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BIOLOGICAL ACTIVITIES OF *Citrullus colocynthis* ETHANOLIC SEED EXTRACT

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ABSTRACT: The bitter gourd plant *Citrullus colocynthis* (L.) is a cucurbit that is widespread in desert areas. It has many pharmaceutical, medicinal, and nutritional uses due to its high content of biologically active compounds. In this study, the total phenolic compounds (TPCs) and total flavonoids (TFs) of ethanolic *C. colocynthis* seed extract were estimated. High-performance liquid chromatography (HPLC) was used to identify the bioactive phenolic compounds. On the other hand, the antioxidant and anticancer activities of this extract were evaluated. The extraction yield from *C. colocynthis* seed using ethanol was recorded 10 g/100g seed. The total phenol (TPCs) content of the ethanolic extract obtained from *C. colocynthis* seed flour was 21 mg GAE g^{-1} dry extract, while the total flavonoid (TFCs) content was 15 mg QE g^{-1} dry extract. As well as the major phenolic and flavonoid compounds in *C. colocynthis* seed ethanolic extract, as determined by HPLC. Ellagic acid and cinnamic acid have the highest concentrations (894 and 746 µg/g dry extract, respectively) of all the components. The anticancer activity in vitro of the HCT116 and A-549 human cancer cell lines was evaluated using the MTT assay. The results of the MTT assay demonstrated that the *C. colocynthis* seed extract inhibited the proliferation of HCT116 and A-549 cells in a dose-dependent manner. *C. colocynthis* seed extract had an IC_{50} against HCT116 and A-549 (454, and 416 µg/mL, respectively). Therefore, *C. colocynthis* can be used as a source to produce pharmaceutical and medicinal products.

Key words: Bitter gourd, total phenolics, total flavonoids, DPPH-assay, MTT-assay.

INTRODUCTION

Cancer is one of the mortal disease and important public health subjects in both developed and developing countries **(Shokrzadeh *et al.*, 2010)**. Cancer belongs to a group of diseases which cells abnormally are proliferating and extending in the adjacent tissues. Uncontrolled division of cells can lead to create tumors **(Bray *et al.*, 2012)**. Breast and gastric cancer are the five most common types of cancer which diagnosed in 2012 **(Fattahi *et al.*, 2013)**. According to the World Health Organization comments, breast cancer is the most prevalent cancer among women worldwide and very rare in men. It is the second most common cancer in women after skin cancer in USA. Each year there are about 2,300 new cases of breast cancer in men and about 230,000 new cases in women. Stomach cancer known as gastric cancer is the fifth leading cause of cancer and the third leading cause of death from cancer making up 9% of deaths. It infected 950,000 people and caused 723,000 deaths, in 2012 **(Fattahi *et al.*, 2013).** Therefore, offering pivotal treatment options will play an important role in development society health **(De Martel *et al.*, 2012)**. Surgery, radiation therapy, chemotherapy, hormone therapy and targeted therapy are used for treatment of cancer, but due to the lack of selective toxicity have negative side effects **(Kim *et al.*, 2015)**. Nowadays, medicinal plants have been considered as a natural source of anti-cancer agents due to some advantages for example, having antioxidant compounds and anti-mutagenic effects against

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chemicals and environmental factors, low side effect, low cost, and being easily accessible, may play a significant role in the treatment of the cancer **(Shokrzadeh *et al.*, 2010 and De Martel *et al.*, 2012)**.

Reactive oxygen species (ROS) such as hydroxyl radicals, hydrogen peroxide, singlet oxygen and superoxide anions are typically produced because of endogenous and exogenous stimuli, and are often routinely neutralized by living organisms using well established endogenous antioxidant defense systems **(Nwachukwu and Aluko, 2019)**. When produced in excess, ROS can overwhelm natural defense systems resulting in a state of oxidative stress. Sustained and cumulative oxidative stress has the potential to cause deleterious oxidative damage to cellular macromolecules such as proteins, lipids and nucleic acids resulting in irreversible alteration of cellular functions **(Nwachukwu *et al.*, 2019)**. In the case of oxidative damage to DNA for instance, ROS can react with cellular components such as phospholipids and proteins to form secondary reactive intermediates, which can irreversibly bind to DNA bases to form DNA adducts. Since DNA adducts can promote miscoding and even mutations if they evade cellular repair mechanisms, their formation is important in the carcinogenic process **(Fuchs-Tarlovsky 2013).** Studies have linked oxidative stress with the pathogenesis of inflammation-related cancers, and agents with the capacity to protect cells against ROS attack by quenching free radicals, are thought to be potent chemo preventive candidates **(Chi *et al.*, 2015)**. In fact, the correlation of antioxidative function with anticancer property has traditionally been reported in a range of biological and chemical agents, including dietary kelp and plant phenolics **(Dia and de Mejia, 2010)**.

