

INDUCTION OF BAX AND ACTIVATION OF CASPASES BY ETHANOLIC LEAF EXTRACT OF CITRULLUS COLOCYNTHIS(L)- MEDIATED APOPTOSIS IN HUMAN LIVER CANCER CELLS HEP G2

Running Title: Leaf extract of *Citrullus colocynthis* on human liver cancer cells

ABSTRACT:

Aim of the study:

To study the induction of bax and activation of caspases by hydro ethanolic leaf extract of *Citrullus colocynthis* (L)- mediated apoptosis in human liver cancer cells hep G2

Introduction:

Citrullus Colocynthis is a desert plant of the cucurbitaceae family. *C. Colocynthis* grows in many countries with semi dry lands and that seeds are rich in oil which can encourage their sustainable agriculture to produce an economic biodiesel with a competitive price. Apoptosis, also termed as programmed cell death this is a key regulator of tissue homeostasis

Materials and methods

In the present study, a human liver cancer cell line was procured from the National Centre for Cell science (NCCS) ,Pune,India. The cytotoxic potential of plant extract was analysed by MTG assay. mRNA expression of Bax mRNA and caspase-mRNA were analysed by Real-Time PCR using gene specific primers and a comparative CT method was used to analyse expression of genes. The data were analysed statistically using Graph pad prism software Version 5.

Results

MTT analysis showed that *Citrullus colocynthis* a significant increase in proliferation of human liver cancer cells ($P < 0.05$) compared to untreated cells. However, 400 and 500 /g of the extract showed maximum effect in controlling cell growth. Results of the mRNA expression analysis showed that *Citrullus colocynthis* reduced the expression of Bax mRNA/ caspase-mRNA in human liver cancer cells at 400 and 500 /g ($P < 0.05$).

Discussion

The anticancer effect of *Citrullus colocynthis* has been via a variety of pathways which includes apoptotic pathway, anti-inflammatory traits, and antioxidant pathway, etc (1). Effect of HEAM on the mRNA level of tumor related genes (p53, Bcl-2), inflammatory (IL-1 β , IL-6 and c-jun) and anti-inflammatory (IL-4) cytokines. Representative photographs of ethidium-bromide-stained 2% agarose gels for each gene after 7 months of treatment.

Conclusion

The hydroethanolic leaf extract obtained from the *Citrullus colocynthis* exhibited anti-proliferative effect on human liver cancer cells HepG2 cell line by affecting the expression of the Bax mRNA and Bcl-2 mRNA and hindering the cell viability. Hence, it can be a great scope for using the extract into medicinal properties with further studies to improve herbal medicine for cancer over expensive drugs.

KEYWORDS:

Citrullus colocynthis, human liver cancer cells

INTRODUCTION:

Cordyceps militaris is fungus which parasites lepidoptera larvae that is extensively used as folk tonic food and crude drug.(2) Apoptosis, also termed as programmed cell death this is a key regulator of tissue homeostasis (3) A549 and HepG2 are the perfect cell models for lung and liver cancer(4), it aids in precisely cellular mechanisms and gets modulation (5). Activation of caspases is normally related to induction of apoptosis in many types.(6) Stimulation of normal human T-Lymphocytes with allogeneic antigen or mitogen which leads to cell proliferation and caspases-3 activation (7), *Citrullus colocynthis* plant was traditionally for the remedy of diabetes in Sabzevar city, Iran.(8) *Citrullus colocynthis* plant having a strong laxative property and application of high doses of this herbal drug can cause diarrhea(9), painful cramps and blood diarrhea in humans.(10) It is currently advised as a dried fruit for the patients who are suffering from diabetes in several cities in different dosages from 300 to 800 mg/day (11). *Citrullus colocynthis* oil for biodiesel production.(12) *Citrullus colocynthis* belongs to the cucurbitaceae family.(13) *C. Colocynthis* grows in many countries with semi dry lands and that seeds are rich in oil which can encourage their sustainable agriculture to produce an economic biodiesel with a competitive price (14).

The present work was designed to study the potential cell toxic of some novel lip amino acid coated super paramagnetic iron oxide nanoparticle compared with glycine coated and naked SPION on HepG2 cells and it goes(15), before that toxicity of HepG2 cells and their changes in cell cultures are due to SPION exposure were identified using the MTT assay with comparison of result with previous study (14,16). BH3 mimetic molecules such as HA14-1 or ABT-737 have been shown selectively kill cancer cells by its activating the intrinsic signaling pathway of apoptosis, (17) and leaving the normal cells unharmed (18)

At low doses cell death occurs through two different mechanisms apoptosis (19) characterised by the activation of initiator caspases which trigger effector caspases (-cas -3, cas -6, cas -7) to cleave cellular substrate (20). Human liver cancer cells Hep3b and HepG2 were purchased from a cell bank of the Chinese Academy of science(21). RAB9 is one of the members of the Rab GTPase family.(22) RAB9 has isoforms, members of the

RAB9A and RAB9B (23). It has been remembered the existence, in the liver as of a strict connections linking development, regeneration and carcinogenesis(24) mild injury is mainly repaired through compensatory hyperplasia of hepatocytes, severe damage implies the activation of the liver progenitor cell compartment (25)

