

CYTOTOXIC EFFECT OF HYDROETHANOLIC EXTRACT OF CITRULLUS COLOCYNTH (L) FRUIT AGAINST HUMAN LUNG CANCER CELL LINE

RUNNING TITLE: *Citrullus colocynthis* extract against human lung cancer cells.

ABSTRACT:

Citrullus colocynthis is a traditional medicinal plant that belongs to the cucurbitaceae family. The extract of the fruit is rich in therapeutic phytochemicals. Lung cancer is a disease with high mortality and morbidity rates. Recently the research has been focused on herbal medicine as a treatment approach for cancer because of its no or less side effects. The aim of the study is to know about the anticancer effect of hydroethanolic extract of *Citrullus colocynthis* fruit against human lung cancer cell line through apoptosis pathway. Cell viability test was done by MTT assay. The mRNA expression of Bcl2 and Bcl-xL was done using the real-time PCR. The obtained data was analysed statistically by one way analysis of variance and Duncan multiple range test with graph prism version 5 to analyse the significance. The significance was considered at $p < 0.05$ level in Duncan's test. *Citrullus colocynthis* fruit extract increased the inhibition of growth of lung cancer cells. The activity of Bcl2 and Bcl xL was significantly down regulated at 400 μ g. The study concludes that *Citrullus colocynthis* fruit extract has anticancer activity on A549 human lung cancer cell line through apoptosis pathway.

KEYWORDS: *Citrullus colocynthis*, Lung cancer cell line, innovative techniques, phytochemicals, Anticancer activity.

INTRODUCTION:

Medicinal plants have been a resource for healing in communities. Medicinal plants are the source of anti cancer agents. It has anti mutagenic and antioxidant compounds against chemicals with low cost, low side effects¹. Owing to the strong therapeutic effects, the medicinal plants are being traditionally used to treat several diseases²⁻⁴. Different parts of medicinal plants have numerous nutraceutical values and are enriched with proteins, carbohydrates, vitamins, fibre, potassium, calcium and also the presence of phytoconstituents contributes to its significant medicinal property⁵⁻⁸. A well known traditional medicinal plant is *Citrullus colocynthis* belonging to the Cucurbitaceae family of plants⁹.

The fruit extract of *Citrullus colocynthis* is rich in therapeutic phytochemicals such as alkaloids, flavonoids, glycosides, terpenoids, tannins, flavones, saponins and phenols^{10,11}. Fruit pulp is the main medicinal part of the plant used to report many biological activities^{12,13}. Anti-inflammatory activity, antioxidant activity, antidiabetic activity and hypolipidemic activity are seen in fruit extract of *Citrullus colocynthis*¹⁴⁻¹⁸. The main chemical compound found in *Citrullus colocynthis* is cucurbitacins¹⁷.

The non communicable disease responsible for 63% of worldwide deaths is reported as cancer by the World Health Organization (WHO). Cancer is the second cause of death in Western countries and it was also reported by WHO^{19,20}. The collection of heterogeneous genetic diseases united by common alterations in multiple cellular signaling pathways is known as cancer¹⁹. Lung cancer accounts for the highest mortality worldwide. Lung cancer causes a higher death rate among the population because it is detected at substantial progression of the illness that causes significant reduction in quality of life of the patients²⁰. Possible factors that are responsible for the cause of lung cancer are active cigarette smoking, pipe and cigar smoking, second hand cigarette smoking (passive smoking), exposure to indoor and outdoor air pollution, occupational exposure to agents such as asbestos, chromium, nickel and arsenic and the common cause is exposure to indoor and outdoor air pollutions²¹. Smoking is an important factor in lung cancer²².

Treatment of lung cancer and major sources of new drugs have a long history in plants²³. Herbal medicines have attractive approaches for lung cancer therapy by proving that they prevent the side effects of chemotherapy and improve quality of life in lung cancer patients²⁴. Both Bcl2 and Bcl xL genes are anti-apoptotic genes^{25,26}. Our team has extensive knowledge and research experience that has translated into high quality publications²⁷⁻⁴³. The aim of the study is to know about the cytotoxic effect of hydroethanolic extract of *Citrullus colocynthis* fruit against human lung cancer cell line (A549 human lung cancer cells).

