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ANTICANCER POTENTIAL OF CITRULLUS COLOCYNTHIS FRUIT EXTRACT(L) HUMAN LIVER CANCER CELL IS MEDIATED BY MODULATING THE EXPRESSIONS OF INFLAMMATORY CELLS

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ABSTRACT
Citrullus colocynthis is widely known for its anticancer efficiency, they are also known as desert gourd and bitter apple. Despite considerable efforts taken on its treatments and prevention of liver cancer still it lies as an aggressive killer worldwide. Aim of our study is to figure out the anticancer potential of *citrullus colocynthis* in human liver cells. Human liver cancer cell line (HepG2) was purchased from the National Centre for Cell Sciences, Pune, India. Cell viability test was done by MTT assay. Gene expression analysis was done by Real Time-PCR. The obtained data were analysed statistically by one-way analysis of variance and Duncan's multiple range test with Graph Pad Prism version 5 to analyse the significance The significance was considered at $p<0.05$ level in Duncan's test. *Citrullus colocynthis* caused a marked increase in cell death in dose dependent manner. At the end of 48 hours, maximum inhibition was at 400 and 500 µg/ml. Treatment with 400 and 500 µg/ml concentration of *Citrullus colocynthis* extract has significantly reduced the expression TNF-α-mRNA and IL-1β-mRNA when compared to control cells ($p<0.05$). This study concluded that *Citrullus colocynthis* fruit extract has anticancer activity on liver cancer cell lines (HepG2).
KEYWORDS: *Citrullus colocynthis,* liver cancer cell, IL-1β-mRNA, TNF-α-mRNA, medical plants, disease, health, medicine.

1. INTRODUCTION
Medicinal plants constitute an effective alternative for cancer treatment. More than 3000 plant species had been reported to have anticancer properties (1). Recently the plant derived products for cancer treatment is ranging from 10% to 40% which now rapidly increased to 50% in asiatic patients (2). In Europe the anticancer herbal product expenditure is about 5billion dollar per year (3). Among the medicinal plants, *Citrullus colocynthis* known for its anticancer efficiency, which are also known as desert gourd and bitter apple. These kinds of plants are widely distributed in desert regions of Mediterrnean basin and asia. Medicinal plants have been a resource for healing in communities. Deep cultural and long term historical roots in plant medicine in Asian countries. Traditional practice for medicine in China, Pakistan, India, srilanka and Thailand is widespread. Medicinal plants are the natural source of anti cancer agents. It has anti mutagenic, antioxidant compounds against chemicals with low cost, low side effects. Due to strong therapeutic effects, the medicinal plants have been traditionally used to treat diseases(4) (5) (6). Different parts of medicinal plant 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 property.Their cultivation is rapidly increased over a decade due to their medicinal properties. Various fields such as pharmacology, toxicology and phytochemistry are studied on *Citrullus colocynthis*, crude extracts of its leaves, fruits and roots are known for its therapeutic phytochemicals (7). They are reported as a cure for respiratory, cardiovascular, neurological,musculoskeletal and gastrointestinal problems (8). *Citrullus colocynthis* is a potent antioxidant which is used to treat oxidative stress (8,9). Their antioxidant potential contributes for the treatment of cancer, inflammation, tissue injury and breast cancer cells (10). Recent studies reported that the plant extract inhibits proliferation and metastasis potential of breast cancer cells (11,12).The most widespread fear of the current generation is cancer, which is independent of age, race and sex. Despite considerable efforts taken on its treatments and precaution still it lies as an aggressive killer worldwide. About 60% of drugs which are used as anticancer are extracted from natural products. Currently 16 new plant derived compounds are being tested for clinical trials among which 13 are in phase 1 or phase 2 and 3 are in phase 3. The most prevalent cancer is said to be liver cancer which holds up 2nd position in total death due to cancer. The risk factors are known so the preventive measures can be easily capitulated (11–13), preventive measures also include control of hepatitis B, hepatitis C infection; reduce the consumption of alcohol which reflects a greater impact on reducing the causes of liver cancer in the human population. The risk factor also includes overweight and metabolic syndrome which generates greater impact on acquiring cancer. Related follow up data about incidence and high risk individuals in subsets as compared to HBV, HVC and alcohol are scarce. The future reduction in viral chases like control of HBV and HCV counters balances the increasing etiologic groups. At the final stage of cancer the only left out option will be liver transplant and final diagnosis, accurate and options should be available (14).

Various previous studies were done on anticancer drugs where many plant products like *Urtica membranacea, Artemisia monosperma, Origanum dayi* showed up their anticancer potentials. Many anticancer drugs are developed but treatment is still challenging which in cases of liver cirrhosis limits options for chemotherapy so the possible options lie on surgery, percutaneous interventions, transarterial interventions, radiation therapy and gene-immunotherapy. Even Though treatment options have become diverse in recent years, improvement in HCC survival is lag far behind when compared to other tumours. Synthetic chemotherapy in particular shows a good treatment option but due to its adverse side effects they are not preferred (15). As an alternate treatment option plant extract having medicinal properties can be used . Due to their less side effects and cost effective nature they can be patient friendly. The aim of the present study was to determine the anticancer potential of the planet extract *Citrullus colocynthis* on HepG2 cell line.

