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Research paper

Traditional Palestinian medicinal plant *Cercis siliquastrum* (Judas tree) inhibits the DNA cell cycle of breast cancer – Antimicrobial and antioxidant characteristics

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ABSTRACT

Introduction: *Cercis siliquastrum* (Judas tree) is a traditional medicine with drug discovery potential. The aim of this study was to investigate the antimicrobial and antioxidant activity of *Cercis siliquastrum* L. flowers and leaves for their effect on the DNA cell cycle (proliferation) in a breast cancer cell line.

Methods: Assessment of the DNA cell cycle of breast cancer was conducted using propidiumiodide (PI) while apoptosis activity was estimated by staining with Annexin-V using flow cytometry. The antimicrobial activity was assessed through a broth microdilution method on four bacterial pathogens and one fungal strain. Moreover, the Diphenylpicrylhydrazyl (DPPH) method was utilized to evaluate free radical scavenging efficacy of *C. siliquastrum* flowers and leaves in four different solvent fractions.

Results: The acetone and methanol fractions of *C. siliquastrum* leaves and flowers showed highest antioxidant potentials with IC_{50} values of 8.31 ± 1.36, 4.78 ± 1.84, 1.75 ± 2.03 and 3.31 ± 1.66 µg/ml, respectively compared with Trolox that had an antioxidant potential of IC50 value of 1.41 ± 1.05 µg/ml. The leaf hexane fraction showed potent antibacterial potential versus *Staphylococcus aureus,* Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* with MIC values of 0.007, 0.024, 0.048 mg/ml, respectively. Notably, the *C. siliquastrum* flowers hexane fraction strongly inhibited growth of *Staphylococcus aureus, Pseudomonas aeruginosa,* MRSA, and *Candida albicans* with MIC values of 0.009, 0.039, 0.048 and 0.08 mg/ml, respectively. Moreover, C. *siliquastrum* leaves and flowers hexane fractions caused cell cycle arrest followed by cell death via apoptosis/necrosis due predominantly to defects in the mitotic process.

Conclusion: Our data revealed the significant antimicrobial and antioxidant effects of *C. siliquastrum* that contributed significantly to the cytotoxicity of cancer cells requires further detailed studies.

1. Introduction

Herbal extracts containing polyphytochemical mixtures have a leading role in the discovery and development of medicines with most of the recently used anticancer agents derived from natural sources [1]. Moreover, in the last decade, cancer investigations have made remarkable advancements in cancer fingerprinting, biology and diagnosis that can lead the scientists to search for a suitable treatment for each type of cancer. It is scientifically proved that the imbalance between cell proliferation and death affects normal tissue homeostasis negatively, which is characteristic of all neoplastic diseases [2]. The values of natural compounds in the regulation of cellular proliferation and differentiation have been known, but their roles as regulators of the pharmacological and physiological process of cell death have only recently been suggested [3].

Breast cancer is a serious disease that affects not only women but also men and has a metastatic activity especially in its late stages [4]. It is one of the most five common types of cancer in Palestine with the highest morbidity rate, along with lung, colon, blood and brain cancers. These five cancers contribute to 59.1% of deaths that are caused by cancer among Palestinians, according to the Palestinian Health Information Center (PHIC) of Health Ministry [5].

Breast cancer either could be acquired or inherited because of a certain genetic mutation e.g. mutation of the genes of BRCA1 and BRCA2. BRCA1mutations may increase a woman's risk of developing peritoneal and fallopian tube cancers, in addition, may raise the risk of

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breast cancer, and women with mutations of BRCA1 and BRCA2 are more likely to develop breast cancer and later develop ovarian cancer [6].

Moreover, the fast growth and distribution of bacterial and fungal resistance is occurring worldwide, which is endangering the efficacy of antibacterial and antifungal agents' and putting millions of lives under risk. This global crisis is mainly attributed to the misuse and overuse of these medicines also to the slow release of new generations of antibacterial and antifungal agents by the pharmaceutical industry due to reduced economic incentives and the difficulties in registrations requirements [7]. Additionally, the excess of the reactive nitrogen and oxygen species leads to oxidative damage to cells, tissues and organs, which may lead to the oxidation of proteins and lipids and changing in their functions and structure. Many diseases can be postulated to be due to oxidative stress including certain kinds of cancers, inflammatory conditions, atherosclerosis, aging, respiratory diseases, hemochromatosis, glomerulonephritis, arthritis, vasculitis, heart diseases, acquired immunodeficiency syndrome, hypertension, Parkinson's disease, Alzheimer's disease and many others [8].

In fact, that there is a well-known relationship between oxidative stress with carcinogenesis in conjunction with recent investigations which indicate that cancer-associated with the peroxidation of lipid suggests this might help in defending adjacent non-malignant cells from cancer invasion [9].

