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Original Article

Cytotoxic Effect of the Crude Alcoholic Extract of the Fruits of *Citrullus Colocynthis* on Human Hepatocyte Carcinoma (Hep-G2)

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Abstract

The *Citrullus colocynthis L* is a perennial herbaceous plant belonging to the family *Cucurbitaceae*. Several pharmacological investigations have been performed based on the medicinal application of *Citrullus colocynthis.* The anticancer and antidiabetic activities of fruit and seed extracts of *Citrullus colocynthis* have been studied. Newly developed anticancer/antitumor medications appear to have been developed based on the extracted chemicals from *Citrullus colocynthis* due to the high contents of cucurbitacins. The present study aimed to identify the cytotoxic effect of the crude alcoholic extract of plants of *Citrullus colocynthis* on the growth of human hepatocyte carcinoma (Hep-G2). The results of the chemical (preliminary) examination of the extract indicated that the fruits contain most of the secondary metabolites including Flavonoids, Tannins, Sapiens, Resins, Amino acids, Glycosides, Terpenes, Alkaloids, and Flavonoids. The toxicological effect of the crude extract was investigated by using six half dilutions concentrations of 20,10,5,2.5,1.25, and 0.625 µg/m at three exposure periods of 24,48, and72 h using MTT testing. The toxicological effect of the extract appeared for all six concentrations in the Hep-G2 cell line. The highest concentration of 20 µg/ml had the highest percentage inhibition rate with a significant difference ($P \leq 0.01$) and reached 93.36 ±1.61 after 72 h of exposure. While the lowest concentration of 0.625 µg/ml was recorded rate of inhibition of 23.36 ± 2.34 after 24 h of exposure. The findings of the present study concluded that the *Citrullus colocynthis* is one of the most promising medicinal plants which effectively treats cancer through its inhibitory effect and fatal toxicity on cancer cells.

Keywords: *Citrullus colocynthis,* Cucurbitacin, Human hepatocyte carcinoma, Inhibition Rate

1. Introduction

Complementary or alternative medicine using medicinal plants plays a key role in maintaining human health and improving their life path. Increasing interest in studying medicinal plants is reflected in the number of recent publications as they contain a large number of medically effective ingredients which reflect their great therapeutic potential, and are distinguished from chemical drugs by their therapeutic effects as well as their low side effects (1). Cancer is a leading cause of death worldwide including a group of diseases in which cells abnormally multiply and spread to other nearby tissues (2). Two types of tumors are common in cancer: benign tumors and malignant tumors. Cancer affects most tissues and organs of the body such as the breast, liver, stomach, lung, colon, and skin among which breast, liver, and lung cancer are the most common ones in the world (3).

The *Citrullus colocynthis L* is a perennial herbaceous plant belonging to the family *Cucurbitaceae* which is a semi-arid plant found in Arab countries, along the coasts of the Mediterranean Sea and Caspian Sea, North Africa, Turkey, Iran, Afghanistan, India, Pakistan, and Sri Lanka (4). *Citrullus colocynthis L* has several designations,

including bitter apples, bitter cucumbers, or bitter pumpkins (5) which is used to treat many diseases such as diabetes, ulcers, asthma, urinary tract infections, mastitis, jaundice, and cancers (6). Several parameters such as nutritional facts and data are noticed when considering the quality of food. The fruit of *Citrullus colocynthis* is approved for feeding animals and humans alongside medicinal applications. The nutritional and functional properties of the seeds of *Citrullus colocynthis* have been investigated by the United States Department of Agriculture (USDA) which indicates that these seeds potentially find a place in the food industry. Several pharmacological investigations have been performed based on the medicinal application of *Citrullus colocynthis.* The anticancer and antidiabetic activities of fruit and seed extracts of *Citrullus colocynthis* have been studied. Newly developed anticancer/antitumor medications appear to have been developed based on the extracted chemicals from *Citrullus colocynthis* due to the high contents of cucurbitacins. Therefore, the present study aimed to identify the cytotoxic effect of the alcohol extract from the fruits of *Citrullus colocynthis* on the growth of human hepatocyte carcinoma (Hep-G2) (Figure 1).

