RESEARCH ARTICLE

Editorial Process: Submission:06/17/2025 Acceptance:12/20/2025 Published:12/27/2025

Synergistic Anticancer and Certain Biochemical Events Impact ER+ MCF-7 Breast Cancer Cells by Local "*Citrullus colocynthis-Lemon Fruit*" Hydroethanolic Extract Combination

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Abstract

Objective: Despite of advancements in oncology, classical therapy like chemotherapy, radiotherapy often come with various side effects, including drug resistance and toxicity to healthy cell. Recent studies suggest that various herbal combinations may reinforce therapeutic efficacy through synergism between combined herbs. The present work was carried out to investigate the synergistic effect of Citrullus colocynthis and lemon fruit hydroethanolic combination against ER+ MCF-7 breast cancer cell line in different aspects. **Methods:** The cytotoxic effect of herbal extract combination was studied by MTT, while DNA degradation was detected by Propidium Iodide/Acridine Orange assay. The antiangiogenic effect was evaluated by measuring neuropeptides NPYR1 and NPYR5 using a sandwich enzyme immunoassay (ELISA Kit), as they have been shown to play a role in cancer growth**. Results:** A highly cytotoxic effect of the Citrullus colocynthis and lemon juice combination extract was observed against MCF-7 cancer cell line, with a significant IC_{50} value and extensive DNA fragmentation, indicating intense apoptotic events. On the other hand, results showed a decreased level of both NPYR1 and NPYR5, suggesting inhibition by the herbal extract combination. **Conclusion:** It can be concluded that the Citrullus colocynthis and lemon juice combination extract shows high efficiency as a cytotoxic agent against MCF-7 cancer cell line.

Keywords: Breast cancer- NPYR 1,5- colocynth- *citrus lemon*

Asian Pac J Cancer Prev, **26 (12)**, 4617-4622

Introduction

The global burden of cancer has increased, which leads to significant psychological, physical and economic pressures on patients, community and health system [1]. Many health systems in different countries have limited capacity to manage such a burden. Additionally, a huge number of cancer patients around the world do not receive proper diagnosis or access to high-quality medications in a timely manner [2].

Tumors can arise from virtually any cell type or tissue in the body, which means that cancer can involve all organ systems and cell types (although some tissues are more susceptible than others) and is therefore inherently more complicated than other major diseases [3].

Ancient individuals used a broad spectrum of medicinal plants, and they had a wide understanding not only of their healing powers but also of their herbal toxicity [4, 5]. Medicinal plants have attracted the interest of many investigators as a relatively safe source for anticancer agents. These interest has led to the discovery of many effective, plant-derived chemotherapeutic drugs such as Taxol, vincristine, and irinotecan, which are now widely used as anticancer treatments in humans [6]. The antioxidant compounds of this plants such as Citrullus colocynthis and *citrus lemon*, along with thie antioxidant activity, have been investigated in various countries (China, Korea, Japan, and America.). However, several studies have also explored the anti-cancer and anti-tumor features of the Citrullus colocynthis in different parts of the world [7, 8].

The aim of this study was to evaluate the anticancer potential of our herbal combination by using different approaches.

Materials and Methods

Herbal combination

One fresh local Citrullus colocynthis pulp of (100 grams) was pulverized with 50gm of lemon fruit pulp using a blender. The mixture then macerated for 48 hours in a hydroethanolic solvent (50:50) at 40^{O}C in shaking water bath. It was then filtered first through gauze and secondly through a Millipore filter (0.22 μm pore size). Serial dilutions of 100, 50, 25, 12.5, 6.2, 3.1 μg/ml were prepared using deionized water.

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Maintenance of cell cultures

MEM was used to maintain MCF-7 cancer cell line, supplemented with 10% calf serum. Antibiotics including penicillin (100 units/ml) and streptomycin (100 microgram /ml), were added. Trypsin-EDTA was used for passaging cells, which were reseeded at 50% confluence twice a week, and then incubated at 37°C as described by Freshney (2005) [9].

NOTE: "The MCF-7 human breast adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), where it underwent authentication and quality control. Upon receipt, cells were handled, cultured, and stored in accordance to ATCC's standardized protocols to ensure their identity, viability, and contamination-free conditions."