Cercis siliquastrum L. (family Leguminosae) is a deciduous perennial small tree commonly known as Judas tree. It is covered with deep pink blossoms and has a heart shape with deep green colored leaves [10]. It is rich in chemical compositions; the leaves contain hydroxycinnamic acids (neochlorogenic, chlorogenic, and ferulic acids), flavonoids as myricitoside C, and phenolic compounds. The blossoms of *C. siliquastrum* contain several kinds of volatile oils including heptadecane, nonadecane, pentadecane, linalyl acetate, eicosane, limonene and many others [11].

Cercis siliquastrum is used in different countries and cultures not just for decoration, but also for further traditional medicinal uses, e.g. in Turkey, the blossoms are used as an antiseptic, while in Iran the leaf extract utilized for the treatment of malaria, anemia, and stress [12]. In addition, the flowers are used in Syria as a component of the Unani "Zahraa" herbal tea, this drink is used after meals as a digestive drink and to treat infectious diseases [13]

The use of traditional medicine is widely spreading in Palestine, but only several herbal species have been scientifically investigated for their biological activity from about 2700 flora species distributed in Palestine [14].

In addition, breast cancer is breaking news globally because chemotherapy, mastectomy, and radiation therapy all have many harmful side effects both physiologically (e.g. decrease the immunity) and psychologically and tumor recurrence is a high possibility even after therapy. Moreover, the increase of bacterial and fungal resistance and the decrease of antioxidant molecules in the modern humankind, current diet have all reached critical levels.

Because of the above reasons, the current investigation focused on the assessment of *C. siliquastrum* leaves and flowers using four solvents fractions on breast cancer proliferation. It also aimed to estimate the antibacterial, antifungal and antioxidant effects of these fractions.

2. Material and methods

2.1. Plant materials

The leaves and flowers of *C.*siliquastrum plant were gathered from the Jerusalem region of Palestine in April 2017. The Natural Products Laboratory at An-Najah National University botanically characterized the plant, receiving the (Pharm-PCT-587) voucher specimen code. The leaves and flowers of *C. siliquastrum* plant were cleaned and dried in an air-drying oven (Heratherm ovens 6050TS1, Canada) for five days with a fixed humidity (55 ± 5 RH) and controlled temperature (25 ± 2 °C) prior to use.

2.2. The four solvent fractionation method

The powdered material of *C. siliquastrum* plant was sequentially fractionated by adding four solvents with various polarities: water and methanol (polar protic solvents), hexane (nonpolar) and acetone (polar aprotic). Briefly, plant material (25 g) was suspended in 500 ml of acetone (Loba Chemie, India), hexane (Frutarom LTD, Haifa), methanol (Loba Chemie, India), and Milli-Q water (Millipore-Sigma, USA) separately and each one of these solvents was placed in a shaker apparatus (Nuve, 0012 TK, Turkey) for 96 h at a stable temperature (25 ± 3 °C), with 150 rounds per min. Then, all these fractions were kept in a fridge for 7 days. After that, the methanol, acetone, and hexane fractions were filtered and then evaporated using a rota-vapor (Heidolph-2000VV, Germany) under certain vacuum conditions. The aqueous fraction was lyophilized utilizing a Cryo-Desiccator (Mill-rock technology, BT85, China). Finally, all these crude *C. siliquastrum* fractions were kept in the refrigerator at 4–8 °C until further use [18]. The yield of *C. siliquastrum* leaves aqueous, methanol, hexane and acetone solvents fractions were 2.54%, 2.1%, 1.5%, and 1.44%, respectively. While the yields of *C. siliquastrum* flowers aqueous, methanol, hexane and acetone solvents fractions were 1.54%, 1.1%, 1.7%, and 1.02%, respectively [15].

2.3. DPPH antioxidant activity assay

For the evaluation of four *C. siliquastrum* leaves and flowers fractions and Trolox (Sigma, USA), a solution of a concentration of 1 mg/ml in methanol was initially prepared from these samples and produced the solutions utilized to form 1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80 and 100 µg/ml concentrations. Then, the DPPH reagent (Sigma-Aldrich, Germany) was dissolved in 0.002% w/v methanol and mixed with the prepared previously working concentrations in 1:1:1 ratio. The pure methanol solution was utilized as a blank. All of the solutions were incubated in a dark chamber for 30 min at room temperature. Then, their absorbance values were estimated by utilizing a UV–vis spectrophotometer at a wavelength of 517 nm. The percentage of antioxidant potential of each plant fraction and the Trolox was estimated using the following formula:

$$\text{DPPH inhibition activity (\%)} = (Z - Y)/Z \times 100\%.$$

Where Z is the absorbance of the blank and Y is the absorbance of the sample. The antioxidant half maximal inhibitory concentration (IC_{50}) of each plant fraction and Trolox was determined utilizing BioDataFit edition 1.02 [16].

2.4. Antimicrobial activity

The antibacterial effect was determined using four strains of bacteria which brought from the American Type Culture Collection (ATCC); *Pseudomonas aeruginosa* (ATCC 27,853) *Escherichia coli* (ATCC 25,922) and*Staphylococcus aureus* (ATCC 25,923),as well as against the growth of a diagnostically confirmed clinical isolates of MRSA (Methicillin-Resistant *Staphylococcus aureus*). The antifungal activity of *C. siliquastrum* samples was evaluated against the growth of *Candida albicans*; a diagnostically confirmed clinical isolate. However, the antimicrobial activity of *C. siliquastrum* used in this study was determined using the broth microdilution method [17,18].

