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Original Article

IN VITRO EVALUVATION OF ANTIHEPATOCARCINOGENIC ACTIVITY OF A HERBAL FORMULATION (CHATHURMUKA CHOORANAM) AGAINST HEP G 2 CELLS

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ABSTRACT

Objective: Hepato cellular carcinoma (HCC) is rising alarmingly throughout the globe. The aim of the present study was to investigate the anti-hepatocarcinogenic property of hydro-ethanolic extract of a poly herbal formulation Chathurmuka Chooranam (CMC) in Hep G 2 cell lines.

Methods: *In vitro* apoptosis studies were performed by DNA fragmentation analysis and Clonogenic assay. Gene expression was also carried out on tumor suppressor gene p53 by RT-PCR analysis.

Results: The results revealed that apoptosis was evidenced by fragmentation of DNA and colony formation of apoptotic bodies was modulated by the extract in the Hep G 2 cell lines. The apoptotic process triggered by the hydro-ethanolic extract of the formulation involved the up-regulation of p53 tumor suppressor protein.

Conclusion: The herbal formulation may act as an anti-hepatocarcinogenic drug through the induction of apoptosis.

Keywords: Chathurmuka Chooranam, Apoptosis, Hep G 2 cell lines, p53 and Fragmentation.

INTRODUCTION

Apoptosis is a term used to illustrate the terminal morphological and biochemical events seen in programmed cell death [1]. Cancer commences due to the imbalance between cell proliferation and apoptosis pathway [2]. The plant compound exerts antiproliferative activity in the cancer cells targeting many levels of regulation in cellular growth that induce the apoptotic process.

In vitro cytotoxicity screening models provide an important preliminary data to select, plant extracts with potential antineoplastic properties for future work [3]. HCC is the fifth most common malignancy worldwide and with continuous increasing incidence, the recurrence rate may be as high as 50% at 2 years [4].An extremely promising strategy for cancer treatment today is chemotherapy [5]. However, most of the synthetic chemotherapeutic agents exhibit severe toxicity resulting in undesirable side effects. The discovery of effective herbs and elucidation of their underlying mechanisms could lead to the development of an alternative and complementary method for cancer prevention. Most of the current anti-cancer drugs are derived from plants, which act in different ways, ultimately leading to activation of apoptosis in cancer cells. The herbal formulation has been standardized according to the WHO guidelines, free radical scavenging potential and its cytotoxicity in Hep G 2 cell line was done as demonstrated by Revathi *et al*., (2013) [6] and Revathi *et al*., (2014) [7]. The present study was carried out with an aim of obtaining evidence to confirm that the apoptosis is undeniably a major mechanism mediated through herbal formulation. The anti hepatocarcinogenic potential of the formulation was evaluated by the apoptotic process in HepG2 cells, which was carried out by DNA fragmentation analysis, Clonogenic assay and analysis of gene expression in tumor suppressor protein p53 associated with cell death.

MATERIALS AND METHODS

Preparation of poly herbal extract

The poly herbal formulation contains *Semecarpus anarcadium* (seed), *Curculigo orchioides* (rhizome), *Asparagus racemosus* (root), *Plumbago zeylanica* (root) and *Tinospora cordifolia* (whole plant).The plants were authentified by Botanical survey of India, Coimbatore and ABS botanical garden, Salem.

The herbal plants were equally weighed, shade dried and coarsely powdered. 100 g of dried powder was cold macerated with 50 % hydro ethanol with occasional stirring for 3 days. After 3 days, the suspension was filtered through a fine muslin cloth and the filtrate was evaporated to dryness at low temperature (<40^0 C) under reduced pressure in a rotatory evaporator. The yield of plant extract was found to be 9.64%. The sample was stored in an airtight desiccator and used for further analysis.

Cell culture

HepG2 cells were grown in monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, and 50 IU/ml penicillin and 50 µg/ml streptomycin. Cells were maintained at 37°C in 95% air and 5% CO2 atmosphere, with 95% humidity.

DNA fragmentation analysis

Agarose gel electrophoresis was used to detect the characteristic ladder pattern of DNA fragmentation as described by Yang *et al*. [8] with slight modifications. Cells (2 × 10^5 cells/ml) were exposed to the drug formulation (500 µg and 1000 µg) and doxorubicin for 24 and 48 yr.The cells were gently scraped and harvested by centrifugation. The cell pellets were incubated for 60 min at 50°C in 100 µl lysis buffer (100 mM Tris-HCl-pH 8, 100 mM NaCl and 10 mm EDTA). Proteinase K (10 µl 20 mg/ml stock solution) was then added to the lysis mixture and further incubated for 30 min at 50°C. RNase (3 µl from 10 mg/ml stock solution) was then added and the mixture was incubated for 2 hr at 50°C. DNA was extracted with phenol-chloroform- isoamyl alcohol, subjected to 2.0% of agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light using a gel-doc system (Quantum- ST4 1100/20 M).

