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Anticancer Efficacy

RESEARCH ARTICLE

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Anticancer Efficacy of *Citrullus Colocynthis* Oil as a Promising Agent in Suppressing Human Lung Adenocarcinoma A549 Cells

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Abstract

Objective: Lung adenocarcinoma is the most common respiratory system tumor worldwide. This type of tumor facing challenges such as multidrug resistance and low selectivity. The need for alternative therapeutic strategies motivates researchers to discover new approaches for tumor treatments. Previous studies indicate that phytochemicals may inhibit cancer development and progression. **Methods:** *Citrullus colocynthis* oil extraction was used as phytochemical therapy to inhibit cell proliferation, invasion, and enhanced cell death. The extracted oil was analyzed using GC-mass, and cell viability was assessed by MTT, apoptotic, and wound healing assay. In this study, human lung adenocarcinoma A549 cancer cell lines and human breast epithelial HBL-100 normal cell lines were used to test the efficiency of *Citrullus colocynthis* oil extraction on inhibiting lung cancer. **Result:** The results demonstrated a significant cytotoxic effect of Citrullus Colocynthis oil extraction against cancer cells, with no observed effects on normal cells. **Conclusion:** Phytotherapy may suppress human lung adenocarcinoma A549 cancer cell lines and offer a new strategy for future cancer therapy.

Keywords: Cytotoxicity- Anticancer therapy- Human lung adenocarcinoma- *Citrullus Colocynthis* medicinal plant

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Introduction

Cancer will remain a major global health issue, leading causes high rates of mortality [1]. Lung cancer has become the leading cause of cancer-related death around the world. According to the World Health Organization (WHO), about 1.8 million deaths and 18.7% of all cancer-related deaths annually (WHO, 2024). Cancer development starts with genetic mutations or with genes that have tumor suppressors [2]. Uncontrolled cell growth resulting from genetic mutation leads to damage in surrounding tissues and organs [3]. Current cancer therapy such as chemotherapy displays several severe adverse effects, nausea, hair loss, digestive problems, and weakened immune system. These adverse effects are due to poor selectivity affected tissues overall normal tissues. [4,5]. As a consequence, alternative therapies, including natural products are needed that could offer more effective treatments with less toxic for cancer treatments [6,7].

As a protection strategy, several plants have developed various strategies to protect themselves from outside threats through producing metabolites and natural bioactive compounds [8, 9]. For instance, *Citrullus colocynthis*, also known as bitter apple or desert gourd, is a valuable plant in the Cucurbitaceae family found indigenously in the Mediterranean Basin and West Asia regions [10-12]. *C. colocynthis* has several active secondary metabolites including phenols, glycosides, flavonoids, alkaloids, and fatty acids. The oil extracted from the plant contains a high concentration of these compounds, and have been found to have therapeutic effects [13], for example, anti-viral, anti-bacterial, antifungal, anti-inflammatory, antimutagenic, anticarcinogenic, and antioxidant implications [14,15]. The potential therapeutic benefits of *C. colocynthis* oil have not been entirely discovered, especially in its regions [16]. Based on our knowledge, as yet there are no pharmacology and toxicology studies focused on the benefits of *C. colocynthis* oil in Iraq. The aim of this study is to evaluate the cytotoxicity of extracted oil from *C. colocynthis* on lung A549 cancer cells using MTT assay. As well as, determine the oil potential to induce apoptotic cell death in treated cells with IC_{50} concentration, and investigate the efficiency of the extracted oil to suppress cancer cell migration and invasion via monolayer wound healing assay.

Materials and Methods

Plant samples collections

C. colocynthis was collected from desert areas in the

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Rumailah region, Basrah, Iraq between July to September 2023. The specific locations in both the northern part (722960º.215´E, 3376749º.021´N) and the southern part of Rumailah (727466º.011´E, 3361715º.765´N).

Plant authentication

The samples were certified and documented in the Department of Biology, College of Education for Pure Sciences, University of Basrah, Basrah, Iraq.

Samples preparation for extract

To prepare samples for extraction, the samples were washed with distilled water. After air drying, the samples were cut into small pieces and ground using an electric grinder.

Oil extraction

The soxhlet apparatus method was used to perform the oil extraction process. 75 grams of the grounded plant were added to a 500 mL beaker containing 250 ml of hexane. Samples were thus extracted under reflux with hexane for 8-10 h. After the extraction, the major solvent was eliminated in a vacuum rotary evaporator. Thereafter, the extract was concentrated using a rotary evaporator under reduced pressure and a controlled temperature of 45 ºC - 50 ºC. Then, the extracted oil was filtered using a 0.45 µm syringe filter at room temperature. The yield was stored in a 15 ml sterile dark tube and kept at room temperature until use.