Medicinal plants contain several active principles with specific therapeutic effects. They represent a source of chemical compounds such as tannins, flavonoids, saponins, resins and alkaloids with curative properties, often not provided by synthetic chemical compounds **(Fabricant and Farnsworth, 2001)**. *Citrullus colocynthis* belongs to the family of Cucurbitaceae. Members of this family are generally dioeciously herbs which may be prostate or climbing by means of tendrils. *Citrullus colocynthis* had been used medically since ancient times. Traditionally fruit of was used for the treatment of microbial diseases, jaundice, inflammation, ulcer, diabetes, and urinary diseases in Asian and African countries **(Saganuwan, 2010)**. The aqueous extract of *Citrullus colocynthis* possesses an antidiabetic effect and antioxidant activity **(Gurudeeban and Ramanathan, 2010)**. It had been used as a strong laxative to treat refractory edema, amenorrhea, nerves pain fever, snake bite, antiparasite (worms), muscle pain in hand and feet effects are its other important features. Recent research shows that consumption of *C. colocynthis* with radioactive radiations has effects on growth of cancerous tumors **(Al-Zahrani and Al-Amer, 2006).** The objectives of this work are to demonstrate the antioxidant and anticancer activities in correlation to phytochemical constituents of ethanolic crude extracts from seeds of *C. colocynthis*.

MATERIALS AND METHODS

Plant Materials

The seeds of *Citrullus colocynthis* (L.) were purchased from a local herb market in Zagazig City, Egypt.

Preparation of Seed Ethanolic Extract

The *Citrullus colocynthis* seeds were cleaned manually and powdered. Twenty grams of ground seeds were extracted with ethanol 80% (200 ml) using a magnetic stirrer at 25°C±3°C for 2 h, followed by filtration by filter paper Whatman No.1 The remains were re-extracted twice under the same conditions. Ethanol was separated in a vacuum rotary evaporator (BüCHI -water bath-B-480), while remained water was lyophilized (Thermo- electron Corporation–Heto power dry LL 300 Freeze dryer). The extract was preserved in a refrigerator for further analysis **(Shawkey *et al.*, 2014)**.

Total Phenolic Compounds (TPCs) Determination

The TPCs were estimated by Foline-Ciocalteu reagent as described by **Singleton *et al.* (1999)**. One ml of sample (1000 µg/mL) was added to 5 ml from Folin-Ciocalteu reagent (previously diluted with water 1:10, V/V) and 4 ml sodium carbonate (75 g/l). The tubes were

vortex mixed for 15 s and allowed to stand for 30 min at 40°C for colour evolution. Absorbance was determined at 765 nm. Gallic acid was applied to gain the standard curve (20 – 200 µg/mL), and the lowering of Folin-Ciocalteu reagent by the samples was expressed as mg of gallic acid equivalents (GAE) per g of extract. The calibration equation for gallic acid was y = 0.001x + 0.0563 (R2 = 0.9792), where y is absorbance and x is concentration of gallic acid in µg/mL.

Total Flavonoids (TFs) Eetermination

Total flavonoids (TFs) were estimated according to the protocol of **Ordonez *et al.* (2006)**. Two ml aliquot of 20 g/L $AlCl_3$ ethanol solution was blended with 1 mL of the extract (1000 µg/mL solvent). After 60 min, the absorbance at 420 nm was estimated. Quercetin was applied to gain the standard curve (20–200 µg/ml), Total flavonoids contents expressed as quercetin equivalent (QE), which was calculated based on the calibration curve. The calibration equation for quercetin was y = 0.0012x + 0.008 (R^2 = 0.944), where y is absorbance and x is concentration of quercetin in µg/mL

Polyphenolic Compounds Identification

High-Performance Liquid Chromatography (HPLC) was used to identify polyphenolic compounds in the ethanolic extracts of the *Citrullus colocynthis* seeds **(Abd Elhamid *et al.*, 2022)**. HPLC-Agilent 1100 apparatus is composed of a C18 column (125 mm × 4.60 mm, 5 µm particle size), two LC- pumps pump, a UV/Vis detector. Phenolic compounds were isolated by using a gradient mobile phase of two solvents was applied- Solvent A (methyl alcohol) and Solvent B (Acetic acid in water (1:25).