Figure 1. The fruits of the *Citrullus colocynthis L*

2. Materials and Methods

2.1. Preparation of Plant Materials and Extracts

The crude alcohol extract of the fruit of *Citrullus colocynthis* was prepared by adding 50 g of plant powder to 250 ml of 70% ethanol, and the mixture was placed on a magnetic stirrer at room temperature for 3 days. Then the mixture was filtered with gauze and paper (Whatman No.1). The filter was placed in the incubator for drying. The extract was then distributed after drying and stored in glass bottles at 4 ° C. Then, 0.1 g of dry extract was dissolved in 10 ml of serum-free RPMI medium when preparing the original extract (stock). Finally, the required concentrations of 20,10,5,2.5,1.25, and 0.625 µg/ml were prepared after sterilizing with 0.4 and 0.22 µm filter paper.

2.2. Cancer Cell Line (Hep-G2)

This cell line was cultured with passage 25 by the Faculty of Nursing, the University of Babylon, Babylon, Iraq. The cell line was developed on RPMI-1640 medium supplied with 10% bovine serum. After complete monolayer formation, cells were treated with Trypsin-Versene solution to divide them on another secondary culture.

2.3. Preparation of Cancer Cell Line Medium

The culture medium was prepared according to Freshney (7) by mixing its components to prepare one liter of it and sterilizing using 0.22 µm filter paper. The culture medium was then distributed in 200 ml airtight glass bottles and samples were kept at -20° until use. Where the steps for tissue culture were performed under sterile conditions including the addition of 2 ml of Trypsin-Versene solution, a tissue culture vial of 25 cm^3 containing cells was performed after emptying it from the old culture medium. The vial was then gently moved and incubated at 37 ℃ for 15 min to decompose the adherent cells into single cells, after that approximately 15 ml of new growth medium (RPMI-1640) was added to the vial containing the loose cells. The vial was stirred well and then emptied. The contents of the vial containing the new culture medium with the cells were transferred to another new vial so that the level of the culture medium with the cells was equal between the two vials in which approximately the same volume of the culture medium was placed with the cells. The bottles were incubated for many days at 37°C after writing full information about the type of cells on them. Then, the secondary culture and the follow-up were performed.

2.4. Preparation of Methyl Thiazolyl Tetrazolium (MTT) dye

The solution was prepared according to the method of Betancur-Galvis, Morales (8) by dissolving 0.005 g of dye powder in 1 ml of Phosphate Buffered Saline (PBS) in a beaker placed on a magnetic stirrer. Then, the dye was filtered through a 0.22 µm filter paper to remove the blue crystals. The dye was stored under sterile conditions and in a dark place to prevent oxidation in the light.

2.5. Detection of the Toxic Effect of Plant Extracts by MTT Assay

• The suspended cells were prepared by treating the layer of cells grown in a tissue culture bottle of 50 cm^3 with a solution of Trypsin-Versene and 20 ml of culture medium containing serum was added. Then, 10% of the cell suspensions were mixed well and 0.2 ml was transferred after each well mixing into a pit of a plate Calibration using Micropipette.

• The container remained in the incubator at 37 ° C for 12-18 h until the cells adhered to the hole, after which the old culture medium was removed from the holes. Then, 0.2 ml of the previously prepared concentrations of the extract was added with four replicates for each concentration also to four replicates for control.

After the exposure time specified for the incubation, the plate was removed from the incubator. The cells were washed with prepared phosphate buffer saline (PBS) after removing the culture medium. Then, 0.1 ml of MTT stain was added to each hole and left for 3 h. The contents of the plate in the incubator were neglected for some time and cells adhered to the bottom of the hole turned yellow. Finally, 0.1 ml of DMSO solution was added. The results were read using an ELISA Microplate Reader at 492 nm.

• The previous steps were performed on the cancer cell line with three exposure times of 24, 48, and 72 h.

• The growth inhibition percentage was calculated for each concentration of the extract by calculating the growth inhibitory rate using the following equation (9-11).

Inhibition rate (IR) = A- B / A * 100

Where:

IR= percentage of inhibition rate

A = optical density of control samples

B = optical density of the test samples

2.6. Statistical Analysis

The effect of differences in the parameters of the study was identified using SAS (2012) and a significant difference was used to compare the means in this study by the LSD test.

