Original Research Article

Induction of Bax and activation of caspases by hydro ethanolic leaf extract of *citrullus colocynthis* (L)-mediated apoptosis in breast cancer cell line (MCF-7)

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

Abstract:

Cancer is the most deadly disease that causes death among the world. It is caused by genome and epigenome abnormalities. Herbal plants are used for treatment of cancer. *Citrullus colocynthis* is valuable medicinal plants used for treatment of cancer. The plant as antidiabetic, anticancer activity, and antioxidant activity.The aim of the study is to find induction of bax and activation of caspases by hydro ethanolic leaf extract of *Citrullus colocynthis* (L)-in Human breast cancer cells (MCF-7). Materials and methods, Human breast cancer cell was purchased from the national centre for cell sciences. Cell line and cell culture were performed and cell viability was done by MTT assay. Total RNA isolation ,cDNA conversion and gene expression analysis by real time PCR. This study clearly indicates that plant extract has a significant role in modulation of intrinsic apoptotic signaling in human breast cancer cells which might be due to the presence of cucurbitacin present in *Citrullus colocynthis*. It is conducted that *Citrullus colocynthis* extract inhibits the growth of the human breast cancer cells (MCF-7) by regulating the expression of pro apoptotic (Bax) and caspases -3 genes. Hence *Citrullus colocynthis* may be served as a potential drug for treatment of human breast cancer cells.

Key words : cancer, genome, *Citrullus colocynthis*, human breast cancer cells, bax, caspases, potential drugs, innovation, novel

Introduction:

Cancer is the most deadly disease that causes death among the world (1), (2). Genome abnormalities cause cancer (3), (4) . It is also caused by epigenome (5).there are anticancer drugs for direct effects anthracyclines, cyclophosphamide, cisplatin and for indirect effects taxanes, trastuzumab, sorafenib (6)(7)(8). Cancer cells are resistant against chemotherapeutic drugs. Metformin is a chemotherapeutic drug that kills four different types of breast cancer (9)(10). Breast cancer is the most common type of cancer among women all around the world. New tumours are formed from the breast cancer cells.

Sequential mutation in genes causes cancer (11) (12). Breast cancer is either inherited or acquired through mutations (13)(14). In some countries patients use several herbal plants for cancer treatment (15)(16). *Citrullus colocynthis* plant and its family cucurbitaceae.And its common name is bitter apple.

Citrullus colocynthis is a valuable medicinal plant. The plant contains several bioactive compounds such as cucurbitacin glycosides, flavonoids. Antioxidant activity is also present in these plants (15,17). It is also an antidiabetic property and treated for hemorrhoids (18). The cucurbitacin glycosides have therapeutic value against human breast cancer (19). There are various studies done by scientists by extraction of fruit, root, stem, pulp etc. There is inhibition of cell viability and migration of various cancer cells during pulp extraction. Anti-metastatic potential along with apoptotic activity is present in pulp extraction (20)(21).

The beneficial effect of cucurbitacin glycosides as an effect on chemo prevention of human breast cancer cells(22). In another study fish oil rich in n-3polyunsaturated fatty acids has beneficial effects in many diseases including cancer also (23). The aim of study is to find induction bax and activation of caspases by hydroethanolic leaf extract of *Citrullus colocynthis* (L)-in Human breast cancer cells (MCF-7)

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 - tetraethylbenzimidazolocarbocyanine iodide) and Real Time PCR kit was purchased TAKARA (Meadowvale Blvd, Mississauga, ON L5N 5S2, Canada).

Cell lines and cell culture

Human breast cancer cell line (MCF-7) 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% CO_2.

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 (1×10^4/well) were exposed to different concentrations of *Citrullus colocynthis* leaf extract (100-500µg/ml) with MCF-7 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 = [A570 nm of treated cells/A570 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 ddH_2O. 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:

This study clearly indicates that plant extract has a significant role in modulation of intrinsic apoptotic signaling in human breast cancer cells which might be due to presence of cucurbitacin present in *Citrullus colocynthis*. The ethanolic extract of *Citrullus colocynthis* inhibits the growth of human breast cancer cells (MCF-7) by regulating the pro apoptotic expression and caspases-3 genes and the result shows that *Citrullus colocynthis* can be used has potential drug for treatment of human breast cancer cells

