arka lavana* of was prepared by running sample using Soxhlet extractor.
- The concentrated solvent mixture was dried over using china dish and net weight of the extract was noted.
- The concentrated solvent mixture was dried over china dish and net weight of the extract was noted and sample was stored at -20°C and further used for anticancer studies.

Table 1: Details of extract value of *Arka lavana*

Details	**Extract value**
Aqueous extract of Arka lavana	3.663g

Chemicals and Reagents:

- Antibiotic 100 X (A001-100ML)
- Cellulose Nitrate Membrane (SF98A-1x 1000NO)
- Cisplatin
- Fetal bovine serum (FBS) (RM1112-100ML)
- Ham's F12k+ FCS
- KCI-(G12A/1012/1707/08) (SDFCL)
- KH2PO, (61754605001730)
- Leibovitz-15+FCS
- L-glutamine (RM049-100GM)
- Minimum Essential Medium with NEAA +FCS MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (TC191-IG)
- NazHPO (61795105001730)
- NaCl (GRM853-500G)
- Nutrient Mixture F-12 Ham, Kaighn's Modification (AT106A)
- Sodium bicarbonate (RM849-500G)
- Tryphan blue (TC193-100G)
- Trypsin (TCL08-1×100ML)

Instruments and Equipment used:

- ESCO Biosafety cabinet Class-II Type A2, Galaxy 170S CO2 Incubator (New Brunswick), Inverted microscope (Motic AE31), Multiplate Reader (TECAN), Water bath (ROTEK RSW 03), Centrifuge (REMI R-8C), Refrigerator, Deep freezer (ROTEK), Hemocytometer, Liquid nitrogen cryocan (BA-35), pH meter (EUTECH), Autoclave (ROTEK).
- Cell culture flasks (T-25 and T-75 flasks), petri dishes, cell culture grade sterile 96 well plates, 2-20 µl, 20-200 µl, 100-1000 µl pipettes, Easy pet, Multichannel pipette, sterile tips, sterile pipette, beakers, microfuge tubes etc., waste container, aluminium foil.

Details of steps involved in the experimental procedure:

- Preparation of medium for cell line study.
- Growing, trypsinization of confluent cells and cell seeding to 96 well plates.
- Screening of cytotoxic and anticancer activity of aqueous extract of *Arka lavana* against normal and cancer cell line for 48 hours followed by addition of MTT dye.
- Calculation of percentage of viable cells with the following formula.

% of viable cells = [(Test sample-blank) / (Control-blank)] x 100

Methodology

Screening for the anticancer activity of aqueous extract of *Arka lavana* against HepG2 cell lines by MTT Assay (Mosmann, T.1983)

Experiment

- The screening for the anticancer activity of aqueous extract of *Arka lavana* against HepG2 cell line by MTT assay (Mosmann, T. 1983)
- Reference: MTT Assay

Procedure

- HepG2 cell line was procured from NCCS Pune and sub cultured using MEM (E) with NEAA + Na Pyrv. and fetal bovine serum
- Around 70-80% confluent HepG2 cell line was taken and medium from the culture flask was removed.
- The cells were washed twice with sterile Phosphate buffer saline (PBS) without disturbing the cells. The wash solution from the culture flask was removed.
- Around 50-100 µl of trypsin (0.25 %) was added to flask and uniformly spread over the cells and culture flask was incubated in incubator at standard condition for approximately 2-5 minutes until cell starting detached from the flask.
- After completion of incubation time, the excess trypsin was removed and flask was gently tapped and observed under inverted microscope to check the activity of trypsin on cells.
- Once the cells are detached from the flask, around 1-2 ml of fresh medium (medium with 10% fetal bovine serum) was added to the flasks.
- Based on the cell density around 1 to 2 ml of medium containing cells transferred to 15 ml sterile centrifuge tube and centrifuged at around 800 to 1000 rpm for 5 to 6 minutes.
- After centrifugation, the pellet was carefully washed twice with PBS and re suspended with growth medium (medium with 10% FBS).
- About 100 µl of tryphan blue (0.04 %) was pipetted to a vial and equal volume of cell suspension was added. Both are mixed carefully and loaded to haemocytometer and counted under inverted microscope.
- After counting the cells, seed the cells to 96 well plate so that, each well having around 10,000 cells/well in 100 µl of medium.
- After completion of seeding the 96 well plate was incubated in CO2 incubator for 24 hours.
- After 24 hours, the old medium from 96 well plate was carefully discarded.
- Cells were carefully washed once with PBS using multichannel pipette.
- Different concentrations of aqueous extract of *Arka lavana* was dissolved in serum free medium and added to different test groups and incubated for 48 hours respectively. Control cells are supplemented with routine growth medium.
- Treat the cells with Cisplatin separately as a positive control.
- After completion of incubation time 20 µL of MTT dye (5 mg/mL. in PBS) was added to all wells in dark.
- Plate was covered with aluminium foil and incubated in CO_2 incubator at 37°C for 4 hours.
- After 4 hours, 100 µL of 0.4 N HCl and isopropanol (1:24) was added to all the wells and mixed carefully to dissolve the crystals.
- By using multi plate reader, the absorbance was recorded at 570 nm and 640 nm reference range.
- The percentage of viable cells were calculated using the formula:

  % of viable cells = [(Test sample-blank) / (Control-blank)] x 100

OBSERVATION

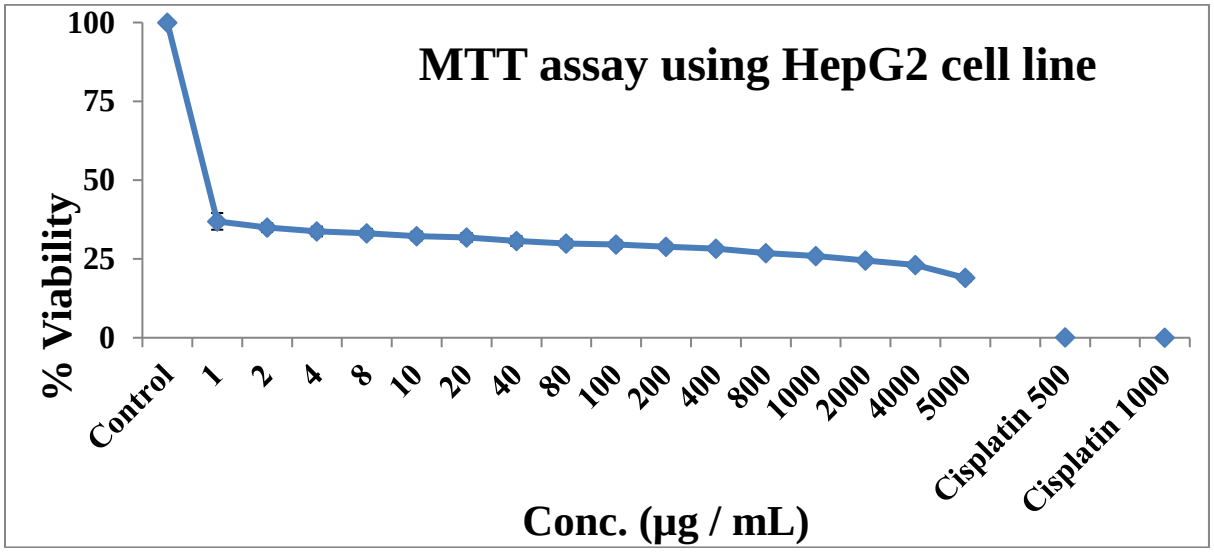

Figure 2: Anticancer activity of *Arka lavana* against HepG2 cell line

Conc. (µg / mL)	% Viability	SD	SE
Control	100	0	0
1	36.919	3.742	2.646
2	34.961	1.969	1.392
4	33.71	2.129	1.505
8	33.188	1.88	1.33
10	32.273	1.96	1.386
20	31.788	2.025	1.432
40	30.655	2.188	1.547
80	29.883	1.897	1.342
100	29.594	1.799	1.272
200	28.891	1.72	1.216
400	28.306	1.645	1.163
800	26.863	0.356	0.251
1000	25.9	0.872	0.617
2000	24.535	1.279	0.904
4000	23.044	1.017	0.719
5000	18.982	1.711	1.21
Cisplatin 500	0.099	0.007	0.005
Cisplatin 1000	0.04	0.007	0.005

Table 25: Results showing viability of HepG2 cell line.