We dissolved each of the flowers and leaves of *C. siliquastrum* fractions in 5% DMSO (Dimethyl sulfoxide) (Riedeldehan, Germany) at a concentration of 132 mg/ml. The produced solution was filter-sterilized and then was serially micro-diluted (2 folds) 11 times in sterile nutrient broth (Himedia, India). The dilution processes were performed under aseptic conditions in 96 well plates (Greiner bio-one, North

Table 1
DPPH inhibitory activity and IC_{50} values of the reference compound (Trolox) and *C. siliquastrum* leaves fractions.

Conc.	Trolox, ± SD	Hexane fraction, ± SD	Acetone fraction, ± SD	Methanol fraction, ± SD	Aqueous fraction, ± SD
0	0	0	0	0	0
1	47.13 ± 1.19	4.98 ± 1.8	20.19 ± 1.25	17.7 ± 2.21	7.73 ± 1.25
2	66.08 ± 1.18	13.7 ± 1.87	24.93 ± 1.36	25.18 ± 1.36	7.73 ± 1.25
3	73.06 ± 1.21	40.14 ± 1.65	33.41 ± 1.35	47.63 ± 2.25	7.73 ± 1.25
5	84.03 ± 1.31	41.14 ± 1.31	35.41 ± 1.28	52.61 ± 2.11	7.73 ± 1.64
7	92.01 ± 1.11	42.14 ± 1.84	37.15 ± 1.25	75.06 ± 1.97	17.7 ± 1.69
10	96.01 ± 0.97	42.89 ± 1.36	51.12 ± 1.65	77.55 ± 1.98	25.18 ± 2.31
30	97.01 ± 0.91	42.89 ± 1.22	60.34 ± 1.65	80.04 ± 1.95	50.12 ± 2.31
40	97.25 ± 0.9	46.13 ± 1.98	89.52 ± 1.36	97.5 ± 1.25	55.86 ± 1.58
50	98 ± 0.85	48.87 ± 1.32	91.27 ± 1.1	97.7 ± 1.38	77.55 ± 1.98
80	98.5 ± 0.88	49.87 ± 1.38	97.25 ± 1.36	98.5 ± 1.84	80.04 ± 1.65
IC_{50}, µg/ml	1.41 ± 1.05	39.8 ± 1.57	8.31 ± 1.36	4.78 ± 1.84	23.44 ± 1.65

America). In the micro-wells that were assigned to evaluate the antibacterial activities of the *C. siliquastrum* leaves and flowers extracts, the concentration of the plant extracts ranged from 0.129 to 66 mg/ml. At the same time, the concentrations of this plant in the microwells assigned to evaluate their antifungal activities ranged from 55 to 0.065 mg/ml. In these plates, micro-well number 11 contained plant-free nutrient broth, which was used as a positive control for microbial growth. On the other hand, micro-well number 12 contained plant -free nutrient broth that was left un-inoculated with any of the test microbes. This well was used as a negative control for microbial growth. Microwells numbers 1–11 were inoculated aseptically with the test microbes. At the time of inoculation, the final concentrations of microbial cells were about 5×10^5 and 0.5–2.5 $\times 10^3$ colony-forming unit (CFU)/ml for the tested bacterial pathogens and *Candida albicans*, respectively. Each of the included microbes in this study was examined in duplicate for being inhibited by the obtained plant extracts.

All the inoculated plates were incubated at 35 °C. The incubation period lasted for about 18 h for those plates inoculated with the test bacterial strains and for about 48 h for those plates inoculated with *Candida albicans*. The lowest concentration of *C. siliquastrum* at which no visible microbial growth in that micro-well was observed, and was considered as the minimal inhibitory concentration (MIC) of the examined *C. siliquastrum* plant fractions [18].

2.5. Cytotoxic activity

2.5.1. Cell line

Cytotoxicity assessment was conducted utilizing the MCF-7 and MCF-10A cell lines, which were obtained from the American Type Culture Collection. MCF-7 is a breast cancer cell line with overexpression of estrogen receptor, while MCF 10A is a non-tumorigenic epithelial breast cell line. MCF7 condition was carried out using RPMI-1640 medium (sigma RO883-6X, Germany) supplemented with 10% fetal bovine serum (Sigma 10733056001, Germany), 1% L-glutamine (Sigma G7513, France), 1% streptomycin and 1% penicillin (Sigma 03-031-113, USA), and adjusted to pH 7.2 by Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma 79383, USA). Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C in ESCO cell-culture incubator. In addition, the MCF10A condition established utilizing DMEM medium (Sigma D5546, Germany) supplemented with 10% fetal bovine serum,1% L-glutamine, 1% streptomycin, and 1% penicillin, and adjusted to pH 7.2 by Dulbecco's Phosphate Buffered Saline (DPBS). Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C in ESCO cell-culture incubator [19].