Gas Chromatography-Mass Spectrometer

Gas chromatography-mass spectrometry (GC-MS) was used to determine the bioactive phytochemical structures of *C.colocynthis*. The analyses of the GC-MS were carried out at Basrah Oil Company Laboratory, Department of Quality Control, with Agilent Technologies (Aanta Clara, CA, USA), the GC system was 7890B coupled to 5977A MSD an Agilent Technologies with EI Signal detector, by using HP-5ms 5% phenyl 95 %, methyl siloxane 30 m * 250 µm * 0.25, the temperature of the oven had set at 40 ºC hold for 5 min, then raised to 8 ºC per min to 300 ºC for 20 min. The flow rate of the carrier of Helium gas was 1 ml per minute and the purge flow of 3 ml/min. The injection mode was pulsed split less with injection temperature 290 ºC and the injection sample volume was 0.50 µl. The mass spectrometer used an Ion Source Temperature of 250 ºC, with a scan speed of 1562(N2), and a mass range 44-750 m/z.

The identification of oil's compounds

The identification and characterization of phytochemical compounds in *C. colocynthis* fruit samples have been carried out using GC-MS. Data was run through the NIST 2014,2020 Library database to confirm the identity of compounds. The percentage composition of the sample constituents was expressed as a percentage by peak area.

Cell lines maintenance

The A549 non-small lung adenocarcinoma and HBL-100 normal human breast epithelial cell lines were obtained from the cells bank of tissue culture laboratory in the College of Education for Pure Sciences, Department of Biology, University of Basrah. The cell lines were maintained in fresh RPMI 1640 (US Biological, USA) culture medium, supplemented with 10% fetal bovine serum (Gibco, USA), 100 units/mL penicillin, and 100 µg/mL streptomycin, under humidified conditions at 37°C with 5% CO2 until they reach confluence (75% - 85%). The cells were washed twice with phosphate buffer solution (PBS) (Chemical Point, Germany), trypsinization by the addition of 1 ml of Trypsin-EDTA solution (Capricorn, USA), and resuspended in RPMI with 10% FBS in a new tissue culture flask. Then, the cells were reseeded at the same above conditions. This process was repeated twice a week or when the density of culture appeared under inverted microscopy as a monolayer [17].

Cytotoxicity assay

The cytotoxicity of *C. colocynthis* fruit oil extract was measured on HBL-100 normal cell line and lung A549 cancer cells using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Bio World, USA). Cell viability was assessed using a modified colorimetric protocol that determined live cells through the change of a tetrazolium compound into purple formazan crystals by mitochondrial reductase enzymes [18]. Briefly, A 96-well microplate was used to seed cells at a density of 1×10^4 cells/well and incubated at 37 °C with 5 % CO_2 for 24 hours. Both A549 cells and HBL-100 cells were exposed to different concentrations of *C. colocynthis* fruit oil extract (0.0625%, 0.125%, 0.250%, 0.500%, and 0.750%) for 24, 48, and 72 hours. 100 µl/ well MTT solution was added to each well and incubated for 2 hours at humidified conditions. Then, formazan crystals were dissolved in DMSO and incubated for 15 minutes. A Microtiter ELISA plate reader was used to measure absorbance at 490 nm. The percentage of inhibition was calculated relative to control cells without the extract. The cytotoxicity percentage (%), was calculated as follows: Cell inhibitory = [ODcontrol – ODtreatment / ODcontrol] × 100 %, Where, ODcontrol Optical density of untreated cells (control); ODtreatment Optical density of treated cells [19].

Inhibition concentration (IC_{50}) determination

After three experimental replicates, the IC_{50} values for each cell line were determined the inhibition rate was calculated using data collected from three replicates per concentration recorded by ELISA Microtiter (Thermo Fisher Scientific, United States). GraphPad Prism Software (version 10.1.2 (324)) was used to obtain the IC_{50} values (GraphPad Software, San Diego, California, USA) [20].

In vitro morphological changes

To study cell morphological changes in vitro, cells were incubated at a density of $5*10^5$ cells/well in a slide chamber-8 well, in a humidified incubator at 37 ºC with 5% CO_2 for 24 hours. The cells were treated with *C. colocynthis* oil at the IC_{50} dose. Untreated cells served as control. The cells that were treated with only 0.1%

methanol medium and serum-free medium served as negative control. The slide was incubated at 37 ºC with 5% CO2 for 72 hours. The cells were washed with PBS. Then, cells were viewed and captured using a camera attached to an inverted microscope (Mieji, Japan).