Antioxidant Activity Evaluation (DPPH-assay)

The antioxidant activity of *Citrullus colocynthis* seed extract was estimated by using DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay according to **(Hatano *et al.*, 1988)**. 500 µl of extract at different concentrations (100, 250, 500, 1000, 1500 and 2000 µg extract/1ml solvent) were added to 2500 µl of 0.1 mM DPPH dissolved in ethanol according to the solvent used for extraction. After incubation period of 30 min at 27 °C ± 3 °C, the absorbance was recorded with the control at 517 nm **(Gülçin *et al.*, 2004).** The antioxidant potential of DPPH radicals (%) was studied as follow:

$$\text{Inhibition (\%)} = [(\text{Abs control} - \text{Abs sample})/\text{Abs control}]\text{x } 100$$

Where Abs. control is the absorbance of the control and Abs. sample is the absorbance in the presence of *Citrullus colocynthis* seed ethanolic extract.

Cell Viability *in vitro* (MTT-assay)

At different concentrations (31.25, 62.5, 125, 250, 500, and 1000 µg/mL), the impact of *Citrullus colocynthis* seed ethanolic extract on the cell viability *In Vitro* was estimated using MTT-assay. The human colon cancer cell line (HCT 116), and adenocarcinoma human alveolar basal epithelial cells (A549) were obtained from Sigma-Aldrich. HCT 116, and A549 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (10 U/mL, Sigma-Aldrich), and streptomycin (1 mg/mL, Sigma-Aldrich). The cultures were incubated in the presence of 5% CO_2 at 37°C and 100% relative humidity. The cells were seeded in 96-well microplates at a density of 10×10^3 cells/well and were grown for 24 h at 37°C in 5% CO_2 before the addition of the samples. The cells were treated with various concentrations (31.25, 62.5, 125, 250, 500, and 1000 µg/mL) of extract dissolved in ethanol. Cell viabilities were determined after 48 h incubation using the colorimetric MTT assay (Promega, Madison, WI, USA) (**Hansen *et al.*, 1989**). The cell viability (%) was estimated according to the absorbance at 550 nm. Triton X-100 (10 µL of a 10% solution) was used as the positive control, whereas untreated cells (0 µg/mL vehicle only) were used as the negative control. The percentage of cell viability was calculated by the following formula:

$$\text{Cell viability (\%)} = (\text{Ab sample}/\text{Ab control})\text{x } 100.$$

Cytotoxic activity (%) of extract was calculated using the following formula:

$$Cytotoxic\ activity\ (\%) = 100\ \% - cell\ viability\ (\%).$$

The extract concentration which gives 50% growth inhibition is referred to as the IC_{50}.

RESULTS AND DISCUSSION

Yield and Identification of Phenolics and Flavonoids Constituents

In experimental study of *C. colocynthis* seeds ethanolic extract, the total phenol content, total flavonoid, and extraction yield are given in Table 1. The extraction yield from *C. colocynthis* seed using ethanol was recorded 10 g/100 g seed. The total phenol (TPCs) content of the ethanolic extract obtained from *C. colocynthis* seed flour was 21 mg GAE g^{-1} dry extract, while the total flavonoid (TFCs) content was 15 mg QE g^{-1} dry extract.

From obtained results, Yemeni *C. colocynthis* has the highest total phenols content (6.1 g GAE /100 g extract) compared with *C. colocynthis* obtained from Pune, India (3.2 g GAE/100 g extract) **(Talole *et al.*, 2013)**, Haryana, India (0.74 g GAE/100 g extract) **(Kumar *et al.*, 2008)** and west Algeria (0.166 g GAE/100 g extract) **(Benariba *et al.*, 2013)**. As a result, *C. colocynthis* seed extract showed good antioxidant capacity values. This result asseverates that the antioxidant activities of extract are associated with their polyphenolic content **(Thamer *et al.*, 2023)**.