2.5.2. Flow cytometry analysis

Following cultures, harvested MCF-7 and MCF-10A cells were adjusted to 10^6/ml in staining buffer (in saline containing 1% bovine albumin; Biological Industries, Israel). For apoptosis and viability measurements, propidium-iodide (PI) staining of fragmented DNA and phosphatidylserine staining by annexin V-conjugated to FITC (R&D Systems, Minneapolis, MN) were used according to the manufacturer's instruction. Apoptosis was defined as annexin-V (+) but propidium-iodide (-). Viable cells were defined as annexin-V (-) but propidium-iodide (-). In each experimental setting, unstained controls, IgG isotype controls as well as FMO controls were used. Cell cycle analysis by quantitation of DNA content was performed by using the propidium-iodide. MCF-7 and MCF-10A cells were fixed in cold 70% ethanol for at least 30 min at 4 °C. Then the cells were washed 2X in PBS. Spin at 2000 rpm and discard supernatant. To ensure that only DNA is stained, cells were treated with ribonuclease (50 µl of 100 µg/ml RNase), stained with 5 µl of 50 µg Propidium iodide/100 ml and analyzed with the flow cytometer (Becton-Dickinson LSR II, Immunofluorometry systems, Mountain View, CA) [20].

2.6. Statistical analysis

Statistical differences were analyzed either with the 2-tailed unpaired Student's *t*-test (For comparison between two groups) or one-way analysis of variance (one-way ANOVA with Newman-Keuls' post-tests among multiple groups) using Graph Pad Prism 5.0 (GraphPad Software, La Jolla, CA). Data are shown as means ± SEM.

3. Results

3.1. Antioxidant characteristics

The antioxidant DPPH method was utilized for estimation the ability of *C. siliquastrum* flowers and leaves aqueous, acetone, n-hexane, and methanol fractions to scavenge the free radicals in comparison with the reference antioxidant compound Trolox, in various concentrations that ranged from 0 to 80 µg/ml.

Tables 1 and 2 depict the inhibition of DPPH for each *C. siliquastrum* leaves and flowers fractions that were utilized, to make a comparative in-vitro evaluation of antioxidant activities. The current *in-vitro* experiment outcomes showed an excellent antioxidant potential with IC_{50} 1.75 ± 2.03 µg/ml and 3.31 ± 1.6603 µg/ml, was exhibited by *C. siliquastrum* flowers acetone and methanolic fractions, respectively, followed by *C. siliquastrum* leaves methanolic and acetone fractions with IC_{50} 4.78 ± 1.84 µg/ml and 8.31 ± 1.36 µg/ml, respectively. All these findings were compared to a Trolox reference that has IC_{50} 1.41 ± 1.05 µg/ml. The detailed results are shown in Figs. 1 and 2.

3.2. Antimicrobial assessment

By utilizing the broth microdilution method, the antimicrobial activity was assessed as depicted in Table 3. Among the studied fractions of *C. siliquastrum* leaves, the hexane fraction showed the highest antibacterial activity against *S. aureus, P. aeruginosa, E. coli* and MRSA with MIC values of 0.007 mg/ml, 0.048 mg/ml, 0.39 mg/ml and 0.024 mg/

Table 2
DPPH inhibitory activity and IC_{50} values of the reference compound (Trolox) and *C. siliquastrum* flowers four solvents fractions.

Conc.	Trolox, ± SD	Hexane fraction, ± SD	Acetone fraction, ± SD	Methanol fraction, ± SD	Aqueous fraction, ± SD
0	0	0	0	0	0
1	47.13 ± 1.19	7.23 ± 0.51	30.17 ± 0.92	12.71 ± 2.15	2.7 ± 1.36
2	66.08 ± 1.18	9.97 ± 0.66	57.6 ± 2.15	47.63 ± 1.95	2.7 ± 1.36
3	73.06 ± 1.21	13.21 ± 1.55	72.56 ± 1.98	67.58 ± 1.36	2.7 ± 1.36
5	84.03 ± 1.31	16.21 ± 2.41	95.01 ± 2.21	75.06 ± 2.55	7.73 ± 1.65
7	92.01 ± 1.11	36.16 ± 2.27	97.75 ± 2.15	75.06 ± 2.15	20.19 ± 1.65
10	96.01 ± 0.97	37.15 ± 2.28	98.25 ± 2.32	75.06 ± 1.98	22.69 ± 1.98
30	97.01 ± 0.91	38.15 ± 2.25	98.25 ± 2.1	98.5 ± 1.32	45.14 ± 1.65
40	97.25 ± 0.9	47.13 ± 2.28	98.5 ± 1.98	99 ± 1.22	72.56 ± 1.65
50	98 ± 0.85	53.11 ± 1.55	99.25 ± 2.11	99.5 ± 0.98	75.06 ± 1.97
80	98.5 ± 0.88	54.61 ± 1.68	99.7 ± 2.36	99.5 ± 0.98	77.55 ± 1.45
IC_{50}, µg/ml	1.41 ± 1.05	47.86 ± 2.14	1.75 ± 2.03	3.31 ± 1.66	22.9 ± 1.61