Cytotoxicity Assays

To estimate the cytotoxicity, a cell viability assay using MTT was carried out on 96-well micro plates. MCF-7 cell line was cultivated at 1×10^4 cells/well. After 24 hours, or once a confluent monolayer was reached; cells were exposed to hydroethanolic extract of the herbal combination (Citrullus colocynthis and lemon fruit). Cell viability was assessed after 72h of exposure by discarding the medium and adding 28µL of MTT solution (2 mg/mL). The cells were incubated for about 1.5 hours at 37°C. After incubation, the MTT solution was discarded, and the formazan crystals remaining in the wells were dissolved by adding 130 µL of dimethyl sulfoxide, followed by incubation at 37c for 15 min with shaking [10]. The optical density was measured on a micro titer plate reader at wave length 492 nm; the experiment was done as triplicate. The rate of Inhibition for cell growth was measured as below equation: -

% cell viability = (Absorbance of treated cell/ Absorbance of non-treated cell × 100

% Cytotoxicity = 100- cell viability

Apoptosis Estimation (Acridine orange/ propidium iodide/ assay) in culture

The only highly apoptotic events in culture (infected and control) were evaluated using (AO/PI). A total of 7,000 cells/well were cultivated in plate. Cells were exposed to (X) at the IC_{50} concentration and incubated at 37°C. For dual staining. 50µl of the AO/PI stain mixture were added to wells (at 25C) for 30 seconds. After that, the stain was removed, and a Leica fluorescent microscope was used to capture images [11].

Neuropeptides receptors (NPYR1 and NPYR5) estimation

Both Human Neuropeptide Y Receptors (Y5 and Y1) were estimated using sandwich enzyme immunoassay ELISA Kit of (My BioSource). The standards, test samples (Citrullus colocynthis and lemon fruit) combination extract added to the wells subsequently, and NPYR5, NPYR1 were measured depending on protocol of MyBioSource. com. The standard curve was generated depending on our experiment. The cell culture supernate was centrifuge for 20 minutes to discard insoluble impurity at 1,000×g at 2- 8°C., the clear supernate then Collect and done the assay immediately.

Statistical analysis

The obtained results were subjected to statistical analysis using GraphPad Prism 5. The data are presented as mean ± SD from triplicate measurements.

Results

The cytotoxic effect of different concentrations of ultra-filtrated crude (Citrullus colocynthis and lemon fruit) hydroethanolic extract of herbal combination on ER+ MCF7 breast cancer cell line at one exposure time (72h) were examined. Figure (1, 1a and 2) showed all concentrations of interested crude extract inhibited the viability of MCF-7 breast cancer cell line. The inhibition was in different degrees depending on the concentration of the extract, with high significant differences at $P \leq 0.05$. However, a lower concentration of crude juice (3.1 µg/ml) was achieved a less antiproliferative activity on MCF-7 cancer cell line. In addition, (IC_{50}) is a little exceed one hundred (IC_{50}= 100.3).

The cytogenetic degradation events due to apoptosis were labeled by propidium iodide/Acridine orange assay using Leica fluorescent microscope showed highly significant difference between treated and control group of MCF7 breast cancer cell line ($P<0.0003$), as cleared in

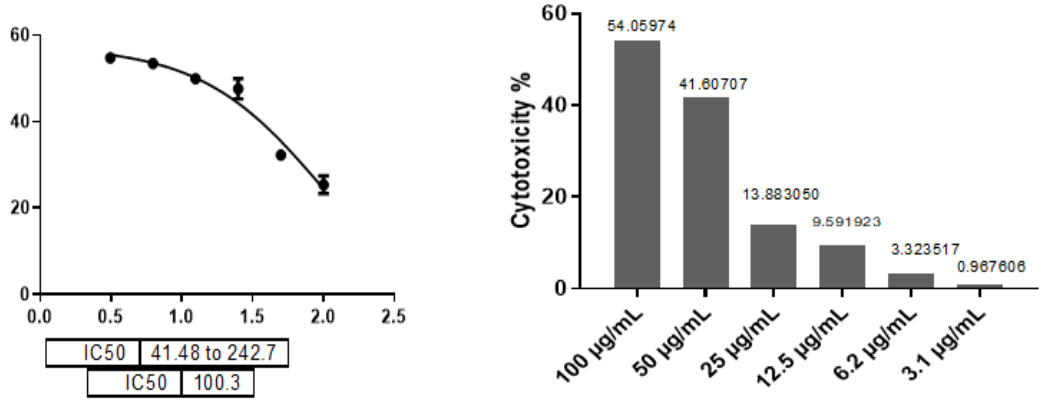

Figure (1, 1a). *in vitro* Cytotoxicity and IC_{50} of Extract against MCF-7 after 72 h Exposure