The aim of the study is induction of bax and activation of caspases hydro ethanolic leaf extract of *Citrullus colocynthis*(L)- mediated apoptosis in human liver cancer cells HepG2

MATERIALS AND METHODS

Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Pvt Ltd, USA. Trypsin-EDTA, fetal bovine serum (FBS), antibiotics-antimycotics, RPMI 1640 medium and phosphate buffered saline (PBS) were purchased from Gibco, Canada. (5,5,6,6-tetrachloro-1,1,3,3 - tetraethylbenzimidazolocarboyanine iodide) and Real Time PCR kit was purchased TAKARA (Meadowvale Blvd, Mississauga, ON L5N 5S2, Canada).

Cell lines and cell culture

Human liver cancer cell line(Hep G2) was purchased from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in DMEM medium (Thermo Fisher Scientific, CA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific, CA, USA) at 37°C with 5% CO₂ .

Cell viability by MTT assay

Cell viability was assayed using a modified colorimetric technique that is based on the ability of live cells to convert MTT, a tetrazolium compound into purple formazan crystals by mitochondrial reductases (Mosmann, 1983). Briefly, the cells (1 ×10⁴ /well) were exposed to different concentrations of *Citrullus colocynthis* leaf extract (100-500µg/ml) with HepG2 cells for 48 h. At the end of the treatment, 100 µl of 0.5 mg/ml MTT solution was added to each well and incubated at 37 °C for an hour. The formed crystals were dissolved in dimethyl sulfoxide (100 µl) and incubated in dark for an hour. Then the intensity of the color developed was assayed using a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed as the percentage of control cells cultured in serum-free medium. Cell viability in the control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability = [A_{570 nm} of treated cells/A_{570 nm} of control cells] × 100.

Gene expression analysis by Real Time-PCR

Samples from each group were submerged in 2 ml Trizol (Invitrogen, Carlsbad, CA, USA) for RNA extraction and stored at -80°C until further processed. cDNA synthesis was performed on 2 µg RNA in a 10 µl sample volume using Superscript II reverse transcriptase (Invitrogen) as recommended by the manufacturer. Real-time PCR array analysis was performed in a total volume of 20 µl including 1 µl cDNA, 10 µl qPCR Master Mix 2x (Takara, USA) and 9 µl ddH₂O. Reactions were run on an CFX96 Touch Real-Time PCR Detection System (Bio-

Rad, USA) using universal thermal cycling parameters (95°C for 5 min, 40 cycles of 15 sec at 95°C, 15 sec at 60°C and 20 sec at 72°C; followed by a melting curve: 5 sec at 95°C, 60 sec at 60°C and continued melting). For quality control purposes, melting curves were acquired for all samples. The specificity of the amplification product was determined by melting curve analysis for each primer pair. The data were analyzed by comparative CT method and the fold change is calculated by 2- $\Delta\Delta$ CT method described by Schmittgen and Livak (2008) using CFX Manager Version 2.1 (Bio Rad, USA).

Statistical analysis

The obtained data were analyzed statistically by one-way analysis of variance (ANOVA) and Duncan's multiple range test with a computer-based software (Graph Pad Prism version 5) to analyze the significance of individual variations among the control and experimental groups. The significance was considered at $p < 0.05$ level in Duncan's test

RESULTS AND DISCUSSION

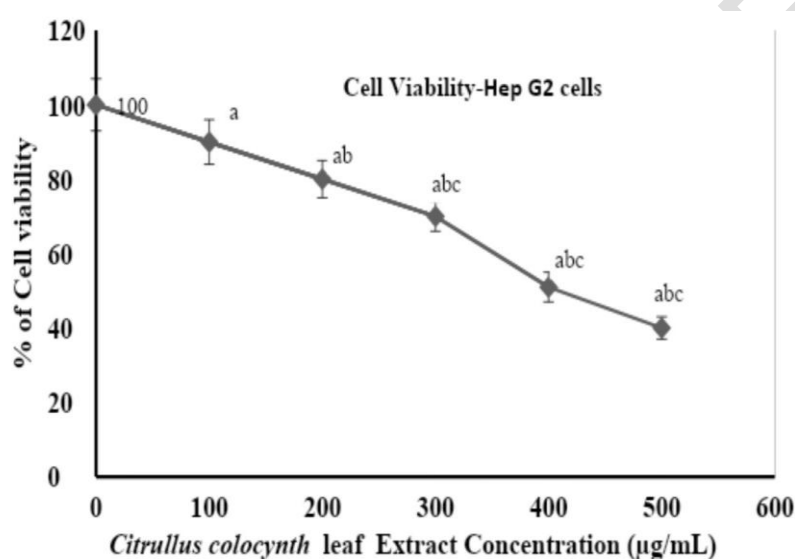


Fig 1: Effect *Citrullus colocynthis* leaf extract on cell viability in HepG2 cells. Each bar represents a mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells, b-compared with 1nM treated Hepg2 Cells.