2. 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 purchasedTAKARA (Meadowvale Blvd, Mississauga, ON L5N 5S2, Canada).

2.1 Cell line 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% CO_2.

2.2 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* fruit 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. Then the formed crystals were dissolved in dimethyl sulfoxide (100 µl) and incubated in the 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.

2.3 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 bySchmittgen and Livak (2008) using CFX Manager Version 2.1 (Bio Rad, USA).

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2.4 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

3. RESULTS

3.1 Effect of *Citrullus colocynthis* on cell viability in HepG2 cells.In the present study, Citrullius colocynthis extract significantly (p<0,05) inhibited the growth of the liver cancer cells dose-dependently compared to untreated control cells. However, 400 to 500 µg/ml concentration of the extract showed maximum inhibition of the viability of the liver cancer cells suggesting that Citrullus colocynthis induces apoptosis in HepG2 cells (Fig.1).

3.2 Effect of *Citrullus colocynthis* on IL-1β-mRNA expression in HepG2 cells.In the present study, IL-1β-mRNA expression was found to be increased in untreated control cells. Citrullius colocynthis extract significantly (p<0,05) inhibited the growth of the liver cancer cells dose-dependently compared to untreated control cells. Treatment with 400 and 500 µg/ml concentration of Citrullus colocynthis extract reduced the expression IL-1β-mRNA when compared to control cells (p<0,05). (Fig.2).

3.3 Effect of *Citrullus colocynthis* on TNF-α-mRNA expression in HepG2 cells.In the present study, TNF-α-mRNA was found to be increased in untreated control cells. Citrullius colocynthis extract significantly (p<0,05) inhibited the growth of the liver cancer cells dose-dependently compared to untreated control cells. Treatment with 400 and 500 µg/ml concentration of Citrullus colocynthis extract reduced the expression of TNF-α-mRNA when compared to control cells (p<0,05) (Fig.3).

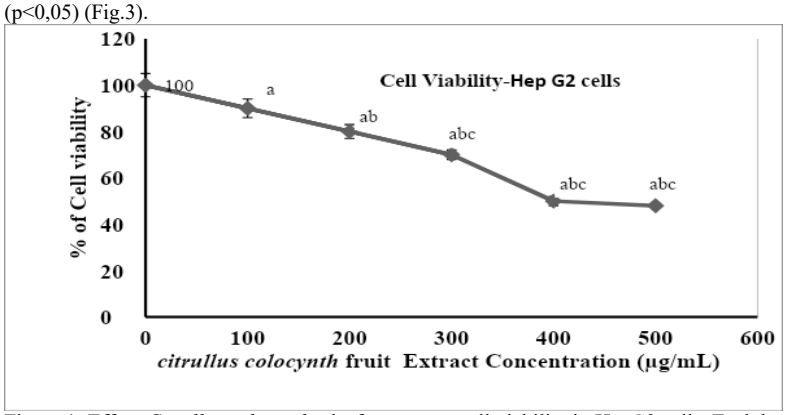

Figure 1: Effect *Citrullus colocynthis* leaf extract on cell viability in HepG2 cells. Each bar represents a mean ± SEM of 6 observations. Significance at p< 0.05, a-compared with untreated control cells, b-compared with 1nM treated HepG2 cells.

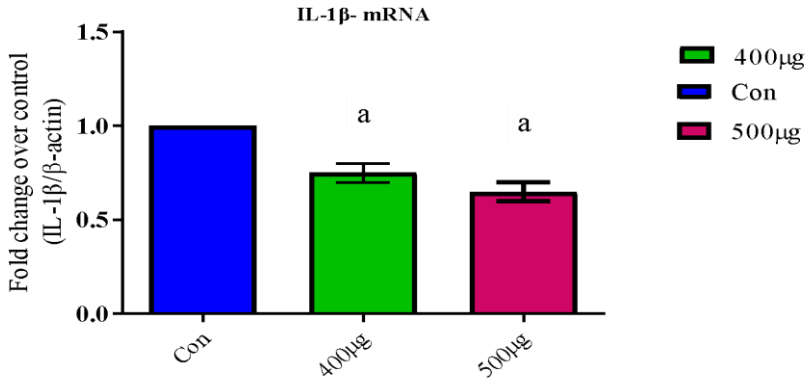

Figure 2: Effect of *Citrullus colocynthis* fruit extract on IL-1β mRNA expression in HepG2 cells. Each bar represents the mean ± SEM of 6 observations. The X-axis represents different concentrations of *Citrullus colocynthis* and the Y- axis represents the fold change over control. There is a statistically significant difference between the control and treated groups with p value < 0.05. a-compared with untreated control cells.