A representative chromatogram of the HPLC phenolic compounds analysis of *C. colocynthis* seeds ethanolic extract was shown in Fig. 1. Twelve peaks (Gallic acid, Chlorogenic acid, Syringic acid, Pyro catechol, Ellagic acid, Vanillin, Ferulic acid, Naringenin, Daidzein, Quercetin, Cinnamic acid, and Hesperetin) dominated in the chromatograms from the separation of C. colocynthis seeds ethanolic extract. Table 2 summarizes the contents of phenolic compounds 1–12 in the *C. colocynthis* seeds ethanolic extract. It was shown that Ellagic acid and Cinnamic acid were the main phenolic compounds in the *C. colocynthis* seeds ethanolic extract. Ellagic acid and cinnamic acid have the highest concentrations (894 and 746 µg/g dry extract, respectively) of all the components.

Antioxidant activity

Antioxidants activity (% inhibition) for *C. colocynthis* seeds ethanolic extract using DPPH assay are presented in Figure 2. *C. colocynthis* seeds ethanolic extract have antioxidant activity. These results are compatible with our results recorded in total phenolic compounds. It can be noted that the antioxidant activity of *C. colocynthis* seeds ethanolic extract increased gradually with increasing concentrations of TPCs and TFCs. When the concentration of extract was increased from 100 to 2000 µg/mL, the DPPH radical scavenging activity increased from 22 % to 88 %. In ethanol, DPPH has a maximum absorbance of 517 nm, making it a stable free radical. The radical is scavenged and the absorbance is reduced when DPPH comes into contact with a substance that donates protons **(Yang *et al.*, 2008)**. This result asseverates that the antioxidant activities of extracts are associated with their polyphenolic content **(Nessa and Khan, 2014).**

Anticancer Activity

C. colocynthis seeds ethanolic extract at different concentrations (31.25, 62.5, 125, 250, 500, and 1000 µg/ mL) were tested *in vitro* for their anticancer activity on the HCT 116, and A-549 human cancer cell lines using the MTT assay. Figures 3 and 4 show estimates of the cell viability percentages as well as their cytotoxic activity. There was a linear relationship between cell viability (%) and extract concentration, and overall, cell viability (%) decrease with increasing concentration. The MTT assay revealed that the extract inhibited the proliferation of the human cancer cell lines (HCT 116 and A-549) in a concentration-dependent manner. *C. colocynthis* seed. extract had an IC_{50} against HCT116 and A-549 (454, and 416 µg/mL, respectively).

Mechanisms by which some secondary metabolites of plant extracts are involved include the stimulation of cancer cells to apoptosis **(Rajabi *et al.*, 2021)**, 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

Table 1. Yield of extracted substances (g), TPCs (GAE g^{-1} dry extract), and TFs (QE g^{-1} dry extract)

Parameters	Concentration
Extraction yield	10 g/ 100g seed
TPCs	21 mg GAE g^{-1} dry extract
TFs	15 mg QE g^{-1} dry extract