Wound healing assay

HBL-100 normal cell line and lung A549 cancer cells were seeded in a 6-well plate at a density of 1x105 cells per well in RPMI-1640 medium supplemented with 10 % FBS, and allowed them to achieve 80-90% confluence. Then, the monolayer cell cultures were scratched using a sterile pipette tip. The cells were treated at the same manner as mentioned before to study morphological changes. The slide was tightly covered and incubated at 37 ºC with 5% CO2 for 72 hours. Then, the cells were washed with PBS. After 72 hours of exposure, the medium was replaced with PBS and photographed [21].

Statistical analysis

All data from this study were analyzed using a one-way ANOVA test using. Three replicates were used.

GraphPad Prism v10.1.2 (324) was used to apply Tukey's post hoc test for multiple comparisons and identifications of IC_{50} values. Significant data was defined as those with a significance level of $p < 0.05$ or below. The means ± standard error of the mean from three replicates [22].

Results

GC-MS analysis findings

GC-MS was used to analyze the extracted oil from *C. colocynthis*. The results showed the highest peaks were decreased over time (Table 1). Five compounds were detected and identified retention over 48 minutes (Figure 1). The retention time of a compound was 24 minutes, a percentage 13% n-Hexadecanoic acid with formula C16H32O2. The retention time of another component was 25 minutes with a percentage of 77.3% 9,12-Octadecadienoic acid (Z,Z) methyl ester with formula C18H32O2.

Anti-tumor activity of C. colocynthis fruit oil

MTT assay results showed that the extracted oil exhibited a slight cytotoxic on the lung adenocarcinoma A549 cell line after 24 hours of incubation. The inhibition rate was 56.4% at the highest concentration (0.75%), while at lower concentrations was 7.3% (Figure 2a). In contrast, HBL-100 cells showed minimal effects with an inhibition rate of 33.6% including high concentrations (Figure 2b) and 2.9% at lower concentrations (Table 2).

Table 1. Show phytochemical compounds in *C.colocynthis* Fruit Oil by GC-MS Test

			GC-MS analysis of phytochemical compounds		
PK	RT	Area pct	Library/ID	CAS.NO/ID	Molecular formula
1	24.054	13.043	n-Hexadecanoic acid	57-10-3	$C_{16}H_{32}O_2$
2	25.153	2.4831	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	112-63-0	$C_{19}H_{34}O_2$
3	25.839	77.3	9,12-Octadecadienoic acid (Z,Z)-	2420-56-6	$C_{18}H_{32}O_2$
4	30.124	2.3925	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester	123-94-4	$C_{21}H_{42}O_4$
5	33.8086	4.7806	dl-.alpha.-Tocopherol	10191-41-0	$C_{29}H_{50}O_2$