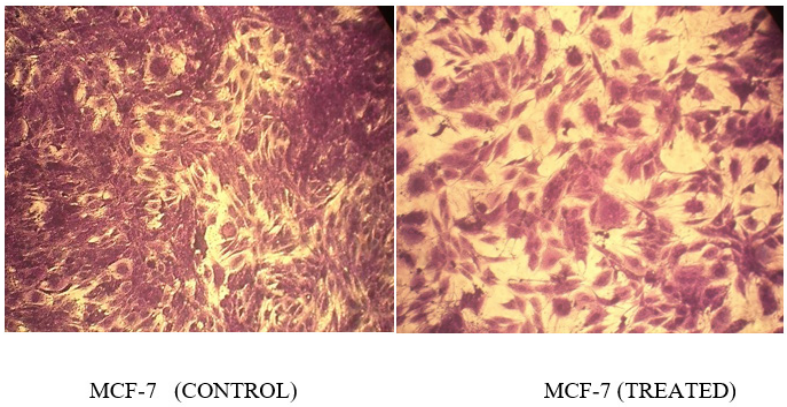

Figure 2. MCF-7 Cell Line before and after Treating with Extract

Table 1. Comparison between Viable and Dead Cell Line after Exposure to Herbal Combination

Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significance	Summary	P- Value
Treated - Control					
Viable	-103.2	-150.7 to -55.75	Yes	***	0.0007
Dead	114.8	67.31 to 162.2	Yes	***	0.0003

Figure 3 and Table 1.

The treated cells were having an orange fluorescence by PI/AO staining and gathered in concentration and located in bias (Figure 4). Ribble and colleagues at 2005 speculated that Acridine orange mostly penetrated healthy and early apoptotic cells with perfect membrane, fluorescing green when bound to DNA. Propidium iodide (PI) absolutely penetrate damaged cells, at late stage of apoptotic and dead cells, secretion orange-red fluorescence when link to fragments of concentrated DNA or apoptotic bodies [12].

In Figure 4, cells undergoing apoptosis showed reduced fluorescence because the dye is no longer able to aggregate in the mitochondria. Instead, it becomes dispersed throughout the cell which dilutes the fluorescent signal.

The present study focused on evaluating the cytotoxicity of herbal combination extract (*C. colocynthis* and lemon fruit) on MCF-7 cell line. These extracts show highly significant inhibition against MCF-7 cell line in compared with no treated MCF-7 cell line.

Additionally, we thoroughly assessed the antagonistic effects of hydroethanolic extract of the herbal combination that included Citrullus colocynthis and lemon fruit on the neuropeptide Y receptors NPY1R and NPY5R. In a variety of cancer models, most notably breast carcinoma, NPY1R and NPY5R are both well-characterized facilitators of oncogenic metabolic pathways that play a major role in tumor growth, survival, and the potential for metastasis. Specifically, NPY5R has been linked to the paracrine-mediated release of vascular endothelial growth factor (VEGF) and its upregulation, which in turn promotes angiogenesis [13, 14]. The potency and effectiveness of this formulation were confirmed by data shown in Figures 5, which show a statistically significant, dose-dependent inhibition of NPY1R and NPY5R

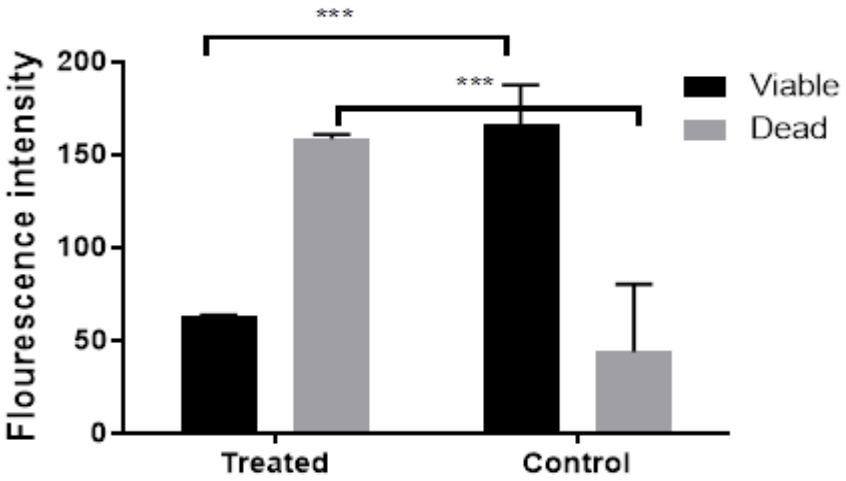

Figure 3. Fluorescence Intensity in MCF-7 Cell Line Culture after Extract Treatment