RESULT

- In the present study, different concentrations of *Arka lavana* ranging from 1-5000 µg /ml were selected and screened for anticancer activity with respect to 48 hours.
- Based on the percentage viability the IC_{50} value is found to be around less than 1µg /ml.
- The dose was extended upto 5000 µg /ml and treated cells to verify the efficacy of drug.
- Overall the higher percentage viability value found was 36.919% after 48 hours with minimum drug concentration. (1 µg /ml).
- The lowest viability value found was 18.982% after 48 hours with maximum drug concentration of 5000 µg /ml.
- Overall, it showed that dose dependent decrease in cell viability with respect to different doses of *Arka lavana*.
- Cell viability at 10 µg /ml, 100 µg /ml and 1000 µg /ml are 32.273%,29.594 % and 25.9 % respectively.
- In the present study as a positive control, we treated a group of cells with Cisplatin at a concentration of 500 µg /ml and 1000 µg /ml and observed for 48 hours with the control and treated cells.
- Cisplatin at concentration of 500 µg /ml and 1000 µg /ml showed maximum cell death of 99.01% and 99.9% respectively.
- The overall anti-cancerous activity of *Arka lavana* was found to maximum at a drug concentration of 5000 µg /ml after 48 hours.

DISCUSSION

Human beings constantly struggle against the changing environmental conditions to maintain optimum health and vigour throughout their life. The allegory of the disease called cancer those feeling and meaning that our society has attributed to it and indeed the folklore surrounding it. The treatment of cancer has increased in complexity. Surgical procedures are often less extensive than in proceeding decades however, to limit the extent of surgery, the patient receives adjuvant chemotherapy or radiation therapy, which increases the length and the toxicities of treatment. The modern cancer therapy which is known to burden by drug induced toxic side effects hoping in cancer management health related quality of life is a multi-dimensional construct that includes the subjective appraisal of the patient's physical and mental well-being. Quality of life out comes are also key goal of contemporary cancer management. So, this is our endeavour to handle these suffering.

India is amongst the few contributors in the development and practice of well documented indigenous systems of medicine, the more important being Ayurveda, Unani and Siddha. Ayurveda in specific has incorporated a number of natural products in the treatment of a variety of cancers. In fact the widely used common anti-cancerous drugs in modern medicine like *taxol* and *vinca* alkaloids are obtained from medicinal plants.

Arka lavana is a unique formulation, and specific indication is for *Yakrit, Pleeha Rogas*. The formulation is not popularly used in clinical practice, but has to consider it after seeing its therapeutic indications.

In the present study, different concentrations of *Arka lavana* ranging from 1-5000 µg /ml were selected and screened for anticancer activity with respect to 48 hours. Based on the percentage viability the IC_{50} value is found to be around 4 µg /ml. The dose was extended upto 5000 µg /ml and treated cells to verify the efficacy of drug. Overall the higher percentage viability value found was 62.144 % after 48 hours with minimum drug concentration. (1 µg /ml). The lowest viability value found was 8.664% after 48 hours with maximum drug concentration of 5000 µg /ml. Overall it showed that dose dependent decrease in cell viability with respect to different doses of *Arka lavana*. Cell viability at 10 µg /ml,100 µg /ml and 1000 µg /ml are 43.758%,36.787 % and 21.14 %.In the present study as a positive control we treated a group of cells with Cisplatin at a concentration of 500 µg /ml and 1000 µg /ml and observed for 48 hours with the control and treated cells. Cisplatin at concentration of 500 µg /ml and 1000 µg /ml showed maximum cell death of 98.85% and 99.54% respectively. The overall anti cancerous activity of *Arka lavana* was found to maximum at a drug concentration of 5000 µg /ml after 48 hours.

CONCLUSION

Changing life style has affected us in manifold ways. People are not aware of the do's and don'ts regarding food consumption habits, but are only concerned about the nutritional value and along with this precise improper physical and mental activities. As cancer has ambiguous causative factors, comprehensive consideration of exact origin at the level of digestion and metabolism and the treatment principles is essential. It is often claimed that the malignancy is a disorder produced due to life style modifications. Ayurvedic literature provides plenty of information regarding the references of *Arbuda* .Hepatocellular carcinoma is the most common type of primary liver cancer in adults and is currently the most common cause of death in people with cirrhosis. HCC is the third leading cause of cancer-related deaths worldwide. *Arka lavana* is a formulation indicated for *Yakrit, Pleeha rogas* and its hepatoprotective activity has already been proven. In the study, *Arka lavana* was prepared according to the classical method mentioned in Rasa tarangini. MTT Assay conducted with aqueous extracts of *Arka lavana* in HepG2 cell lines revealed that the drug possess higher anti-cancerous property in Hepatocellular carcinoma. Further trials have to be carried out to bring out this formulation which possess a promising therapeutic effect against Hepatocellular carcinoma.

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