MATERIALS AND METHODS:

Materials

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).

Preparation of plant material

Hydroalcoholic extract (70:30 alcohol:water) of fruit was prepared by maceration method (3 times), supernatants were filtered through double layer of Whatman filter paper. A Rotary Evaporator was used to extract the solvent. The obtained extract was stored at airtight bottles at 4 °C.

Cell lines and cell culture

Human lung cancer cell line (A549) 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 (1×10^4 /well) were exposed to different concentrations of *Citrullus colocynthis* fruit extract (100-500 μ g/ml) with A549 cells for 48 h. At the end of the treatment, 100 μ l of 0.5mg/ml MTT solution was added to each well and incubated at 37°C. Then the formed crystals were dissolved in dimethyl sulfoxide (100 μ l) and incubated in a dark room. 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 extract 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] \times 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 μ g RNA in a 10 μ l 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 μ l including 1 μ l cDNA, 10 μ l qPCR Master Mix 2x (Takara, USA) and 9 μ l 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 $2^{-\Delta\Delta\text{CT}}$ 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.

RESULT:

Effect of *Citrullus Colocynthis* on cell viability in A549 cells.

In the present study, *Citrullus colocynthis* extract significantly increased ($p < 0.05$) inhibiting the growth of the lung cancer cell dose dependently compared to untreated control cells. However, 400 to 500 μ g/ml concentration of the extract showed maximum inhibition of the viability of the lung cancer cells suggesting that *Citrullus colocynthis* induces apoptosis in A549 cells (Figure.1).

Effect of *Citrullus colocynthis* on Bcl2 mRNA expression in A549 cells.

The activity of Bcl2 was assessed in a dose dependent manner. In the treated A549 lung cancer cell line, the Bcl2 was significantly down regulated by *Citrullus colocynthis* at a dose of 300 μ g. The decrement reached a

higher amount when the cells were treated with 400 μ g. Thus, the decrease of Bcl2 was in dose dependent manner.(Figure.2).

Effect of *Citrullus colocynthis* on Bcl xL mRNA expression in A549 cells.

The activity of Bcl xl was assessed in a dose dependent manner. In the treated A549 lung cancer cell line, the Bcl xL was significantly down regulated by *Citrullus colocynthis* at a dose of 300 μ g. The decrement reached a higher amount when the cells were treated with 400 μ g. Thus, the decrease of Bcl xL was in dose dependent manner.(Figure.3).