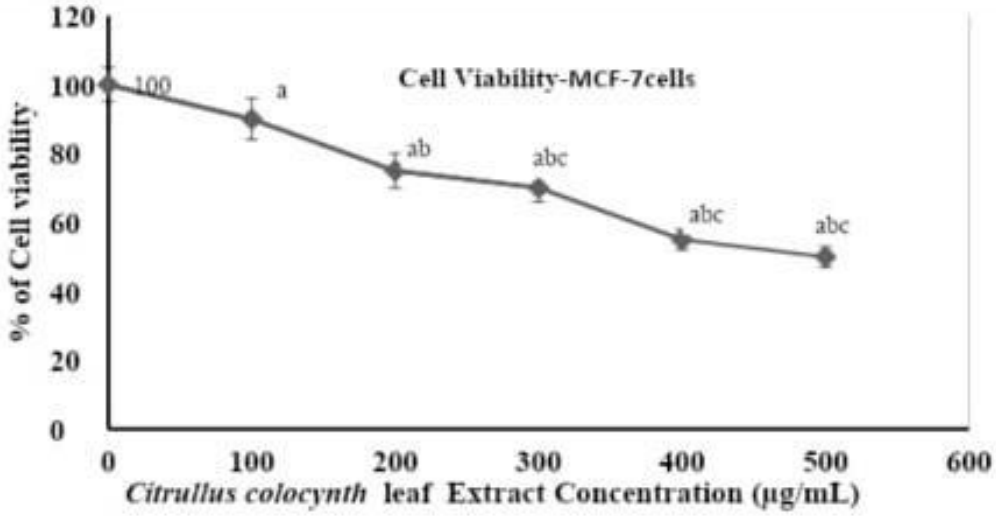

Figure 1: Effect *Citrullus colocynthis* leaf extract on cell viability in MCF-7 cells. Each bar represents a mean ± SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells, b-compared with 1nM treated MCF-7 cells.

Gene expression analysis

Bax mRNA expression (Fold change over control)

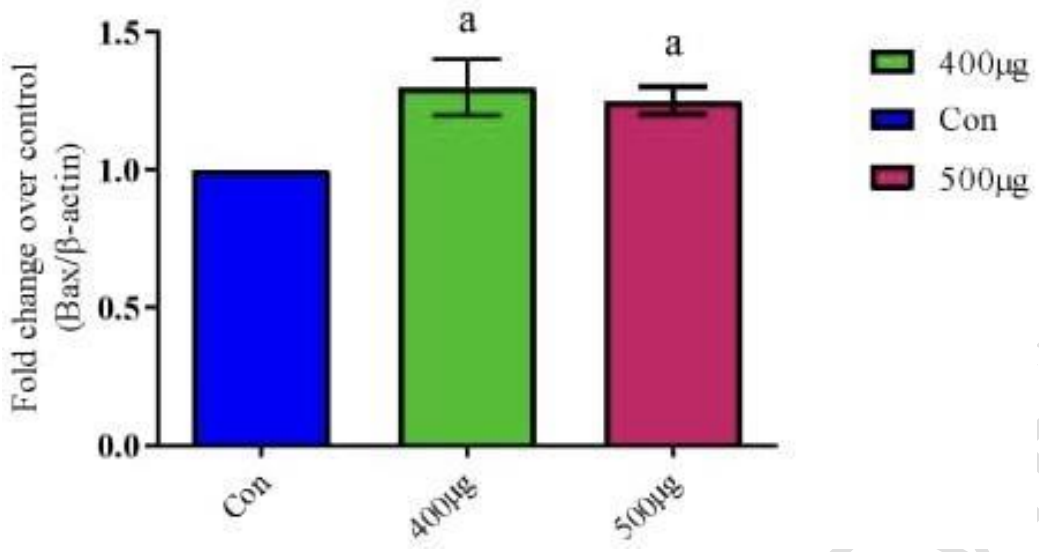

Figure 2: Effect of *Citrullus colocynthis* leaf extract on Bax mRNA expression in MCF-7 cells. Each bar represents a mean ± SEM of 6 observations: green color represents 400 microgram, blue color represents concentration and red color represents 500 microgram Significance at p< 0.05, a-compared with untreated control cells

Caspase-9- mRNA expression (Fold change over control)