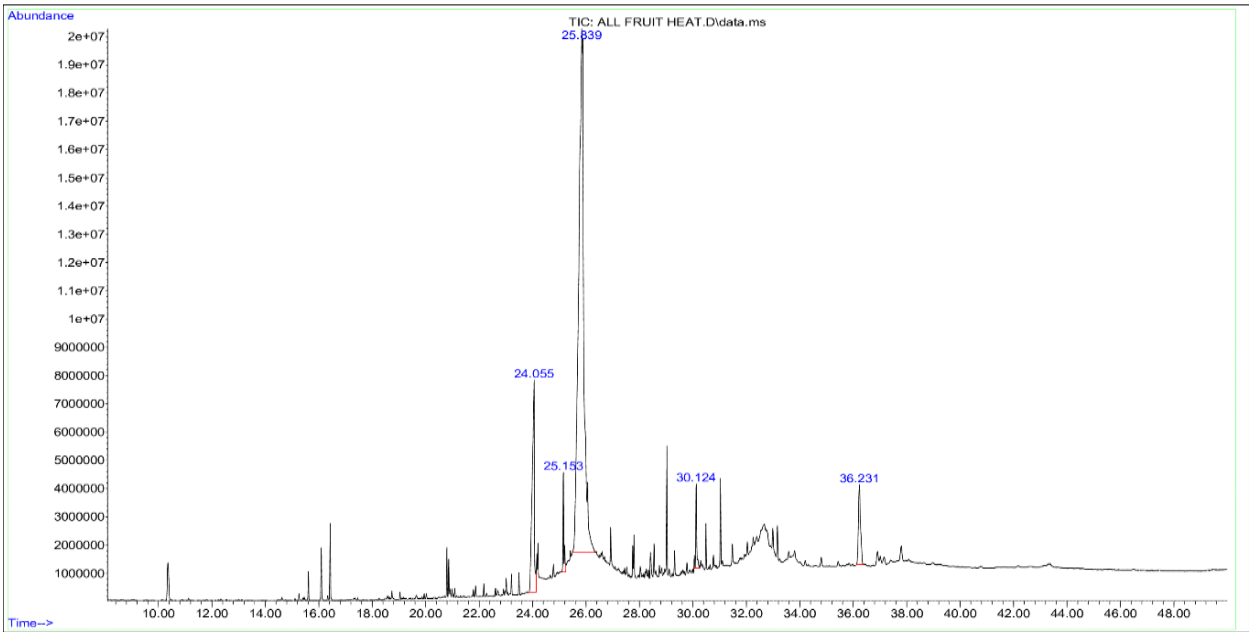

Figure 1. Illustrate Phytochemical Compounds in AFHO of *C.colocynthis* Analyzed by GC-MS Measurement in Rotation Time

Table 2. Show the Cytotoxic Effect and IC_{50} Dose of Oil on Cell Lines after 24 h of Treatment

	MTT assay results Concentrations (%)					
Cell lines	0.06%	0.13%	0.25%	0.50%	0.75%	IC_{50} dose
A549 inhibition rate	7.3	18.1	32.1	42.9	56.4	0.25%
HBL-100 inhibition rate	2.9	9.8	18.2	26.6	33.6	Non available

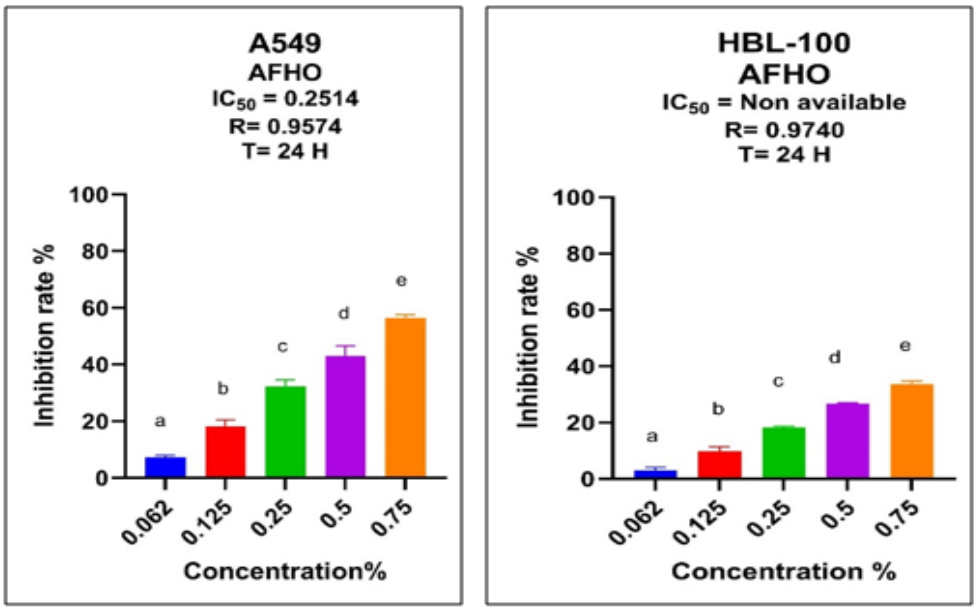

Figure 2. The results of MTT Assay Shows the Inhibitory Effect of *C.colocynthis* Oil for 24 h Treatment (A) The cytotoxic effect of oil against cancer cell line A549; (B) The cytotoxic effect of oil against normal cell line HBL-100. The data found to be statistically significant different ($p<0.05$ N=4, SD ± Mean).

The results revealed a significant difference ($p<0.05$) between the effect of the extracted oil on A549 and HBL-100 cells. The results also, showed that the inhibition effect increased in a dose-dependent manner. The IC_{50} values were started after 24 hours for the cancer cells but were not determined for the normal cells. This pattern of inhibition was observed at both 48 and 72 hours, with the inhibition rate increasing in a time-dependent manner (Tables 3 and 4) and (Figures 3 and 4).