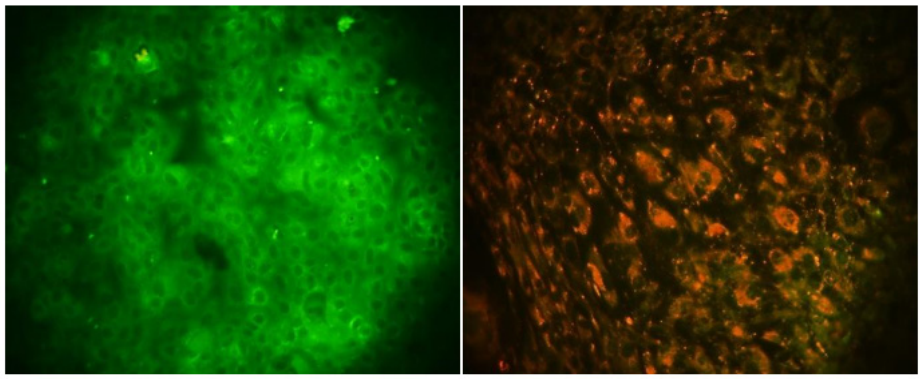

Figure 4. Cytogenetic Degradation Events Due to Apoptosis Treated MCF-7

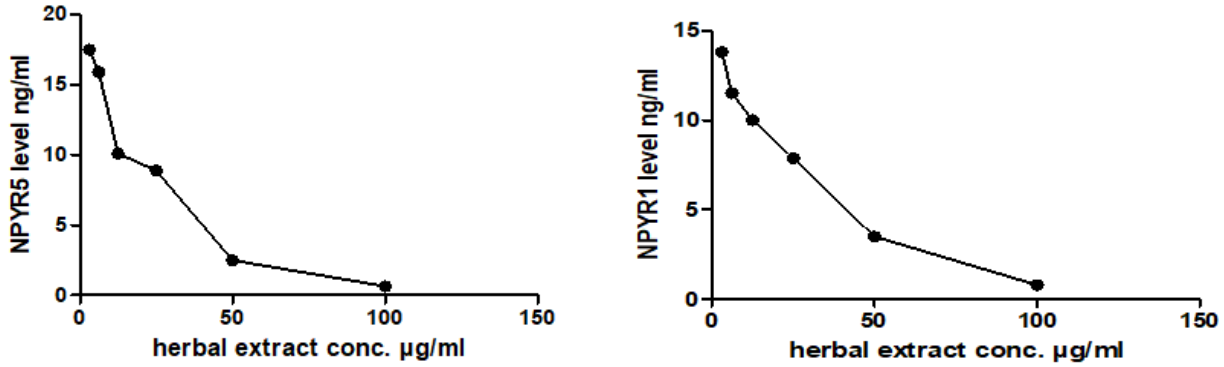

Figure 5. Significant Inhibition of Both NPYR5 and NPYR5 in MCF-7 by Herbal Combination

activity as concentrations of the herbal combination were gradually increased.

Discussion

The present study was carried out to evaluate the cytotoxic, cytogenetic degradation and antiangiogenic effects of a hydroethanolic extract of a herbal combination containing Citrullus colocynthis and lemon fruit, against the MCF-7 breast cancer cell line.

These extracts show highly significant inhibition against MCF-7 cell line in compared with no treated MCF-7 cell line.

The treated cells were having a brilliant orange-red fluorescence by PI/AO dyeing and aggregated in concentration and position in bias (Figure 4). Ribble and colleagues at 2005 speculated that Acridine orange mostly penetrated healthy and early apoptotic cells with perfect membrane, fluorescing green at binding to DNA. Propidium iodide (PI) absolutely penetrate damaged cells, at late stage of apoptotic and dead cells, secretion dark orange fluorescence when link to fragments of concentrated DNA or apoptotic bodies [12].

Furthermore, in a variety of cancer models, most notably breast carcinoma, NPY1R and NPY5R are both well-characterized facilitators of oncogenic metabolic pathways that play a major role in tumor growth, survival, and the potential for metastasis. Specifically, NPY5R has been linked to the paracrine-mediated release of vascular endothelial growth factor (VEGF) and its upregulation, which in turn promotes angiogenesis [14].