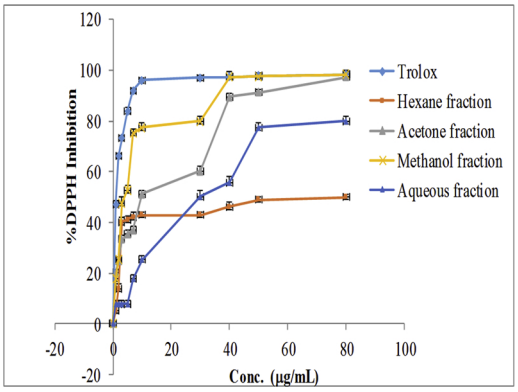

Fig. 1. Inhibition activity of *C. siliquastrum* leaves four solvents fractions and Trolox.

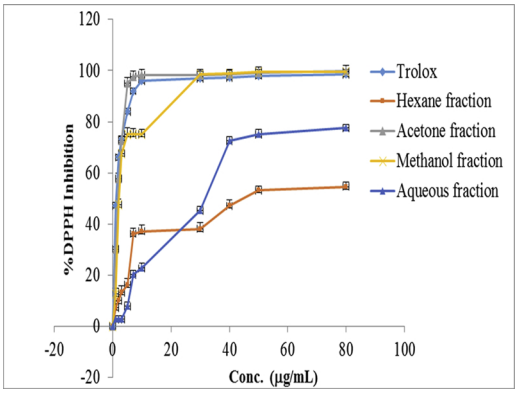

Fig. 2. Inhibition activity of *C. siliquastrum* flowers four solvents fractions and Trolox.

Table 3
Microbial growth inhibition MIC values of C. siliquastrum leaves and flowers various fractions.

	Fractions	*S. aureus,* (mg/ml)	*P. aeruginosa,* (mg/ml)	*E. coli,* (mg/ml)	MRSA, (mg/ml)	*C. albicans,* (mg/ml)
C. siliquastrum leaves	Aqueous	1.25	3.125	25	3.125	–
	Methanol	1.25	3.125	50	0.781	–
	Acetone	6.25	3.125	50	0.195	12.5
	Hexane	0.007	0.048	0.39	0.024	3.125
C. siliquastrum flowers	Aqueous	3.125	12.5	12.5	6.25	–
	Methanol	6.25	3.125	0.165	50	–
	Acetone	1.25	12.5	50	3.125	3.125
	Hexane	0.009	0.039	0.39	0.048	0.08

ml. In addition, the *C. siliquastrum* flowers hexane fraction revealed the highest antibacterial activity against *S. aureus, P. aeruginosa* and MRSA with MIC values of 0.009 mg/ml, 0.039 mg/ml and 0.048 mg/ml, respectively. While the *C. siliquastrum* flowers methanolic fraction showed the highest anti- *E. coli* activity with MIC value of 0.165 mg/ml. Moreover, *C. siliquastrum* leaves and flowers hexane fraction revealed the highest antifungal activity against *C. albicans* pathogen with MIC values of 3.125 mg/ml and 0.08 mg/ml, respectively.

3.3. Cytotoxicity

3.3.1. C. siliquastrum inhibit DNA cell cycle of MCF-7 cells

To investigate whether *C. siliquastrum* could induce cell cycle perturbations in breast cancer cells, flow cytometry analyses of propidiumiodide stained nuclei cells were performed. Cell cycle parameters were investigated for both *C. siliquastrum* leaves and flowers that were fractionated with hexane as they showed high antimicrobial efficiency. The hexane fractions of *C. siliquastrum* leaves and flowers were incubated with MCF-7 for 24 h at concentrations of 0.5, 2, 10 and 15 mg/ml). Hexane treatments alone were used as vehicle control while MCF-10A were used as a control cell with no cancer.

Fig. 3A shows a slight increase in the fraction of cells in the G1 phase following treatments with both *C. siliquastrum* extracts (leaves and flowers). Averages of around 63.7 ± 1.6% were obtained in the G1 phase following treatment of the different concentrations of extract of *C. siliquastrum* leaves as compared to 51 ± 1% in untreated cells ($P < 0.05$); while an average of 70 ± 8.1% were obtained following C. *siliquastrum* flowers as compared to 50 ± 3.6% of untreated cells ($P < 0.05$). There was no significant difference between the different concentrations of the extract. In addition, significant elevations in the proportion of cells in the S phase were obtained following 15 mg/ml concentration of *C. siliquastrum* leaves and *C. siliquastrum* flowers (20.3 ± 3.2% compared to 17.1 ± 4.1 and 24.2 ± 3.5 compared to 20.8 ± 1.2, respectively; Fig. 3B; $P < 0.05$).