Cytopathological changes

Cytopathic effects of the extracted oil from

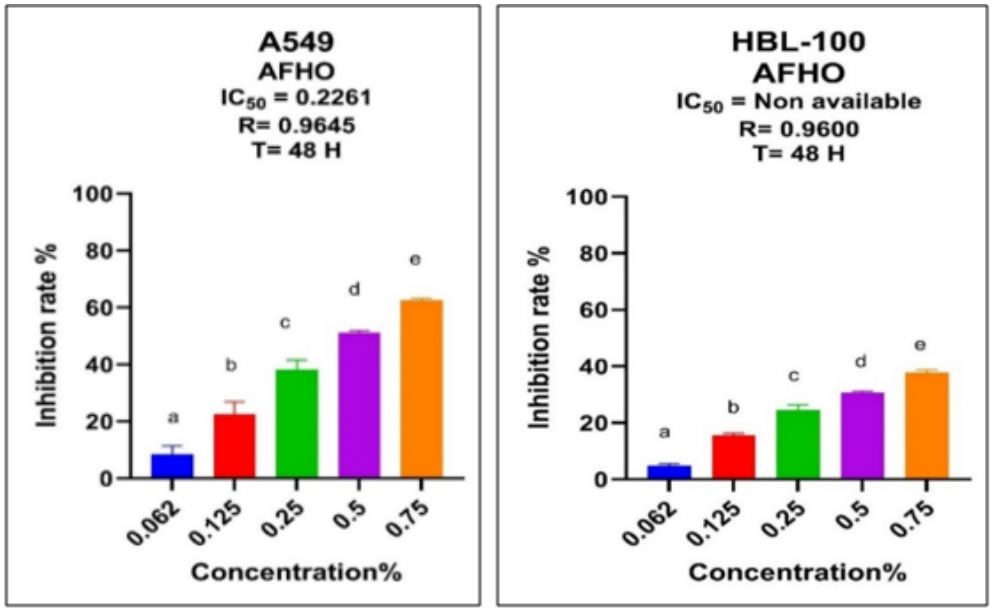

Figure 3. The Results of MTT Assay Shows the Inhibitory Effect of *C.colocynthis* Oil for 48 h Treatment (A) The cytotoxic effect of oil against cancer cell line A549; (B) The cytotoxic effect of oil against normal cell line HBL-100. The data found to be statistically significant different ($p<0.05$ N=4, SD ± Mean).

Table 3. Show the Cytotoxic Effect and IC_{50} Dose of Oil on Cell Lines after 48 h of Treatment

	MTT assay results Concentrations (%)					
Cell lines	0.06%	0.13%	0.25%	0.50%	0.75%	IC50 dose
A549 inhibition rate	8.5	22.6	38.2	51.2	62.5	0.22%
HBL-100 inhibition rate	4.9	15.6	24.5	30.5	37.8	Non available

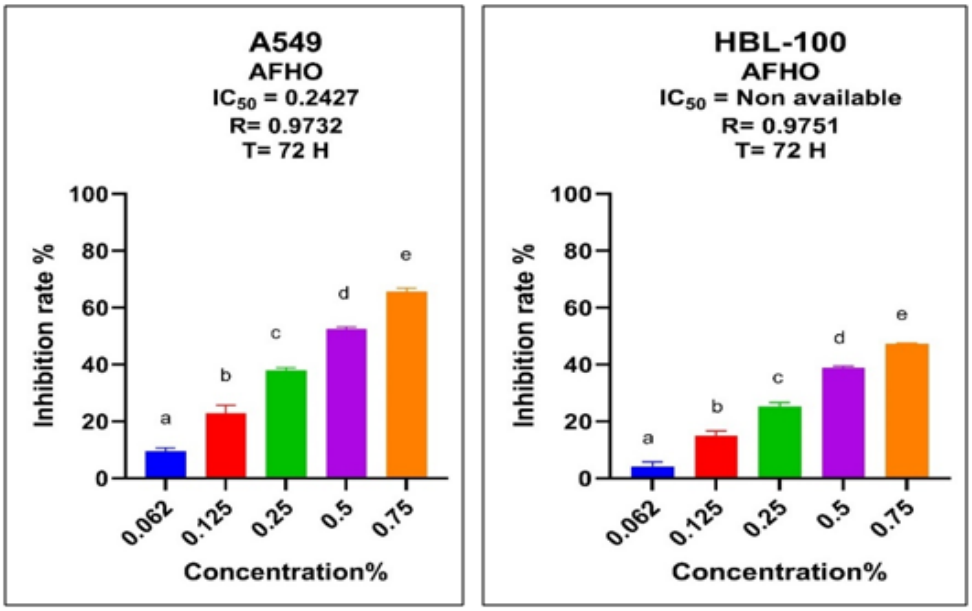

Figure 4. The Results of MTT Assay Shows the Inhibitory Effect of *C.colocynthis* Oil for 72 h Treatment (A) The cytotoxic effect of oil against cancer cell line A549; (B) The cytotoxic effect of oil against normal cell line HBL-100. The data found to be statistically significant different ($p<0.05$ N=4, SD ± Mean).