TPCs: total phenolic compounds; TFs: total flavonoids

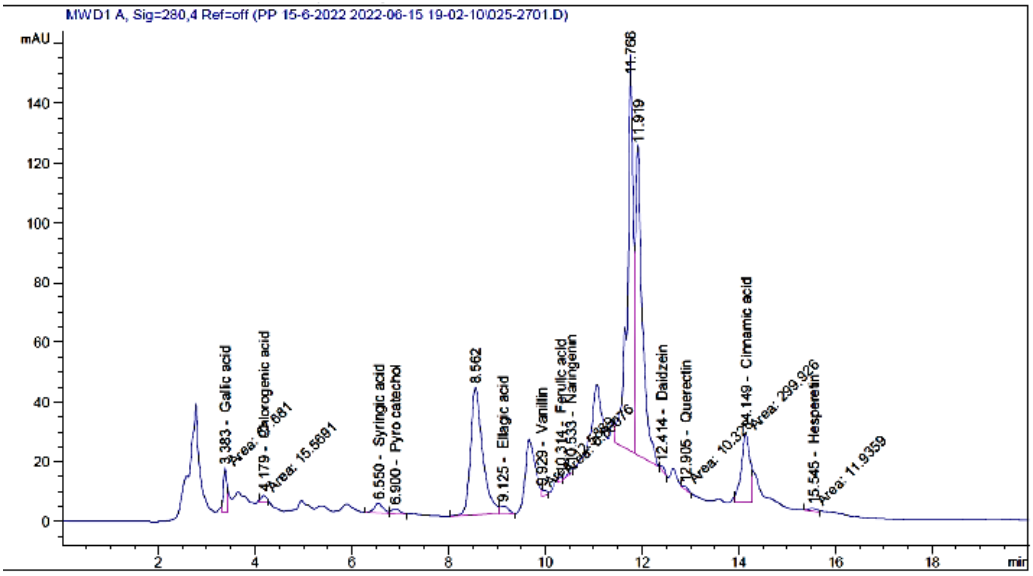

Fig. 1. HPLC-chromatogram of major phenolic compounds in *C. colocynthis* seeds ethanolic extract

Table 2. Major phenolic compounds retention time (min) and concentration (µg/g) in *C. colocynthis* seeds ethanolic extract

Component	Retention time (min)	Concentration (µg/g)
Gallic acid	3.383	554.19
Chlorogenic acid	4.179	209.28
Syringic acid	6.550	324.50
Pyro catechol	6.900	208.05
Ellagic acid	9.125	893.83
Vanillin	9.929	40.40
Ferulic acid	10.314	40.89
Naringenin	10.533	74.14
Daidzein	12.414	49.57
Quercetin	12.905	114.51
Cinnamic acid	14.149	745.65
Hesperetin	15.545	64.90

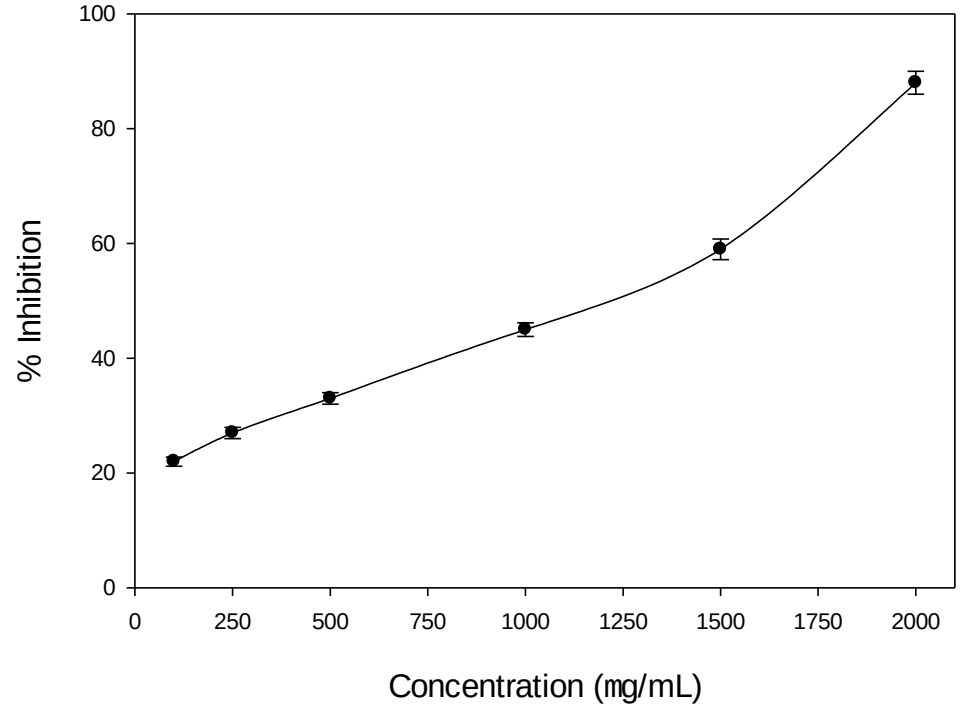

Fig. 2. Antioxidant activity (% inhibition) *C. colocynthis* seeds ethanolic extract at different concentration (100, 250, 500, 1000, 1500, and 2000 µg/mL)