Clonogenic assay

A series of cultures in 60mm petri dishes were prepared by seeding at 5 x 10^4 cells/ml in 3 ml of growth medium, and incubated at 37^0C for 48 h, by which time the cultures will have progressed into the log phase. Cultures were grouped as three petri dishes for each of three nontoxic concentrations and three controls. The supernatant medium was discarded, washed the monolayer once with medium and 3 ml of different selected test dilutions were added to the cultures. The culture flasks were returned to the incubator and incubated for 24 h. The medium in the flask was removed and dislodged the cells by trypsinization and suspended in a medium. Cell viability was measured, diluted the cell suspension with growth medium to adjust the cell population to 5x10^3 cells per petri dish

(60mm). Cultures were incubated at 37^{0}C with 5% CO_2 atmosphere for 10 to 14 days. Fixed the cultures in absolute methanol and stained them for 10 min in 1% crystal violet. The number of colonies with a minimum of 32 cells (5 generations) was counted and plating efficiency was calculated by using the following formula

$$Plating\ efficiency = \frac{No.of\ colonies\ formed}{No.of\ cells\ seeded} \times 100$$

Gene expression by p53 analysis

The mRNA expression levels of p53 were carried out using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, the HepG2 cells was cultured in 60 mm petri dishes and maintained in Dulbecco's Modified Eagle's Medium (DMEM) medium for 24 hrs. The DMEM medium was supplemented with Fetal Bovine serum (FBS) and amphotericin. To the dish was added the required concentration of Test sample (700 µg/ml) and incubated for 24 hr. Total cellular RNA was isolated from the untreated (control) and treated cells using Tri Reagent according to manufacturer's protocol. cDNA was synthesized from total isolated RNA by reverse transcriptase kit according to manufacture's instructions (Thermo scientific). Then 20µl of the reaction mixture was subjected to PCR for amplification of p53 cDNAs using specifically designed primers procured from Eurofins India, (5' CTGAGGTTGGCTCTGACTGTACCACCATCC 3' – Forward and 5' CTCATTCAGCTCTCGGAACATCTCGAAGCG 3'- Reverse).

PCR reactions were carried out in a final volume of 50 µl containing 1x PCR buffer and 5 U/µl Taq-polymerase (Fermentas), 1.5 mM MgCl2, 0.2mM of each dNTP and 0.4 µM of each primer. The template was denatured for 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C for 30 seconds and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 10 min. The PCR products were visualized using 1.2% agarose gel electrophoresis with ethidium bromide staining. In negative control, template cDNA was replaced by DEPC water.

RESULTS AND DISCUSSION

The programmed cell death (Apoptosis) is the major mechanism of the cell in which the cancer cells have evolved multiple actions to resist against them. The modulation of apoptosis signaling pathways by natural compounds have been demonstrated to constitute a key event in anticancer activities [9]. In recent years, it was identified that better biological activity had a mixture of plant compounds in the formulation than a single plant compound. The effect of chemopreventive agent is mainly designed by the extent of inducing the apoptosis by the control cells when treated with anticancer drugs.

In order to evaluate the apoptotic effect of the polyherbal extract, DNA fragmentation assay was carried out in Hep G 2 cells after treatment with two different concentrations of the extract (1000 µg and 500 µg). From Fig-1, it was observed that the Hep G 2 cells treated with the extract showed the characteristic feature of apoptosis due to internucleosomal DNA fragmentation pattern. The fragmented DNA was prominent in Hep G 2 cells treated with doxorubicin and plant extract when compared to the untreated control.

A hallmark feature of apoptosis was that the nuclear DNA released from the apoptotic cells was often degraded in an internucleosomal pattern that occur in chromatin at 200-bp intervals [10] [11]. Breakdown of cellular DNA molecule and the release of enzymes from the cytoplasmic membrane loss is one of the signs of inhibition of DNA replication. This damage may be due to the inhibition of topoisomerase II, a key enzyme in DNA replication [12]. However, few studies have been investigating the apoptotic activity of plant compounds. Some studies demonstrated that the low-polarity organic fractions are responsible for the apoptotic effect on the cell lines [13] coinciding with the results obtained by the poly herbal extract.