C. colocynthis on A549 and HBL-100 cells were assessed. The results revealed that untreated cancer and normal cells were affected and cells appeared elongated like fusiform shapes as compact monolayers. (Figure 5a and

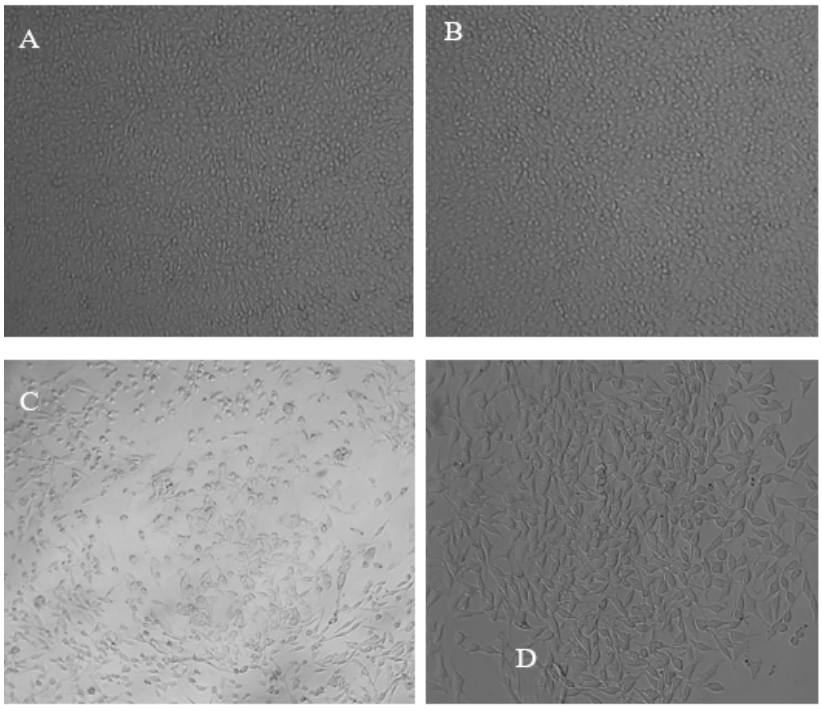

Figure 5. The Pathological Changes of Cells Treated with *C.colocynthis* Oil for 72 h (A) The un treated cancer A549 cell; (B) The untreated normal cells; (C) A549 cells that treated with IC_{50} dose of oil, the cells showed significant implications of cytopathological changes; (D) HBL-100 cells treated with IC_{50} concentration of oil.

Table 4. Show the Cytotoxic Effect and IC_{50} Dose of Oil on Cell Lines after 72 h of Treatment

	MTT assay results Concentrations (%)					
Cell lines	0.06%	0.13%	0.25%	0.50%	0.75%	IC_{50} dose
A549 inhibition rate	9.5	27.8	38.2	52.6	68.4	0.22%
HBL-100 inhibition rate	5.5	15.2	26.6	35	41.8	Non available