3. Results

3.1. Detection of Chemical (Primary) to Secondary Metabolites in the Crude Alcoholic Extract of the Fruit of C*itrullus colocynthis*

Table 1 presents the results of the chemical detection of the effective groups of the alcoholic extract of the fruit of *Citrullus colocynthis* which confirm the presence of many effective compounds including Saponins, Terpenoids, Flavonoids, Amino Acids, Phenols, Glycosides, Tannins, Resalosids, Iridoids. These results are consistent with the findings of many studies, including Hussain, Rathore (12).

Table 1. The results of chemical detection in the crude alcoholic extract of the fruit of C*itrullus colocynthis*

Chemical Group	Results
Alkaloids	+
Amino Acids	+
Flavonoids	+
Terpenes	+
Phenols	+
Tannins	+
Iridoid glycosides	+
Resins	+
Saponins	+
Glycosides	+

(+) the presence of the active substance

When the Hep-G2 cell line was treated with different concentrations of the crude alcoholic extract of the plant for exposure time of 24, 48, 72 h to test the toxicity of the plant extract towards the cancer cells, the results indicated that inhibition was observed in cell proliferation at the six concentrations. This inhibition increases with increasing concentration and exposure time. Table 2 and figures 2 and 3 show that the extract has an inhibitory effect on the cancer cell vitality (Hep-G2) for all concentrations with significant differences from the control group, where the highest rates of cell growth inhibition reached at a concentration of 20 µg/m and for all exposure times of 42, 48, 72 h were 54.58 ± 2.30%, 80.39 ± 0.74 %, and 93.36 ± 1.61 %, respectively with high significant differences with probability level $P \leq 0.01$. When the lowest percentage of growth inhibition of Hep-G2 cells appeared at the lowest concentration of 6.25 µg/m for an exposure time of 24 h, the percentage of inhibition was 23.36 ±2.34 % which is called the dose and time-dependent phenomenon.

Table 2. The effect of alcoholic extract of the fruit of *Citrullus colocynthis* on the growth of human Hepatocellular cancer (Hep-G2) cell line during exposure times of 24, 48, and 72 h

Conc. (µg/ml)	Mean ± SE			LSD value
	24 h	48 h	72 h	
0.625	23.36 ±2.34^{c}	28.55 ±0.86^{b}	38.84 ±0.55^{a}	5.10 **
1.25	27.30 ±1.79^{c}	35.20 ±1.63^{b}	52.51 ±1.59^{a}	5.81 **
2.5	34.39 ±0.29^{c}	40.23 ±1.28^{b}	57.79 ±0.66^{a}	2.94 **
5	40.09 ±0.39^{c}	57.43 ±1.10^{b}	70.38 ±1.98^{a}	4.61 **
10	43.68 ±1.67^{c}	68.04 ±0.63^{b}	87.25 ±2.62^{a}	6.35 **
20	54.58 ±2.30^{c}	80.39 ±0.74^{b}	93.36 ±1.61^{a}	5.80 **
LSD value	5.19 **	3.39 **	5.14 **	---

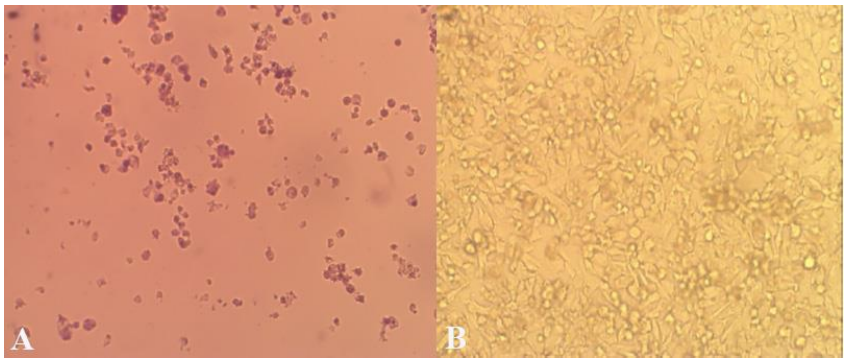

Figure 2. (A) The effect of crude alcoholic extract of *Citrullus colocynthis* on (Hep-G2) of 20 µg/mL, **(B)** compared to control (without extract) in (Hep-G2) cell line, after exposure for 72 h