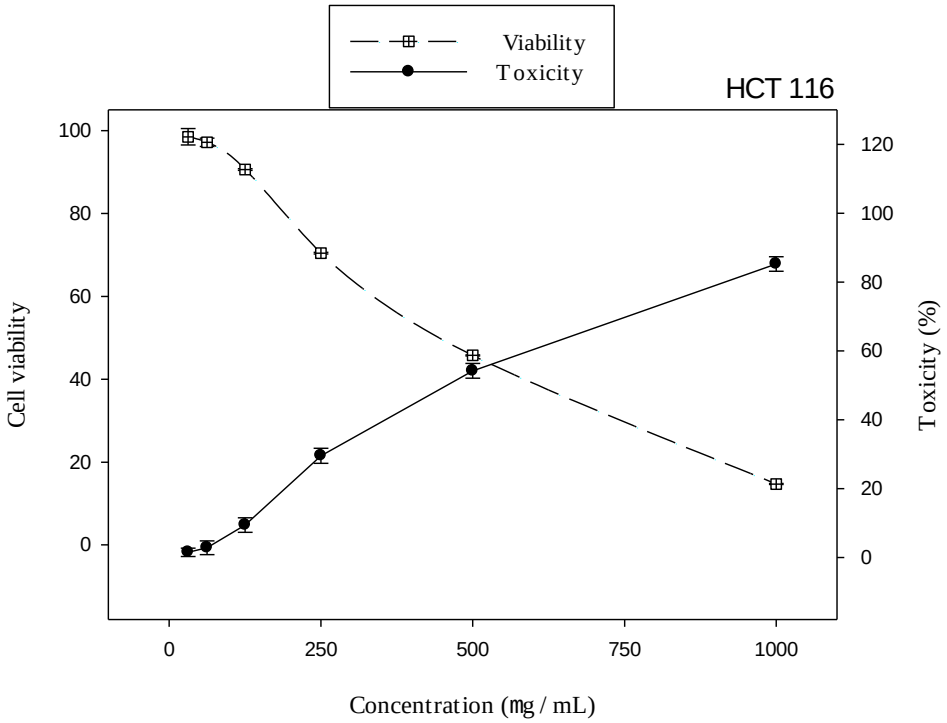

Fig. 3. Toxicity (%) and cell viability (%) of HCT 116 cell line treated with *C. colocynthis* seeds ethanolic extract at different concentration.