Cell cycle parameters were perturbed in MCF-7 cells in the G2/M phase in the 0.5, 2, 10 and 15 mg/ml *C. siliquastrum* leaves to 18.1 ± 2.8%, 20.7 ± 5.5%, 24 ± 5.2% and 13.7 ± 4.0%, respectively, as compared to 31.8 ± 4.3% while with the *C. siliquastrum* flowers to 32.7 ± 4.7, 18.2 ± 0.7, 17.7 ± 4.5 and 5.7 ± 1.1, respectively, as compared to 32.7 ± 3.3% in the untreated cells. P-value was significant between all groups (Fig. 3C).

These results indicate *C. siliquastrum* fractions as a potent inhibitor of cell cycle progression at the G2/M phase and might suggest anticancer properties. *C. siliquastrum* hexane extracts of leaves and flowers had no effects on the cell cycle of MCF-10A cells (data not shown).

3.3.2. C. siliquastrum promotes cell death by apoptosis

We next determined whether the hexane fraction of *C. siliquastrum* of leaves and flowers that perturbs DNA content induce apoptosis

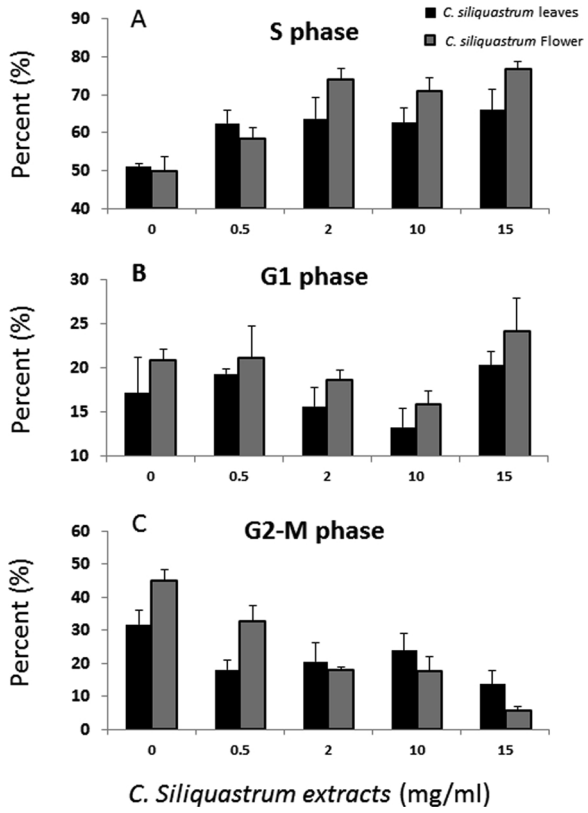

Fig. 3. Cell cycle staining through PI as described in materials and methods. The Figure shows averages of 3 different readings of (A) S phase (B) G1 phase and (C) G2-M phase following treatments with different concentrations of *C. siliquastrum* leaves and flowers hexane fraction.

(programmed cell death). Cells undergoing apoptosis has their phosphatidylserine (PS) phospholipid translocated from the inner face of the plasma membrane to the cell surface; therefore, apoptotic cells can be identified by the presence of PS on the cell surface. As mentioned in materials and methods, detection of PS was estimated by staining with a fluorescent conjugate of annexin-V, a protein that has a high affinity for PS, followed by flow cytometry analysis. Cells were also stained with propidium iodide (PI), which can enter the cell only when the plasma membrane is damaged. Early apoptosis evaluated by positive for PS, but negative for PI and was distinguished from late apoptotic and necrotic cells estimated by positive for both PS and PI.

Fig. 4A shows a representative dot plot of cytometry analysis of MCF-7 breast cancer according to their size (FSC-H) and granularity (SSC-H). Fig. 4B illustrate cells receiving control treatments (untreated or Hexane) possess a baseline apoptotic cell population of 51.7%. Hexane fraction from *C. siliquastrum* leaves significantly decreased apoptosis to 25.33%, while large population of late apoptotic/necrotic cells were observed in Fig. 4C (68.8% as compared to 40.29% in untreated cells). Fig. 4D&E shows the averages of treatment of MCF-7 cells with the different concentrations of *C. siliquastrum* extracts of leaves and flowers, respectively (P- value < 0.05 in all groups). Taken together, the provided data suggest that extracts of *C. siliquastrum* leaves and flowers could have an anti-cancer potential through G2-M cell cycle phase arrest of MCF-7 and shifting the cells to necrosis.

4. Discussion

Throughout history, thousands of plants have been utilized to treat and prevent various diseases and disorders. In addition, a huge number of bioactive compounds have been isolated from plants and more than 25% from available pharmaceutical forms are prepared from plant origin. The plant-derived compounds have a better reputation than conventional synthetic medicines due to their fewer side effects and adverse reactions [21]. For example, in the United States of America, more than 8% of the admissions in hospitals were the result of adverse or side effects of synthetic drugs. Moreover, the acceptance and tolerance of herbal medicine in clinical practice by patients is better than chemically synthetic medications [22].

All over the world, a huge number of research groups have focused on the screening and investigation of antioxidants from medicinal, aromatic and edible plants. In general these studies have found that the plants, which have powerful antioxidant activity, also have anti-inflammatory, antimicrobial, anticancer effects [23].