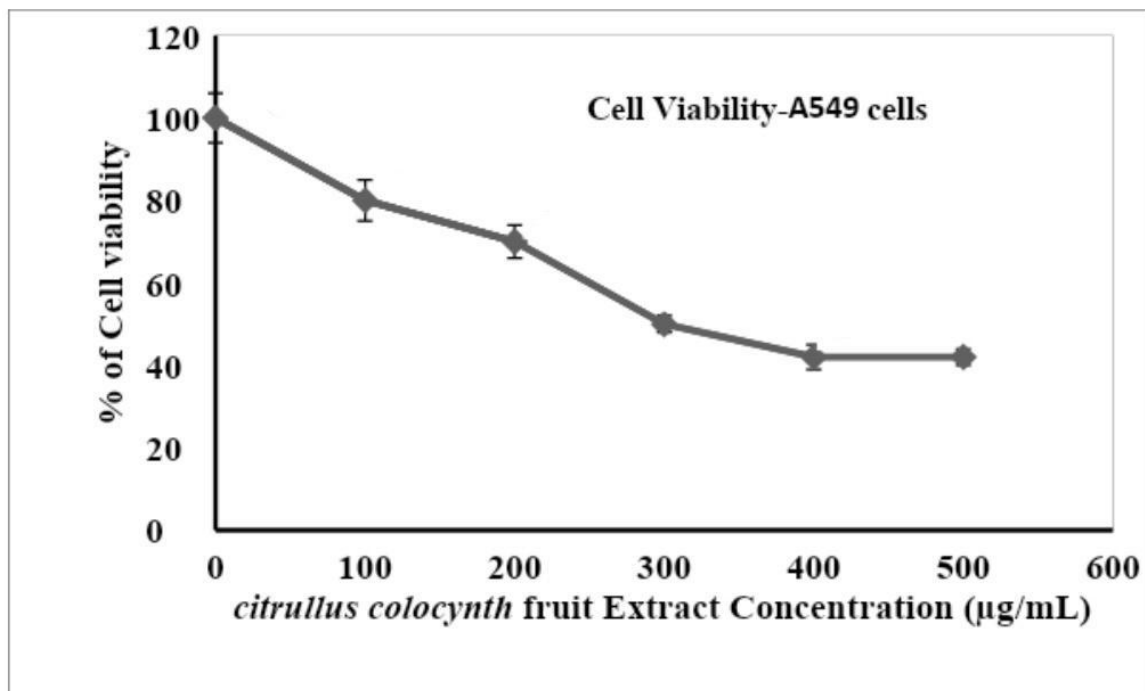


Figure.1: Effect of *Citrullus colocynthis* fruit extract on cell viability in A549 cells. Each bar represents mean + or - SEM of 6 observations. The X axis represents the concentration of *Citrullus colocynthis* fruit extract (μ g/ml) and the Y axis represents the percentage of cell viability. Significance at $p < 0.05$, a-compared to untreated control cells, b-compared with 1nM treated A549 cells.

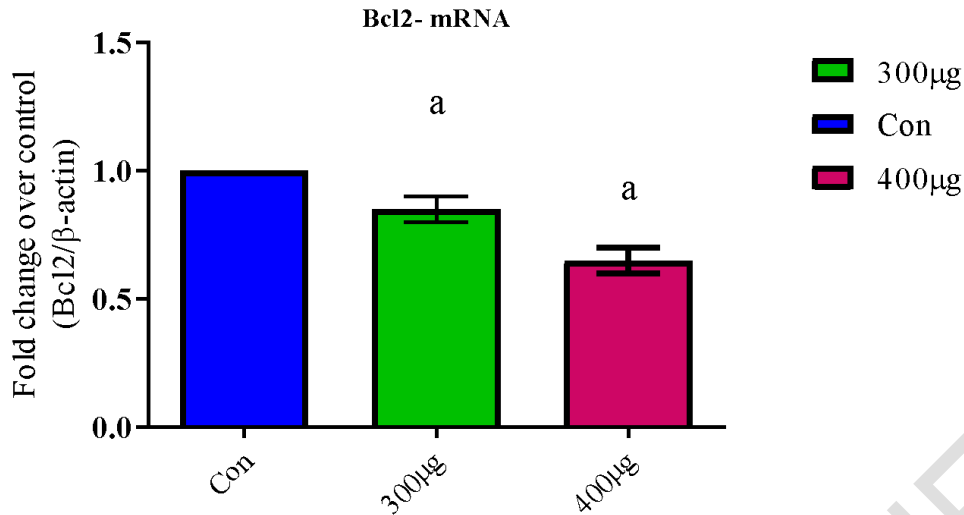


Figure.2: Effect of *Citrullus colocynthis* fruit extract on Bcl2 mRNA expression in A549 cells. Each bar represents mean + or - SEM of 6 observations. The X axis represents different concentrations of *Citrullus colocynthis* and the Y axis represents fold change over control cells. There is a statistically significant difference between the control and treated groups with p value. Significance at $p < 0.05$, a-compared with untreated control cells.