Neuropeptide Y (NPY), which is primarily expressed in the breast tumor microenvironment, exerts its effects through G-protein-coupled receptors (GPCRs). Among the receptor subtypes, NPY1R and NPY5R have the highest relative expression [15]. Dense sympathetic innervation and correspondingly high NPY levels define the breast tissue niche, which together create an ideal environment for aberrant signaling via these pathways. Targeting NPYR subtypes, especially NPY1R and NPY5R, has therefore become a viable therapeutic approach for the treatment of breast cancer [16] notably, despite the limited clinical translation of NPY5R-specific inhibitory modalities to date, recent research supports the pathogenic role of NPY5R in tumorigenesis and metastasis. According to the study's findings, the herbal hydroethanolic extract combination of *C. colocynthis* and lemon fruit exhibits a novel cytotoxic mechanism that involves direct inhibition.

The anticancer effects of *C. colocynthis* may be due to variant pathways and characters, which include apoptosis and its antioxidant and anti-inflammatory effects, also suppression of Wnt/β-catenin signaling pathways and anti-metastatic. The cucurbic acid content in *C. colocynthis* considers anticancer compounds [17]. The leave of present herb revealed noticeable anticancer activity against human breast cancer cell. The biochemical

tests cleared a lowering in growth and multiplication of breast cancer cells that exposure to *C. colocynthis*. The suppression of cyclin-CDK activity leads to a reduction in breast cancer cell proliferation and metastasis [18]. Different phytocomponents in *C. colocynthis* can prevent multiplication and metastasis of breast cancer cell and activation of apoptosis and inhibition of cancer stem cells properties in breast cancer cells [19]. The ability of *C. colocynthis* components in lipid metabolism modulation, this led to high ability of herb as antioxidants in human breast cancer treatments [20].

Previous studies have confirmed that *C. colocynthis* contains numerous bioactive compounds with antioxidant and cytotoxic effects against different types of cancer cell lines. Mohammed Bourhia (2020) documented that *C. colocynthis* ethanolic extract contains Isoorentin 3-O-methylethar, isosaponerin, and isovitexin, which have a potential antioxidant activity [21]. These findings are consistent with those reported by Chowdhury in (2017). The ethylbenzene and tetrachlorethylene could determine the cytotoxicity of *C. colocynthis* on MCF-7 cell line.

As latterly mentioned the potential active compounds in *C. colocynthis* affect different metabolic pathways in cancer cell line and this may led to inhibition of growth and increase the rate of apoptosis in cancer cell line, by the other hand the studies related with effects of active compounds in lemon juice revealed that the lemon juice consist with quite a few of natural chemical compounds like flavonoids, citric acid, vitamin C, and certain minerals, and the antioxidant potential of flavonoids and vitamin C , may consider a very important factor in activity against cancer cell [22, 23], The flavanons and flavon glycoside from flavonoid happened in a huge amount in the lemon flesh [24]. Rafiq and his team reported that the terpenoid groups like limonen might be estimated on lemon rind and lemonoid in the lemon juice [25]. This combination we made between (*C. colocynthis* and lemon flesh) reinforced the cytotoxic and antioxidant potential against MCF-7 and other cancer cell line. In (2022) Valerija and his team found that the stability and availability of polyphenols in lemon juice increase by PH of lemon juice and its content of ascorbic acid. Based on this, we have increased a synergistic effect of antioxidants activity and anticancer efficacy and stability by such herbal combination (lemon flesh with *C. colocynthis*) and in result increasing the antioxidant and anticancer activities of our herbal combination.

Based on our experimental findings, the combination of these herbal extracts demonstrates considerable promise as a potential therapeutic strategy for breast cancer. The data indicate that this blend exerts synergistic cytotoxic and anticancer effects against MCF-7 cells, at least in part through the down-regulation of neuropeptides NPYR1 and NPYR5.

Author Contribution Statement

The second author contributed to the preparation and writing of the manuscript, statistical analysis, and revision of the manuscript.

Acknowledgements

Funding Statement

The present research did not receive grant from any official and unofficial body.

Approval

The research is private and not part of an approved student thesis.

Ethical Declaration

This research does not require medical ethical approvals because the study was not clinical and did not focus on humans or animal models as a research sample, rather that our experiments were outside the body (in vitro) using cell line.

Data Availability

Any researcher can find the data support the present work finding and are available at corresponding author upon suitable request.

Conflict of Interest

There is conflict of interest was reported by the authors.

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