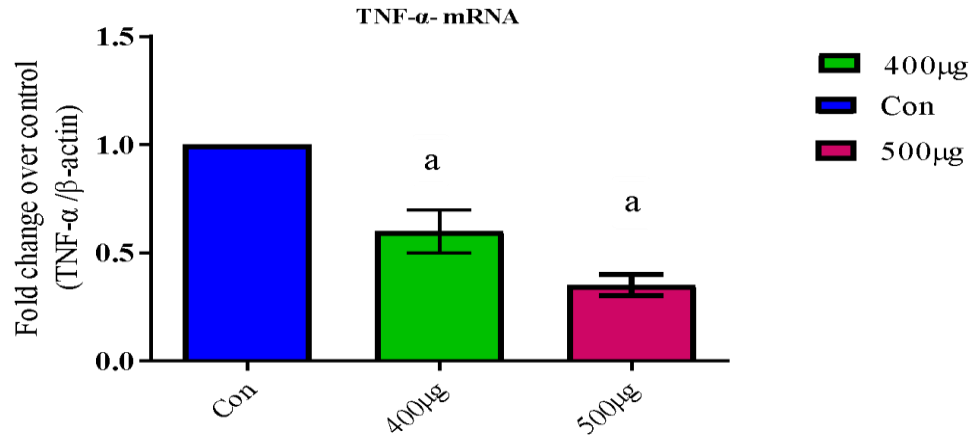

Figure 3: Effect of *Citrullus colocynthis* fruit extract on TNF-α mRNA expression in HepG2 cells. Each bar represents the mean ± SEM of 6 observations.The X-axis represents different concentrations of *Citrullus colocynthis* and the Y- axis represents the fold change over control. There is a statistically significant difference between the control and treated groups with p value < 0.05. a-compared with untreated control cells.

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4. DISCUSSION

Citrullus colocynthis is a wild, perennial, herbaceous, non-tough, hars, plant with alternate leaves and small yellow monoecious flowers. Various studies were done to predict the restorative compatibility of the plant. Plant also contains various phytochemical like alkaloids, carbohydrates, flavonoids, tannins, terpenoids, proteins,saponins and steroids and other pharmacological properties such as diuretics, hypolipidemic, anticancer, antioxidant, microbial (16). In the present study, the cytotoxic effect of *Citrullus colocynthis* was found to be maximum at 400-500 µg/ml (figure 1). Previous research on *Citrullus colocynthis* have reported that it has a cytotoxic effect on breast cancer cell lines which is similar to our study. The methanolic extract of *C. colocynthis* leaves showed anticancer potential in a dose dependent manner against breast cancer cell line MCF-7.

Tumor necrosis factor (TNF), a 17 kDa protein which consists of 157 amino acids, mainly produced by activated macrophages, T lymphocytes, and natural killer (NK) cells (17). The TNF-α is involved in the different stages of tumorigenesis. In our study, the effect of *Citrullus colocynthis* on TNF-α-mRNA in HepG2 cells was determined and found that on treatment with the plant extract at dosage of 400 and 500 µg/ml the expression of TNF-α-mRNA gets reduced which shows that the plant extract helps in downregulation(figure2). Previous research was done on breast cancer cell MCF-7 where the expression of TNF-α-mRNA gets downregulated.

Interleukin- 1β is an important mediator for cancer-related inflammation which can be secreted by the immune system, stromal and tumour cells. IL-1β levels are incensed in various cancers like colon cancer (18,19). In our study, the effect of *Citrullus colocynthis* on IL-1β on HepG2 cells was determined and found that on treatment with the plant extract at dosage of 400 and 500 µg/ml the expression of IL-1β-mRNA gets reduced which shows that the plant extract helps in downregulation (figure 3). Previous studies showed the involvement of IL-1β in the tumor growth and metastasis through the induction of growth factors. Aberrant IL-1β signaling drives tumorigenesis through a variety of pathways (20).
The current study provides anticancer potential of *Citrullus colocynthis* against human liver cancer cells(HepG2) which causes less side effects than usual drugs. But they are not effective as conventional prescription medications and cancer treatments such as chemotherapy and radiotherapy. Recently, public interest and research efforts in medical communities have grown to a greater extent on herbal medication for the cure. So there will be a huge spectrum of scope for herbal drugs against cancer cells.

5. CONCLUSION

The present study concludes that the extract *Citrullus colocynthis* has a significant effect by decreasing the inflammatory IL-1β mRNA and TNF-α RNA in the liver cancer cell line. With further invivo and invitro studies the extract can be formulated into an effective anticancer drug with limited or nil side effects. In future studies we will concentrate on phytoconstituents which are responsible for the anticancer property.

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