In addition, synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole and tert-Butylhydroquinone considered to be the most effective antioxidant compounds currently available but now suspected to be unsafe for human health [24].

Therefore, the search for new herbal products with potential antioxidant capacities is strongly desirable in pharmaceutical, cosmetics and in food industries

The DPPH inhibitory capacity by *C. siliquastrum* leaves and flowers fractions revealed antioxidant activity as shown in Tables 1 and 2. The scavenging property of all of the studied compounds various concentrations samples and Trolox standard compound showed a concentration dependence property in all studied samples. In fact, all the studied samples were compared with Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) which is a water-soluble analog of vitamin E and has powerful antioxidant capacity like vitamin E. Trolox utilized in biochemical or biological applications to decrease oxidative damage or stress [25,26].

All the leaf fractions of *C. siliquastrum* have antioxidant characteristics. The methanol and acetone fractions have the highest antioxidant potentials with IC_{50} values of 4.78 ± 1.84 and 8.31 ± 1.36 µg/ml, respectively in comparison with the standard antioxidant molecule Trolox. Trolox has potential free radical scavenging property with IC_{50} value of 1.41 ± 1.05 µg/ml. Interestingly, the results showed that the acetone fraction of *C. siliquastrum* flowers have almost the same antioxidant power as Trolox.

In a study conducted by Jahromi et al., found that *Cercis griffithii* seeds ethanolic extract which is another species of Cercis genus promoted DPPH inhibitory effect with an IC_{50} value of 131.00 ± 0.57 µg/ml [25].

Throughout the literature review conducted by authors, no previous studies had been carried out on the antioxidant activity of *C. siliquastrum* flowers and leaves. Compared with Trolox (vitamin E analog), the antioxidant capacities of *C. siliquastrum* plant leaves and flowers fractions which were evaluated using DPPH assay and the results concluded that the acetone and methanol fraction have potential free radical scavenging properties. Recently, the total drug resistant and multiple drug-resistant strains are becoming more common in clinical practice and scientists are searching for alternative medicines to treat these infectious diseases. Herbal therapeutic agents are accepted candidates to treat like these infections because they act either by killing or by restricting the microbial growth through the same mechanisms as antibiotics [26].

The current study results revealed that the hexane fraction of *C. siliquastrum* leaves had the best antimicrobial effect against *S. aureus, P. aeruginosa, E. coli*, MRSA and *C. albicans* among other plant fractions. However, the hexane fraction showed the best antibacterial activity against *S. aureus* with MIC value of 0.007 mg/ml also showed potential activity against MRSA growth with MIC value of 0.024 mg/ml and against the growth of *P. aeruginosa* with MIC value of 0.048 mg/ml. Additionally there was weak antifungal activity against *C. albicans* with MIC value of 3.125 mg/ml. On the other hand, *C. siliquastrum* flowers hexane fraction showed the best antimicrobial activity among the other used fractions. Notably the hexane fraction strongly inhibited the growth of *S. aureus, P. aeruginosa,* MRSA, and *C. albicans* with MIC

