

STUDIES ON THE EFFECT OF HYDRO ETHANOLIC LEAF EXTRACT OF CITRULLUS COLOCYNTHIS(L)AGAINST HUMAN MELANOMA CELL LINE (A 375)

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ABSTRACT:

INTRODUCTION: *Citrullus colocynthis* is the most commonly distributed in the desert regions of the world. The main active ingredients of *citrullus colocynthis* are flavonoids, cucurbitacin, phenolic acids and alkaloids, that have been proven to have anti-diabetic, anti-cancer activity, antioxidant, anti-pathogenic microorganisms in modern pharmacological research. So the main aim of this study is to assess the effect of hydroethanolic leaf extract of *Citrullus colocynthis* against the human melanoma cell line. The fruits of *citrullus colocynthis* are usually recognised because of its wide range of medicinal uses as well as nutraceutical and pharmaceutical potential. In spite of multiple medicinal benefits, the most frequently reported complications such as hematochezia, nephrosis and vomiting have placed *Citrullus colocynthis* among top 10 toxic plant. **AIM OF THE STUDY:** To study about the effect of *Citrullus colocynthis* against human melanoma cell line A375. **MATERIALS AND METHODS:** In the present study, a human A375 melanoma cell line was procured from the National Centre for Cell Sciences (NCCS), Pune, India. The cytotoxic potential of plant extract was analysed by MTT assay. mRNA expression of Wnt and beta-catenin were analysed by Real time-PCR using gene specific primers and comparative CT method was used to analyse expression of genes. The data were analysed statistically using graph pad prism software (Version 5). **RESULTS:** The hydro ethanolic leaf extract obtained from *Citrullus colocynthis*, exhibits effect on human melanoma cell line by affecting the Wnt-mRNA expression and Beta-catenin mRNA expression by 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 human melanoma cells over expensive drugs.

KEYWORDS: *citrullus colocynthis*, Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

INTRODUCTION:

Citrullus colocynthis is the most commonly distributed in the desert regions of the world. *Citrullus colocynthis* has used to treat liver diseases, constipation, jaundice, diabetes, typhoid fever, asthma, indigestion, dysentery, joint pain, colic pain, gastroenteritis and in some traditional use (1). The main active ingredients of *citrullus colocynthis* are flavonoids, cucurbitacin, phenolic acids and alkaloids, that have been proven to have anti-diabetic, anti-cancer activity, antioxidant, anti-pathogenic microorganisms in modern pharmacological research (2 and 3). *Citrullus colocynthis* is a valuable cucurbit plant. The fruits of *citrullus colocynthis* are usually recognised because of its wide range of medicinal uses as well as nutraceutical and pharmaceutical potential (4-6). Different parts of the plant such as fruits, seeds, root, stems, leaves are used as either aqueous or oil extracts, and are believed to have laxative, antihyperlipidemic, analgesic, Vermifuge, antifungal, antibacterial and oxidant properties. In spite of the multiple medicinal benefits, the most frequently reported complications like hematochezia, vomiting and Nephrosis have placed *Citrullus colocynthis* among top 10 toxic plants. There are three cases of toxic acute colitis due to ingestion of *Citrullus colocynthis*. Human cutaneous malignant melanoma will remain as the most deadly form of skin cancer and responsible for 75.2 percent of deaths related to skin cancer. Most malignant melanoma cell lines were purchased either from the University of Arizona cancer (UACC) or from American type culture centres (ATCC). In general, the response rate of disseminated melanoma to single agent cancer chemotherapy has been low. This lack of chemotherapeutic agents leads to much frustration in its management. The Metastatic melanoma is considered to be highly resistant to present chemotherapeutic agents, with long term and sustained response among patients. Melanoma on the acral skin is of two types, those of which sun damaged dorsal surfaces of the hands and on the volar surfaces of the acra, by which the hairless areas of the hand and feet. So the main aim of this study is to assess the effect of hydroethanolic leaf extract of *Citrullus colocynthis* against the human melanoma cell line. Cancer in general and especially melanoma are characterized by their various subpopulations and heterogenous nature within the tumor. In most cases, only one subpopulation was targeted by certain treatment while other cells are left unharmed and surviving cells repopulate the tumor. Thus, to address tumor heterogeneity therapeutically, combination therapies are needed in order to eliminate the bulk of tumor and, at same time, the critical subpopulations (7 - 13). Melanoma accounts for the minority of skin cancer diagnoses but at same time results in a great majority of skin cancer deaths. Incidence trends have steadily increased for the last several decades to 2012 estimate of over 76 000 diagnoses and 9000 deaths in the United States alone. *Citrullus colocynthis* Schrad., from the family Cucurbitaceae, popularly named bitter apple in English and it is a well-known medical plant used alone or in compounds for many medical purposes (14–19). Several studies suggest that antioxidant supplementation can influence the response to chemotherapy as well as the development of adverse side effects resulting from treatment with antineoplastic agents (20 - 22). Melanoma is the most aggressive form of skin cancer and is notoriously resistant to most current modalities of cancer therapy. For several years the attempts to treat metastatic melanoma were based on new cytotoxic agents tested alone or in combination, or other biologically active molecules, such as INF- γ (23-25). The aim of the study is to analyse the effect of *Citrullus colocynthis* against human melanoma cell line A375. Our team has extensive knowledge and research experience that has translated into high quality publications (26–30).