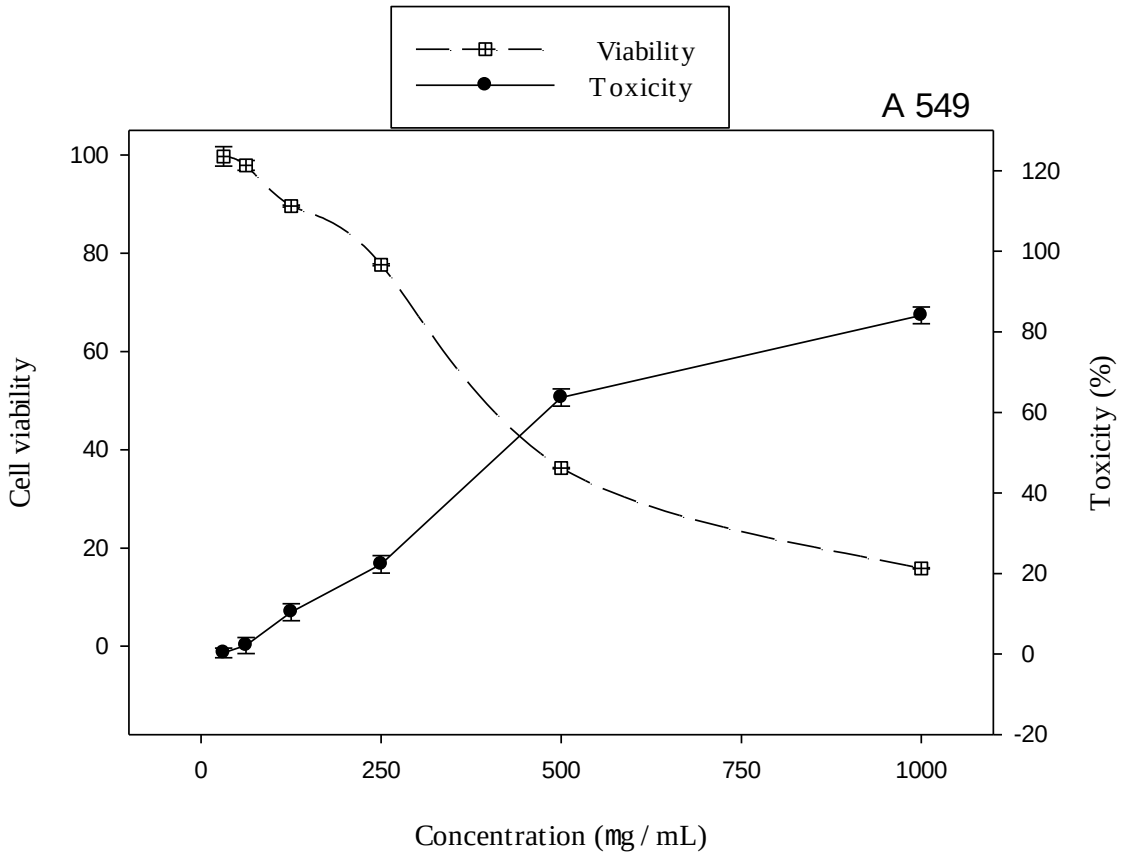

Fig. 4. Toxicity (%) and cell viability (%) of A 549 cell line treated with *C. colocynthis* seeds ethanolic extract at different concentration

exposed **(Mohsenzadeh *et al.*, 2009)**. 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 **(Thelen *et al.*, 2004)**. It also inhibits the enzyme of Topoisomerase and stops the growth of the cancer cell to enter the stage of programmed death **(Chen *et al.*, 2008)**. Field and Schley **(Field and Schley, 2004)** 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.

Conclusions

C. colocynthis seeds are a valuable source of medicinal compounds that have been traditionally used for numerous applications. Based on the results obtained in this study, it can be concluded that *C. colocynthis* seeds extract showed highly effective as an antioxidant and anticancer. Therefore, *C. colocynthis* can be used as a source to produce pharmaceutical and medicinal products.

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الأنشـــطـــة البيـــولـــوجيـــة للمســتخلص الإيثـــانـــولـــى لبـــــذور نبـــات الحنظـــل

وفاء عيد محمد - رجب عبدالفتاح المصري – هند أحمد ممدوح العقاد – على عثمان

قسم الكيمياء الحيوية - كلية الزراعة - جامعة الزقازيق - الزقازيق - مصر

ينتشر نبات الحنظل أو ما يعرف بالقرع المر فى المناطق الصحراوية وله العديد من الاستخدامات الصيدلانية والطبية والغذائية لاحتوائه على نسبة عالية من المركبات النشطة بيولوجيا. فى هذه الدراسة تم عمل مستخلص ايثانولى من بذور نبات الحنظل وتقدير محتواه من المركبات الفينولية ومركبات الفلافونويد الكلية وتعريف المركبات الفعالة بواسطه جهاز التحليل الكروماتوجراى السائل عالى الأداءHPLC. على الجانب الأخر تم تقدير النشاط المضاد للأكسدة والمضاد للسرطان. أوضحت النتائج أن كل 100 جرام من البذور يعطى 10 جرام مستخلص وأن كمية المركبات الفينولية الكلية 21 ملليجرام مكافئ من حامض الجاليك لكل جرام مستخلص بينما كمية مركبات الفلافونويدات الكلية 15 ملليجرام مكافئ من الكيريستين لكل جرام مستخلص. سجلت نتائج التحليل الكروماتوجرافى السائل أعلى تركيز فى حاله حمض الايلاجيك (894 ميكروجرام لكل جرام مستخلص) يليه حمض السينامك (746 ملليجرام لكل جرام مستخلص). تم تقييم النشاط المضاد للسرطان معمليا ضد الخلايا السرطانية البشرية (HCT116 و A-549) باستخدام اختبار .MTT أظهرت نتائج اختبار MTT أن مستخلص بذور الحنظل يثبط تكاثر خلايا HCT116 و A-549 بطريقة تعتمد على التركيز. حيث سجلت قيم IC50 (454 و 416 ميكروجرام لكل ملل مستخلص ضد HCT116 و A549 على التوالى. وبالتالى يوصى باستخدام بذور نبات الحنظل لإنتاج المنتجات الصيدلانية واستخدامها طبيا.

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