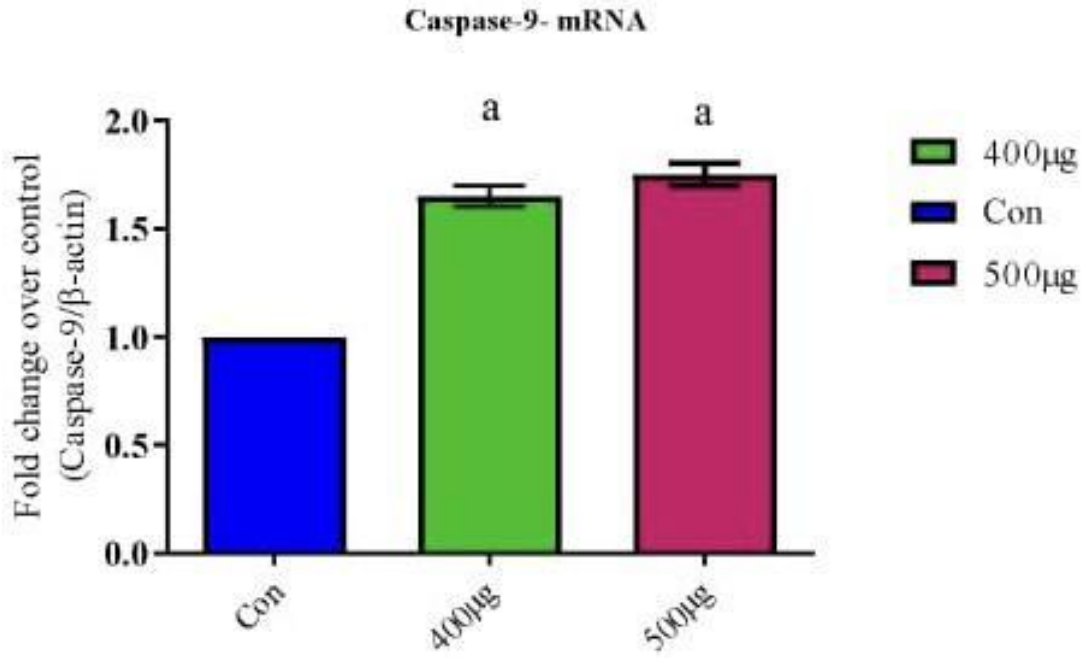

Figure 3:Effect of *Citrullus colocynthis* leaf extract on caspase-9 mRNA expression in MCF-7 cells. Each bar represents a mean ± SEM of 6 observations: green color represents 400 microgram, blue color represents concentration and red color represents 500 microgram . Significance at p< 0.05, a-compared with untreated control cells

Discussion:

This study clearly indicates that plant extract has a significant role in modulation of intrinsic apoptotic signaling in human breast cancer cells which might be due to presence of cucurbitacin present in Citrullus *colocynthis.* This study was aimed to find anti-cancer activity that has been used for diabetic (24)(25). This plant is used as chemotherapeutic drugs for liver and cardiovascular (26).*Citrullus colocynthis* shown as antimicrobial and antifungal effect (27)(28).This plant fruit has cytotoxicity effects (27,29)(30). These are used in chromosomal damage (18)(31). This plant has anti cancer activity against hepatocarcinoma (Hep G2 cells line)(32)(33). It is used in monotherapy (6)(34). *Citrullus colocynthis* fruit is used in treatment of tooth decay (17). Cucurbitacin glycosides extracted from *Citrullus colocynthis* inhibits human breast cancer cell growth(22)(35). *Citrullus colocynthis* has antifungal and antioxidant properties (36). Beta sitosterol present in *Citrullus colocynthis* induces apoptosis in human colon cancer cells and anti-proliferating activity (37)(38) .

Citrullus colocynthis has an anticancer effect, antioxidant and anti metastatic effect(39). The expression of bax gene in *Citrullus colocynthis* and the combination of extract of phycocyanin has higher inhibitory effect on cancer cells (40) but in my research its was found that ethanolic leaf extract showed anicancerous effect on cancerous cell (41)(42) and also has found that *Citrullus lanatus* showed inhibited growth on human leukemia cancer cells (43) (44)but in my research we have used another herbal plants *Citrullus colocynthis* was taken has plant sampling and the study proves that *Citrullus colocynthis* also has anticancer effects. Through this methanolic extract of *Artemisia absinthium* it was found the anticancer effect on colorectal cancer HCT-116 cell line. My research was that ethanolic leaf extract shows not only anticancer effect but also anti inflammatory effect, antioxidant effect and this proves that traditional herbal plants have various medicinal values that can replace chemical drugs (45)(46). It has found that the phytochemical cucurbitacin present in *Citrullus colocynthis* has anti-inflammatory, antihypertensive, anticancer, antihypercholesterolemic and hepatoprotective effects and my research also proves presence of phytochemical cucurbitacin has ability to inhibit cancer cell. *Bryonia dioica* is an herbal plant through which aqueous extract induced apoptosis shows the therapeutic effect against breast cancer cells (47)(48). Limitations of the study are that the time period for the research was too short and Low sample size

Conclusion:

It is concluded that *Citrullus colocynthis* extract inhibits the growth of human breast cancer cells (MCF-7) by regulating the expression of pro apoptotic (Bax) and caspases-3 genes. Hence *Citrullus colocynthis* may be served as a potential drug for treatment of human breast cancer cells.

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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