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

Cell lines and cell culture: Human melanoma cancer cell line (A375) 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 μ g/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 \times 10⁴/well) were exposed to different concentrations of *citrullus colocynthis* leaf extract (100-500 μ g/ml) with A375 cells for 48 h. At the end of the treatment, 100 μ l of 0.5 mg/ml MTT solution was added to each well and incubated at 37 °C for an hour. The crystals formed were dissolved in dimethyl sulfoxide (100 μ l) 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] \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 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 $2^{-\Delta\Delta\text{CT}}$ 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

RESULTS AND DISCUSSION

MTT analysis showed that *Citrullus colocynthis* significant increase in proliferation of human melanoma cells ($p < 0.05$) compared to untreated cells. However, 400 and 500 microgram of the extract showed maximum effect in controlling cell growth. Results of the mRNA expression analysis showed that *Citrullus colocynthis* reduced the expression of Wnt and beta-catenin in melanoma cells at 400 and 500 microgram ($p < 0.05$).

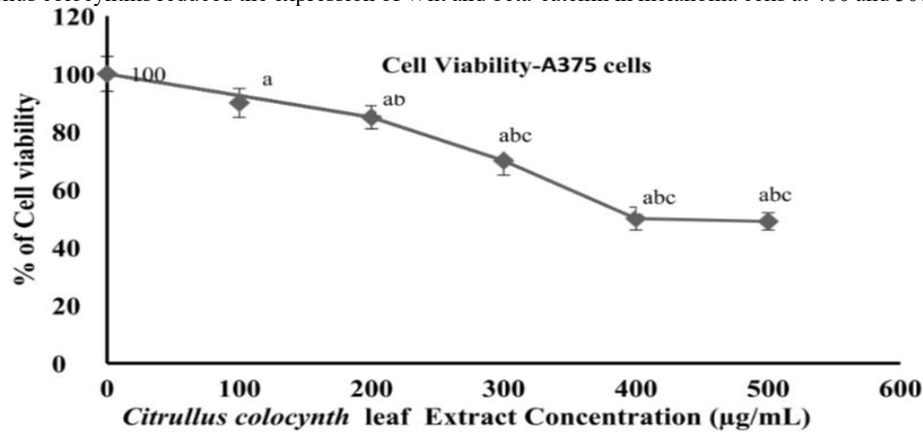


Figure 1: Effect *Citrullus colocynthis* leaf extract on cell viability in A375 cells. Each bar represents a mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells, b-compared with 1nM treated A375 cells. As the cell viability increases the effect of cancer cells decreases.

GENE EXPRESSION ANALYSIS

Wnt-mRNA expression (Fold change over control)