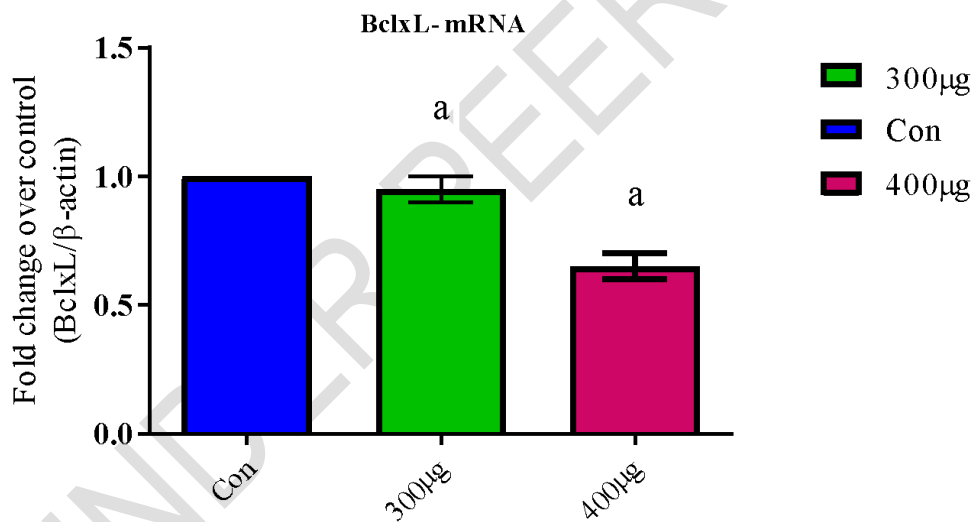


Figure.3: Effect of *Citrullus colocynthis* fruit extract on Bcl xL mRNA expression in A549 cells. Each bar represents mean + or - SEM of 6 observations. The X axis represents different concentrations of *Citrullus colocynthis* and the Y axis represents fold change over control cells. There is a statistically significant difference between the control and treated groups with p value. Significance at $p < 0.05$, a-compared with untreated control cells.

DISCUSSION:

The present study showed that the cytotoxic effect of *Citrullus colocynthis* was found to be maximum at concentration of 400µg. Thus *Citrullus colocynthis* has inhibited the cancer cell proving its beneficial activities. The previous study has shown that alkaloid rich *Citrullus colocynthis* fruit extract has promising anticancer activity in breast and liver cancer cell lines⁴⁴. Then the whole extract seems to have effective anticancer activity on larynx cancer cells⁴⁵. Previously many medicinal plants like *Selaginella tamariscina* were found to have antimetastatic effects against human lung cancer cell lines⁴⁶. *Crocus sativus* L. (Saffron) aqueous extract used in traditional medicine for lung cancer⁴⁷. The anti lung cancer activity of this plant was linked with induction of apoptosis⁴⁸.

Apoptosis is programmed cell death that is mediated by activation of a conserved intracellular pathway. Currently the relation of apoptosis and cancer has been emphasized⁴⁹. Some clues are given by apoptosis for anticancer therapy and a number of chemotherapeutic agents show to exert anti tumor effect inducing apoptosis of lung cancer. In this present study it was found that both Bcl2 and Bcl xL, anti apoptotic genes were increased in control group, but when these cancer cell lines were treated with *Citrullus colocynthis* fruit extract, it has reduced the mRNA expression of both Bcl2 and Bcl xL^{32,34,40}.

This result of the present study was supported by previous study which showed that *Sesbania grandiflora* fruit found to have antiproliferative effects against human lung cancer cell lines by inducing apoptosis⁵⁰. In this present study the phytochemicals present in *Citrullus colocynthis* fruit extract could be responsible for the anticancer activity against human lung cancer cell line (A549 human lung cancer cell).

CONCLUSION:

The present study has concluded that *Citrullus colocynthis* have abundant bioactive phytochemicals that have potential in combating carcinoma by activation of apoptosis. More scientific study must be done to develop our understanding on anticancer activity of *Citrullus colocynthis* fruit extract. Further research is required for the promising results towards treatment of lung cancer.

AUTHOR CONTRIBUTION:

Sowmya - Literature collection,

Preetha - Framing the manuscript, Statistics approval, approval of the manuscript

Dr. Selvaraj J, Dr. Lavanya Prathap - Final approval of the manuscript.

CONFLICT OF INTEREST:

Nil

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