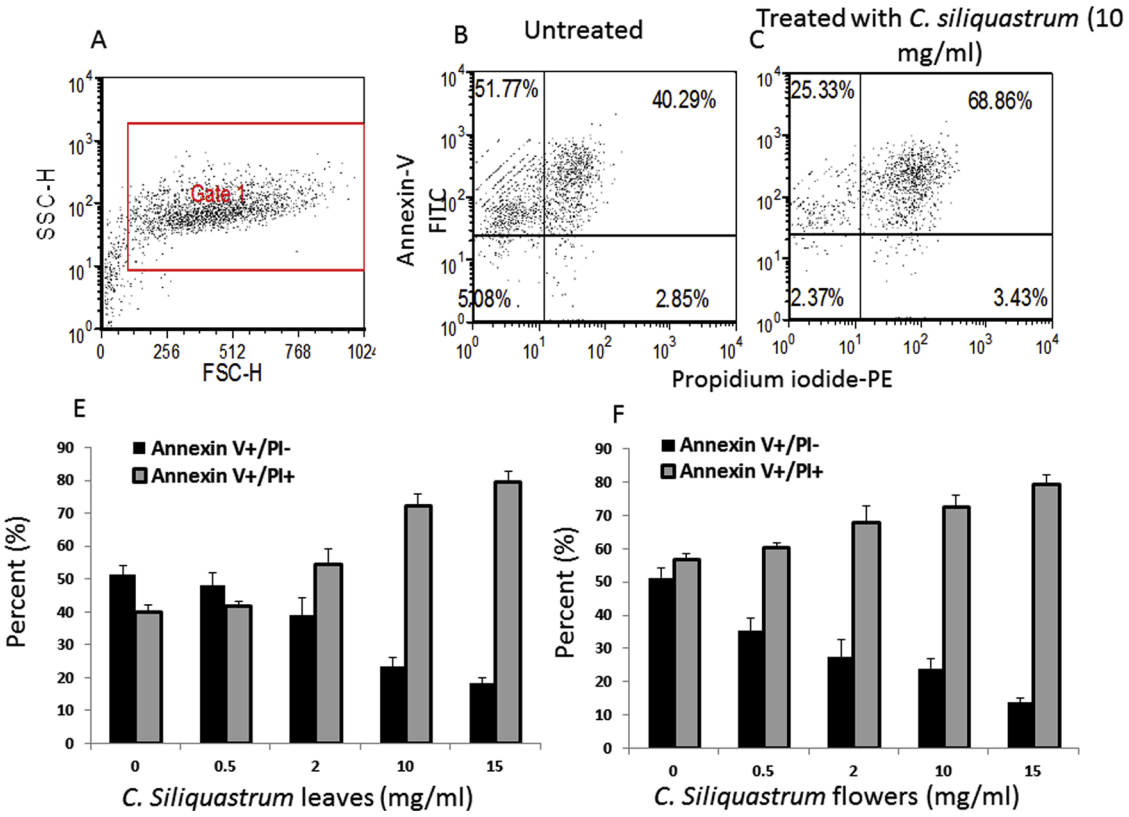

Fig. 4. MCF-7 breast cancer was handled as described in Materials & Methods. (A) Dot plot of cells by size (FSC-H) and granularity (SSC-H). Gate 1 was set on viable cells. Cells were stained for apoptosis markers (Annexin-V-FITC) and necrotic marker (PI-PE). (B) shows a representative dot plot from untreated cells while (C) shows cell following treatment with 10 mg/ml of hexane fraction of a representative *C. siliquastrum* leaves. Upper right quadrant shows percentages of late apoptosis/necrosis cells. Apoptosis and necrosis averages of different concentrations of *C. siliquastrum* leaves (C) and *C. siliquastrum* flowers (D).

values of 0.009, 0.039, 0.048 and 0.08 mg/ml, respectively.

The study conducted by Kaiser et al., found a strong inhibition of the IspC protein from *E. coli* in leaf extract of *C. siliquastrum* [12].

It has also been found that the leaf extract of *C. siliquastrum*, exhibit strong inhibitory activity against 1-Deoxy-D-xylulose 5-phosphate reducto-isomerase (DXR). DXR is the first committed enzyme in the 2-methyl-D-erythritol 4-phosphate terpenoid biosynthetic pathway and is also a validated antimicrobial target [27].

To the best of our knowledge, except for the above-mentioned study, no previous studies have been conducted on the antimicrobial effects of *C. siliquastrum* leaves and flowers four solvents fractions.

Briefly, the hexane fraction of *C. siliquastrum* leaves and flowers showed the powerful inhibition activity the screened bacterial and fungal strains, which promoted the authors to screen the anticancer activity of the hexane fractions of the *C. siliquastrum* leaves and flowers.

Recently, herbal derived molecules play an important role in currently used medicines that utilized for the treatment of cancer. Hence many of the clinically utilized anticancer medications are isolated from plants or semi-synthesized from plants products including vinblastine, roscovitine, epipodophyllotoxin, vincristine, etoposide, flavopiridol, paclitaxel and many others [28]. Several studies have been conducted regarding the effects of herbal extracts on cancer cell lines, but the development and design of anticancer drug are too expensive and take a lot of time. As well as this work is needed on different sample collections, in- vitro and in-vivo evaluations of crude extracts, purification, identification, semi-synthesis of active substances, preclinical and clinical studies. Alcoholic extracts of herbals (herbal medicines such as Star anise fruits, Licorice roots, Frangula bark, Linseeds) have also shown strong antiproliferative activity when tested in vitro on several cancer cell lines [29].

Both hexane fractions of C. *siliquastrum* leaves and flowers have arrested DNA cell cycle of the breast cancer in the G2-phase (mitosis phase) and shifted the cells to G1 (the naïve state). There were no significant effects observed in the S phase (replication phase). *C. siliquastrum* leaves and flowers hexane fractions also decreased apoptosis of the cells while a large population was located in the late/necrotic stages. Additionally in vivo studies are required to prove these effects and to further evaluate all tested fractions on DNA cell cycle and explore the possibilities to use these fractions in clinical trials on human subjects. Moreover, the isolation of active compounds of *C. siliquastrum* plant was not performed in this study, as separation with column chromatography requires large amounts of the plant, which were not available at the time. Isolation, chemical structure elucidation, and determination of structure-activity relationship (SAR) of the most therapeutic active compounds from plant fractions are limitations in this study. However, these experiments will be our future goal.

5. Conclusion

C. *siliquastrum* leaves and flowers were shown to induce cell cycle arrest in the G2/M phase and initiate programmed cell death by apoptosis. The antimicrobial and antioxidant effects of *C. siliquastrum* leaves and flowers may contribute significantly to the biological activities and potential medicinal properties such as treatment of various microbial infections. These data may suggest anti-cancer effects and emphasize the need to generate more data for confirming these potentially therapeutic effects.

Authors' contribution

All research done by the authors.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Ethical approval

N/A.

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