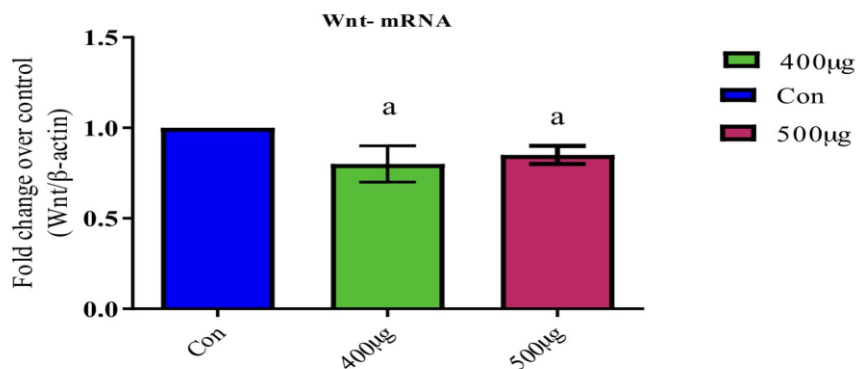


Figure 2: Effect of *Citrullus colocynthis* leaf extract on Wnt mRNA expression in A375 cells. Each bar represents a mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells. Blue colour denotes con, green color denotes 400µg and pink color denotes 500µg. Hence the Wnt-mRNA expression is higher in con and lower in 400 µg.

Beta-catenin-mRNA expression (Fold change over control)

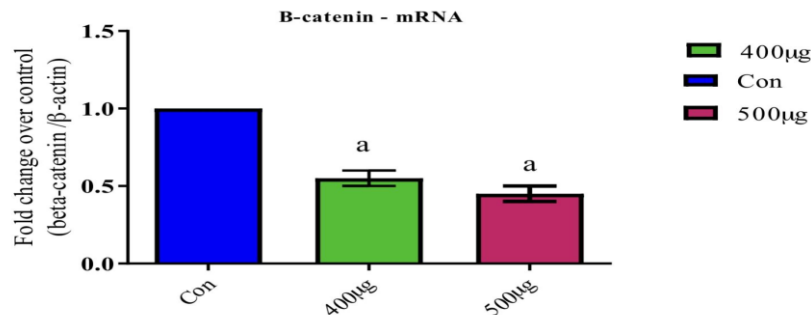


Figure 3: Effect of *Citrullus colocynthis* leaf extract on beta-catenin mRNA expression in A375 cells. Each bar represents a mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells. Blue color denotes con, green color denotes 400µg and pink color denotes 500µg.

Citrullus colocynthis have a very good apoptosis pathway ,antioxidant and anti-inflammatory effect.This also have fighting role against the growth of budding blood vessels from pre-existing vessels and kills the cancer cell growth.But in the present study, leaf extract has a cell viability in A375 cells. As the concentration of leaf extract increases, the cell viability decreases. This showed very good killing effects on cancer cell growth. (Figure 1). (31)

Hydro ethanolic leaf extract has an uneven Wnt-mRNA expression in A375 cells , when the concentration of leaf extract increases (Figure 2).The previous studies done on methanolic extract of leaf *Citrullus colocynthis*, revealed that it has potential anticancer activity by increasing cholesterol and triglycerides (regulating lipid metabolism), (32). Methanolic extract of *Citrullus colocynthis* suppresses the cell cycle of breast cancer cell via regulating inhibitors of cyclin CDK which was similar to this study but it was hydro ethanolic extract (33,34)

Several studies carried out on methanolic extract of different leaves against cancer cell activities. Hideaki etol showed a significant increase in melanogenesis in B16 melanoma cells; they used methanolic extract of piper nigrum leaves (34).

When control was compared with treated hydro ethanolic leaf extract of *Citrullus colocynthis*, it revealed that as the concentration increases (ie) 400 microgram and 500 microgram, the beta-catenin mRNA expression was decreasing.This showed that it arrests the cancer growth cells.(Figure 3).Mangosteen leaf extract has been found to treat for reducing pigmentation and it can also be useful for training cosmetic products.It also showed significant rise of melanogenesis on B16F, melanoma cells. Insufficient sample size for statistical report ,lack of previous research on this topic and time constraints are the limitations of the study. The future scope of the plant was that it can be studied for other medicinal properties and the study can be done better by using more sample size.

CONCLUSION

The hydro ethanolic leaf extract obtained from *Citrullus colocynthis*, exhibits effect on human melanoma cell line by affecting the Wnt-mRNA expression and Beta-catenin mRNA expression by 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 human melanoma cells over expensive drugs.

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CONFLICT OF INTEREST: All authors have none to declare

AUTHOR CONTRIBUTION:

Ruba Devi- Literature collection, article framing
Jothi Priya - Sample Collection, Statistics
Selvaraj - Expert in PCR, article framing
Gayatri Devi - Final approval of manuscript

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