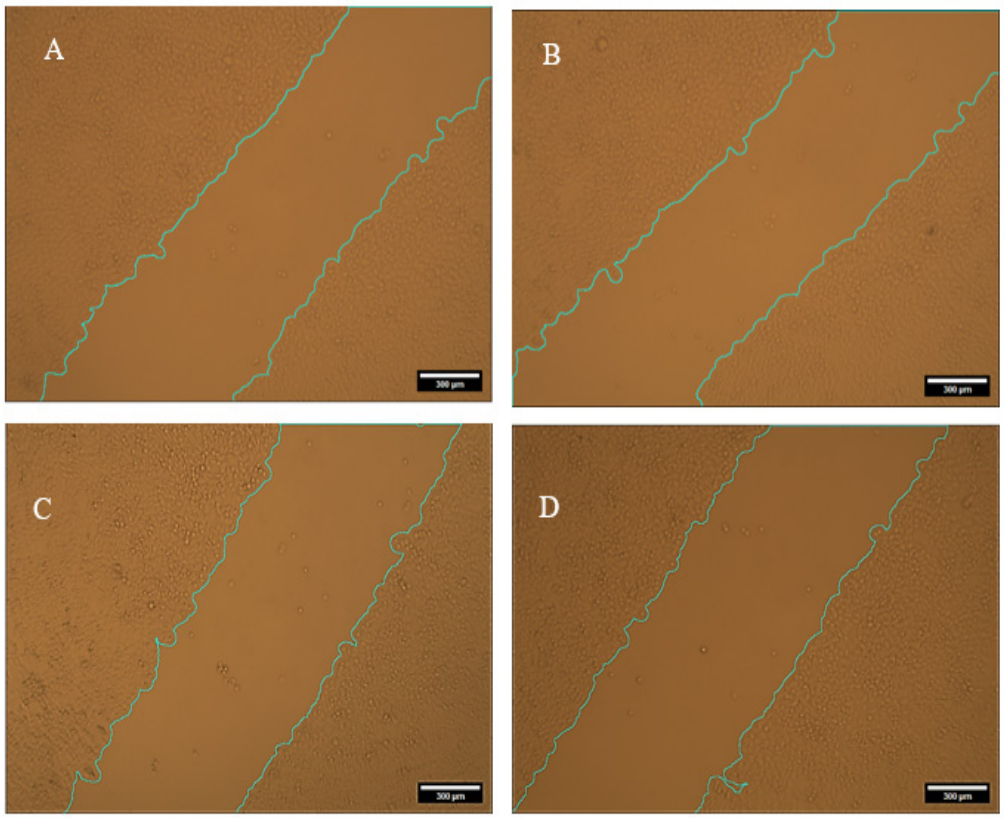

Figure 6. The Analysis of Cancer A549 and Normal HBL-100 Cells Migration by *in vitro* Wound Healing Assay at 0 h (scale bar = 300 µm) magnification 40 x. (A) A549 untreated cells; (B) HBL-100 untreated cells; (C) The cancer A549 cells that treated with *C.colocynthis* oil; (D) The normal HBL-100 cells that treated with *C.colocynthis* oil. The cultures showed same size of area

b). In contrast, the treated cancer cells with the extracted oil exhibited significant pathological changes such as denaturation, necrosis, cell cytoplasmic shrinkage, and bubbles, and also lost contact with surrounding cells (Figure 5c). Normal cells did not show a significant impact (Figure 5d).

Suppression of cancer cell migration

A wound healing assay was conducted to investigate the effect of *C.colocynthis* fruit oil on scratched monolayer cultures of A549 and HBL-100 cells. The results showed that the treated and untreated cells have no effects at 0 h (Figure 6a, b, c, d). However, the normally treated cells showed a decrease in size compared to untreated cells, this clearly indicates that HBL-100 cells were not affected essentially by the extracted oil from *C. colocynthis* fruit. These secondary metabolites play crucial roles in cancer therapy approaches, as illustrated in this assay (Figure 7b and d). After 72 hours, the comparison between the area of treated A549 cancer cells and untreated A549 cancer cells showed no observed effect. This may be due to the extracted oil has a selective effect on the proliferation, migration, and invasion of cancer cells. The results also showed that the A549 cancer cells lost their ability to grow after treatment with the extracted oil.

Discussion

Previously, several studies have found that essential oils have anticancer properties through multiple different mechanisms including interacting with the microenvironment of tumor cells [22,23]. Plant-based phytochemicals have shown the ability to inhibit several cancer signaling pathways and reduce angiogenesis resulting tumor shrinkage [24,25]. Due to the adverse effects of the current cancer treatments, new therapeutic approaches are needed to address current treatment limitations [26]. Chemotherapy and radiation therapy for cancer treatments can produce extreme toxicity to patients. It has been found that angiogenesis can play