Gene expression analysis

Bax mRNA expression (fold change over control)

Fig 2:

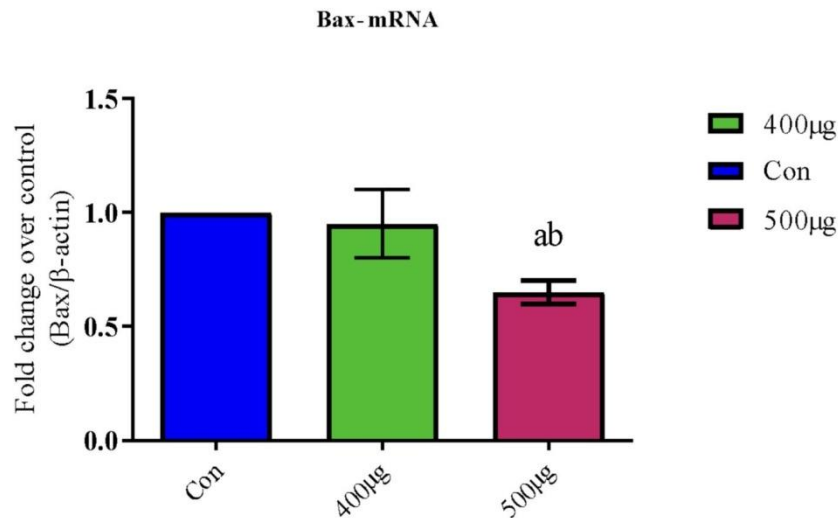


Fig 2: Effect of *Citrullus colocynthis* leaf extract on Bax mRNA expression in Hepg2 Cells. Each bar represents a mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells, b-compared with 400μg treated cells.

Caspase-3- mRNA expression (Fold change over control)

Fig 3:

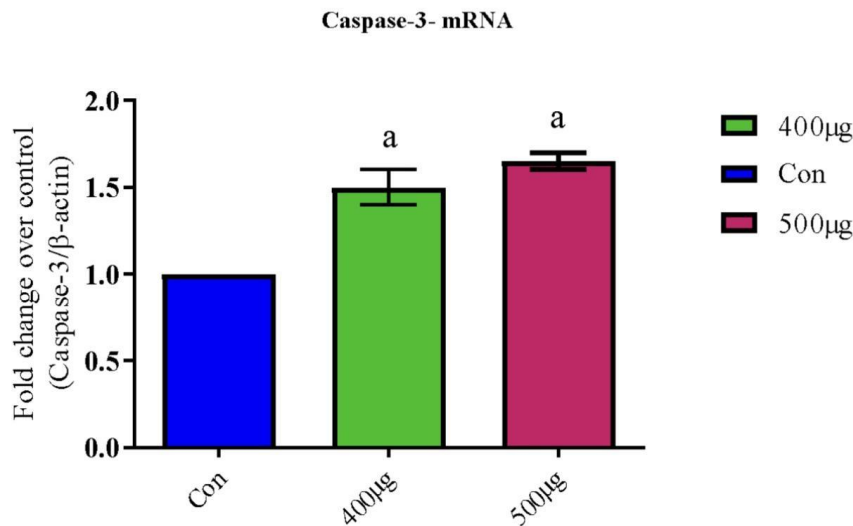


Fig 3: Effect of *Citrullus Colocynthis* leaf extract on caspase-9mRNA expression HepG2 cells. Each bar represents mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells.

DISCUSSION:

The anticancer effect of *Citrullus colocynthis* has been via a variety of pathways which includes apoptotic pathway, anti-inflammatory traits, and antioxidant pathway, etc (1). Effect of HEAM on the mRNA level of tumor related genes (p53, Bcl-2), inflammatory (IL-1β, IL-6 and c-jun) and anti-inflammatory (IL-4) cytokines(26). Representative photographs of ethidium-bromide-stained 2% agarose gels for each gene after 7 months of

treatment.(27).Adult stem cells are the only cells that persist in the tissue for a sufficient length of time to acquire the requisite number of genetic changes for neoplastic development(28). In contrast to intestinal mucosa and epidermis where a steady flux of cells occurs from the stem cell zone to the terminally differentiated cells that are imminently to be lost, (29) liver normally exhibits a very low level of cell turnover.(30)Levels of messenger RNA in COL1A1 & COL3A1 and production of type I procollagen were drastically amplified by AMF and several compounds(31) (especially HDNC) as shown by RT-PCR and ELISA(32) analysisTransition of Fibroblast to Myofibroblast plays a critical role in cutaneous wound healing (33), Fibroblast-to-myofibroblast transition requires a combination of several types of factors, the most important of which are divided into humoural and mechanical factors, as well as certain extracellular matrix proteins (34).

CONCLUSION:

The hydroethanolic leaf extract obtained from the *Citrullus colocynthis* exhibited anti-proliferative effect on human liver cancer cells hepG2 cell line by affecting the expression of the Bax mRNA and Bax mRNA and hindering the cell viability. Hence, it can be a great scope for using the extract into medicinal properties with further studies to improve herbal medicine for cancer over expensive drugs.

NOTE:

The study highlights the efficacy of "herbal medicine" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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