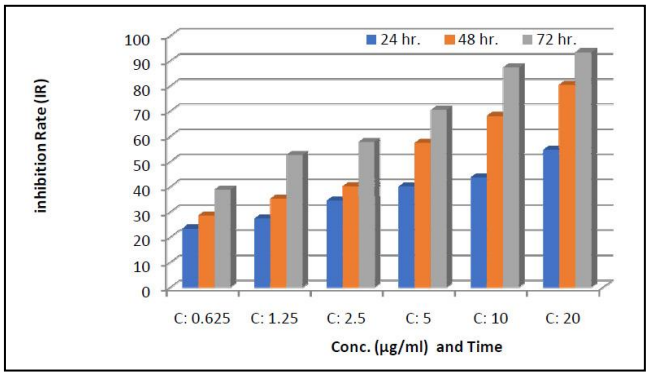

Figure 3. The ***effect*** of alcoholic extract of the fruit of *Citrullus Colocynthis* on the growth of human Hepatocellular cancer (Hep-G2) cell line

4. Discussion

Studies have proven that modern medicine reduces the risk factors for cancer and continues research into a few secondary effects of treating diseases. Accordingly, natural products were used, which were of increasing importance as they have preventive and therapeutic effects for cancer with no side effects (13). The present study reinforced the findings of many researchers in various studies about the anti-cancer activity of plant extracts, and this effect mainly depends on the concentration, the type of extract, and the sensitivity of cancer cells (14).

The fruits of *Citrullus colocynthis* contain effective compounds which inhibit the growth and kill tumor cells by affecting the physiological state of these cells, and some of these compounds also stop the life cycle of cancer cells at a certain stage and prevent their multiplying (15).

Mechanisms by which some secondary metabolites of plant extracts are involved include the stimulation of cancer cells to apoptosis (16), where cancer cells have unique characteristics that their natural counterparts lack, as they are characterized by opportunism, ability to invade, spread, and hyper-need, as well as on the occurrence of changes in their proteins and surface antigens. Also, the permeability of cancer cell membranes facilitates the random and irregular entry of compounds into them which negatively affects these cells and their response to the anti-substances to which they are exposed (17). Also, some factors, genes, or proteins in cancer cells are different from those in normal cells and can be the target of the secondary metabolic compounds affecting cancer cells, including the enzyme Telomerase which is found in the cancer cells and perpetuates the production of DNA (18). It also inhibits the enzyme of Topoisomerase and stops the growth of the cancer cell to enter the stage of programmed death (19).

Field and Schley (20) also found that the alcoholic extract of fruits of the *Citrullus colocynthis* has effective therapeutic effects for many cancers, including liver and breast cancer (Mcf-7) in humans. The linoleic acid in alcoholic extracts increases tumor suppressor genes that regulate Estrogen (21). Another study demonstrated that alcoholic extract of *Citrullus colocynthis* suppresses the growth of cancer cells in colon cancer due to its antioxidants, including Flavonoids (Isosaponarin, Isovitexin, and isoorientin 3-O-methyl ether) (22).

Citrullus colocynthis has attracted the attention of researchers for treating cancer due to having Cucurbitacin and its derivatives which strongly inhibit several types of cancer by influencing pathways such as Janus kinase (JAK)/signal transducers and activators of transcription (STATs), Wnt (integrated or int-1.) signals, and the pathway of Mitogen-Activated Protein Kinase (MAPK) which play an important role in regulating the immune system. Cucurbitacin has been shown to inhibit cancer growth via a wide range of mechanisms, including proapoptosis, induction of autophagy, cell cycle arrest, inhibition of cancer invasion, and migration, and also modulate multiple intracellular signaling pathways (23). Bourhia, Messaoudi (24) found that *Citrullus colocynthis* had toxic effects on two types of cancer lines, colon adenocarcinoma (HT-29) and breast cancer (MDA-MB-231) in humans as the chemical compounds found in bitter melon extracts such as ethylbenzene, tetrachloroethylene act alone or in a possible synergy with other substances found in the plant to have a toxic effect on HT-29 and MDA-MB-231 cells.

Authors' Contribution

Study concept and design: Z. A. A. M.
Acquisition of data: I. H. A.
Analysis and interpretation of data: L. H. S.
Drafting of the manuscript: I. H. A.
Critical revision of the manuscript for important intellectual content: L. H. S.
Statistical analysis: Z. A. A. M.
Administrative, technical, and material support: Z. A. A. M.

Ethics

The study protocol were approved by the ethics committee of the University of Kerbala, Kerbala, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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