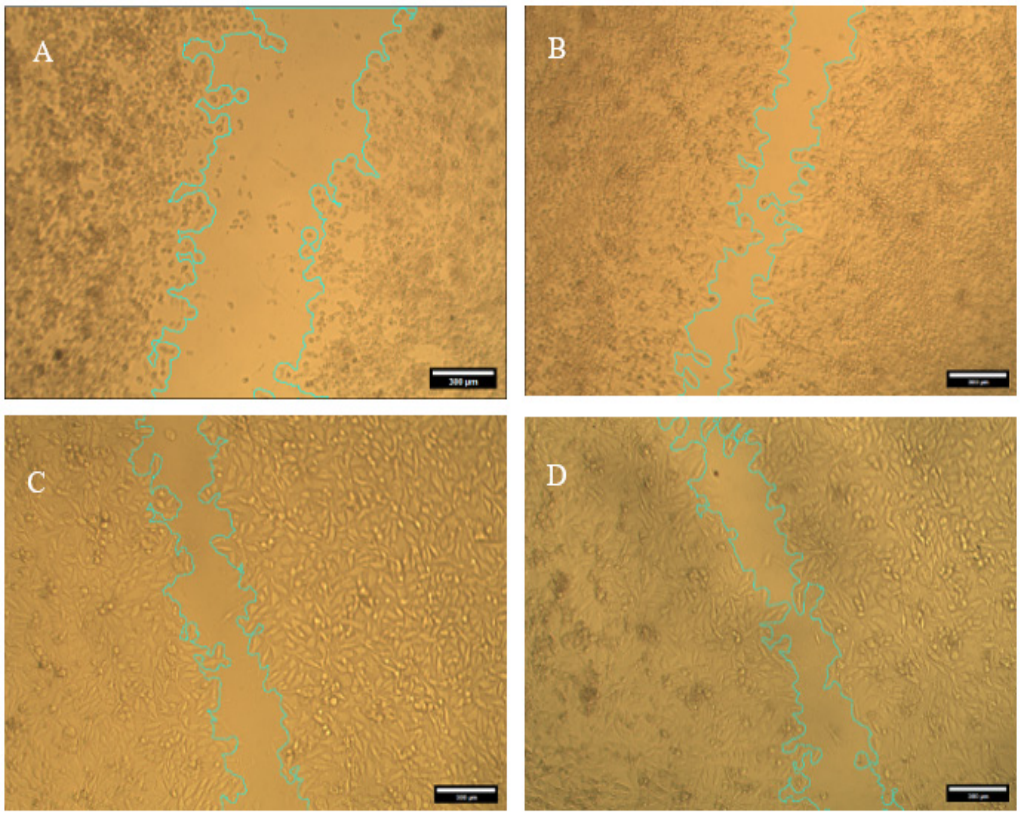

Figure 7. The Analysis of Cancer A549 and Normal HBL-100 Cells Migration by *in vitro* Wound Healing Assay at 72 h (scale bar = 300 µm) Magnification 40 x. (A) A549 untreated cells; (B) HBL-100 untreated cells; (C) The cancer A549 cells that treated with *C.colocynthis* oil; (D) The normal HBL-100 cells that treated with *C.colocynthis* oil.

a role in metastasis development and can contribute in tumor growth leading to death [27,28]. Several previous studies have found that compounds extracted from plants have anti-cancer properties [29]. The anti-tumor activity of *C. colocynthis* can be attributed to various pathways, including apoptotic pathways, antioxidant and anti-inflammatory effects, inhibition of the Wnt/ß-catenin signaling pathway, and antimetastatic effects [30,31]. Fruit pulp preparations containing colocynth can reduce cancer spread, inhibit cell migration, and inhibit the proliferation of breast cancer cells [32,33]. It has been found the extract of *C. colocynthis* contains alkaloids, flavonoids, saponins, terpenoids, and glycosides, which have antimicrobial, antiviral, and anticancer properties [34]. A current study has demonstrated that *C. colocynthis* oil possesses a cytotoxicity effect on lung adenocarcinoma A549 cells, but not affect normal cells. These results suggest that oil extracted from *C. colocynthis* may have significant anti-cancer properties.

In conclusion, due to the side effects of conventional cancer treatments, many studies have been conducted to explore new natural products to improve treatment efficacy and minimize the side effects of current therapies. This study found that the extracted oil from *C. colocynthis* increased cell death in lung adenocarcinoma A549 cells at low concentrations (less than 1%). in vitro, with fewer effects on normal cells. To fully understand the effect of the extracted oil of *C. colocynthis* on different types of cancer, further studies are needed both in vitro and in vivo at different scales.

As a result, we recommend conducting further investigations to better understand the crucial roles and effects of *C.colocynthis* essential oil on other cancer modalities both in vitro and in vivo.

Author Contribution Statement

Ahmad Naeem Al-Khammas carried out the experiments, created the visuals, analyzed and interpreted the data, and produced the report. Ali Abd Alateef AL Ali planned and took photographs of the examinations; provided materials, instruments, or data; interpreted and analyzed the data; penned the initial draft; oversaw the project; and approved the final draft.

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Conflict of Interest

There are no conflicts of interest.

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