Lane 1: Standard (Doxorubcin 5 µg/ml)

Lane 2: Plant extract-1000 µg/ml

Lane 3: Plant extract-500 µg/ml

Lane 4: Control (Untreated)

Lane 1 2 3 4

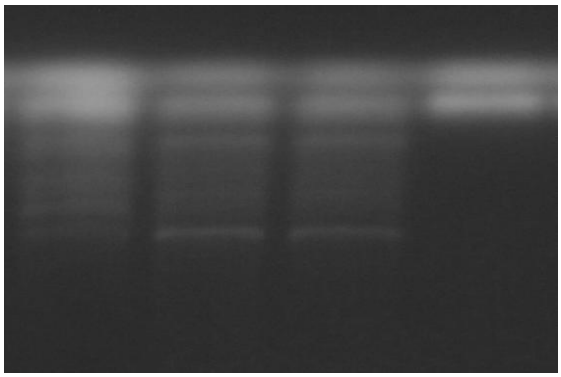

Fig. 1: DNA fragmentation analysis

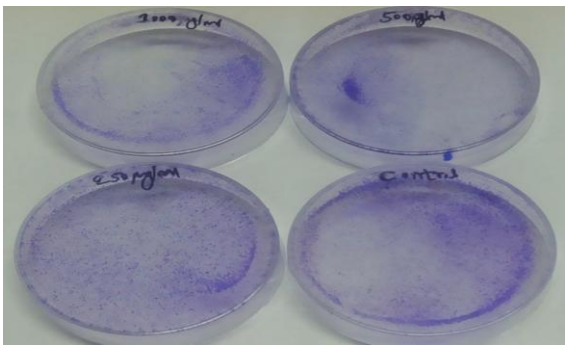

Fig. 2: Clonogenic assay for poly herbal extract in Hep G 2 cell at (14th Day)

From the Fig-2 it was confirmed that the survival of colony formation was 100% in the Hep G 2 cells (control) whereas in the treatment with the polyherbal extract there is a decline in the cancer growth in a dose dependent manner. The cell survival assay requires an ability of a cell to proliferate and have a capacity to form colonies which was evident in the Fig-3, where the percentage of colony formation versus the concentration of the CMC extract showed cell survival turned down after exposure with the plant extract. A cell survival curve describes a relationship between the insult-producing agent and the proportion of cells that survive.

Clonogenic assay is a time consuming assay and it refers to the ability of the cell to proliferate and retains its normal morphological characteristic when the drug was 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 drug exposure. In the present study, it was established that the polyherbal extract has the antiproliferative activity when compared to the control Hep G2 cell line.

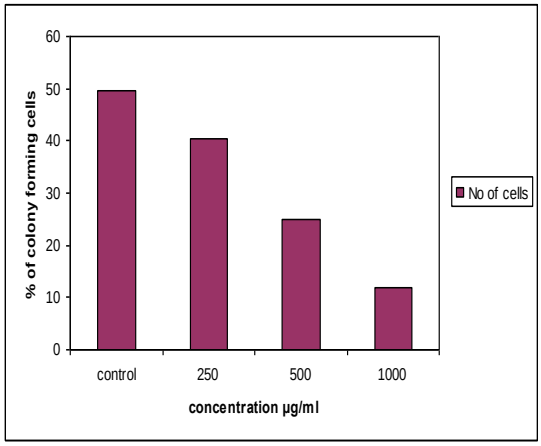

Fig. 3: Colony formation assay of CMC formulation

Lane 1: Marker (1 Kb)

Lane 2 &3: Standard (Doxorubcin 2 µg/ml)

Lane 4 &5:Poly herbal extract(700 µg/ml)

Lane: 1 2 3 4 5

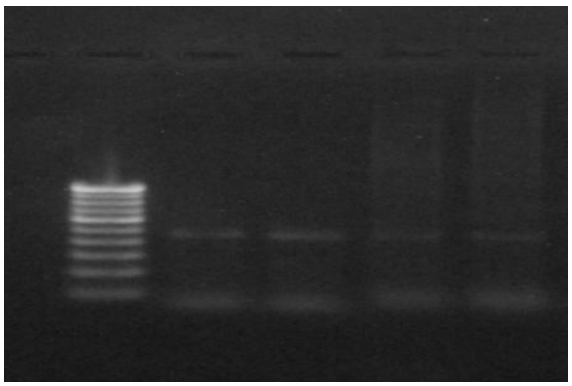

Fig. 4: Gel profile for p53 expression study

The p 53 gene was expressed in doxorubicin and polyherbal extract treated HepG 2 cells. (Fig-4). The p53 is a major defense mechanism of the cell and its function as by two mechanisms one by apoptotic death and the other cell arrest. p 53 plays an important role in cell proliferation and signal transduction pathways. It accumulates in cells after DNA damage, results in cell cycle arrest and induction of apoptosis [14]

Loss of p53 function has been linked to the development of multidrug resistance in many types of cancer [15]. However, exposure to polyherbal extract caused apoptosis in liver cancer cells indiscriminately of p53 status. Further, significantly, it has been shown that up-regulation of p53 can induce apoptosis in the cancer cells.

CONCLUSION

The above said results revealed that the herbal formulation (Chathurmuka Chooranam) has an antiproliferative effect in Hep G 2 cells. The protective effect of the herbal formulation could be due to inhibition of proliferation and induction of apoptosis in Hep G 2 cells through up regulation of p53.

CONFLICT OF INTEREST

Conflict of interest declared none.

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