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Original Article

Induction of apoptosis by *Ruta chalepensis* L. essential oil in human breast cancer cells (MCF-7)

[Inducción de apoptosis por el aceite esencial de *Ruta chalepensis* L. en células de cáncer de mama humano (MCF-7)]

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Abstract

Context: Recent scientific studies have reported that essential oils induce apoptosis in various cancer cell types by interfering with intracellular signaling pathways.

Aims: To evaluate the cytotoxicity, the apoptotic activity of essential oil (EO) of *Ruta chalepensis* L. against MCF-7 cell line.

Methods: Cytotoxicity was determined using methyl thiazol tetrazolium assay. The apoptotic activity of EO was analyzed using annexin V-fluorescein isothiocyanate/propidium iodide binding flow cytometry. The cell morphology was inspected under an inverted microscope. DAPI staining assay was used for the morphological observation. Activation of caspases-3/7, -8, and-9 was assessed using a caspase assay kit.

Results: *Ruta chalepensis* essential oil significantly inhibited the proliferation of MCF-7 cells at 72 h. Moreover, the results showed that cell death is associated with the apoptotic process, and the number of apoptotic cells was significantly increased in the groups treated with EO than in control cells. The main morphological hallmarks of apoptosis in the nucleus were membrane blebbing, chromatin condensation, and nuclear fragmentation. Also, R. *chalepensis* EO-induced apoptosis in the MCF-7 cell line was via the extrinsic caspase-8 dependent pathway in a dose and time-dependent manner.

Conclusions: *Ruta chalepensis* essential oil demonstrated significant apoptotic activity against experimental breast carcinoma. Therefore, it could be introduced as a suitable candidate for breast cancer therapy after further investigation.

Keywords: apoptosis; caspases; DAPI staining assay; flow cytometry; *Ruta chalepensis*.

Resumen

Contexto: Estudios científicos recientes han informado que los aceites esenciales inducen la apoptosis en varios tipos de células cancerosas al interferir con las vías de señalización intracelular.

Objetivos: Evaluar la citotoxicidad, la actividad apoptótica del aceite esencial (EO) de *Ruta chalepensis* L. contra la línea celular MCF-7.

Métodos: La citotoxicidad se determinó mediante el ensayo de metil tiazol tetrazolio. La actividad apoptótica de EO se analizó mediante citometría de flujo de unión de anexina V-isotiocianato de fluoresceína/yoduro de propidio. La morfología celular se inspeccionó con un microscopio invertido. Se utilizó el ensayo de tinción DAPI para la observación morfológica. La activación de las caspasas-3/7, -8 y-9 se evaluó usando un kit de ensayo de caspasas.

Resultados: El EO de *Ruta chalepensis* inhibió significativamente la proliferación de células MCF-7 a las 72 h. Además, los resultados mostraron que la muerte celular está asociada con el proceso apoptótico, y el número de células apoptóticas aumentó significativamente en los grupos tratados con EO que en las células de control. Las principales características morfológicas de la apoptosis en el núcleo fueron la formación de ampollas en la membrana, la condensación de cromatina y la fragmentación nuclear. Además, la apoptosis inducida por EO de *R. chalepensis* en la línea celular MCF-7 fue a través de la vía extrínseca dependiente de caspasa-8 de una manera dependiente de la dosis y el tiempo.

Conclusiones: El aceite esencial de *Ruta chalepensis* demostró una actividad apoptótica significativa contra el carcinoma de mama experimental. Por lo tanto, podría presentarse como un candidato adecuado para la terapia del cáncer de mama después de una investigación adicional.

Palabras Clave: apoptosis; caspasas; citometría de flujo; ensayo de tinción DAPI; *Ruta chalepensis*.

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INTRODUCTION

Natural products and their derivatives have provided humans with a broad set of potent drugs to relieve or eradicate diseases (Bernardini et al., 2018). Regardless of the development of synthetic drugs, some plant-derived drugs still maintain their importance. In recent years, the use of drugs and dietary supplements derived from plants worldwide has accelerated (Yuan et al., 2016). In modern medicine, many ailments or infections for which suitable drugs are yet to be found, necessitating the development of safer medications (humans and the environment) to treat many disorders.

Many studies have recently shown that essential oils induce apoptosis in various cancer cell models by affecting intracellular signaling pathways. Therefore, they represent a promising candidate for the treatment and prevention (Blowman et al., 2018).

Apoptosis (programmed cell death) is necessary for normal tissue development and cellular homeostasis. It is necessary to eliminate unwanted, damaged, and potentially harmful cells. Furthermore, apoptosis is characterized by morphological and biochemical changes such as cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation, apoptotic bodies, rapid phagocytosis by neighboring cells, and the activation of a family of intracellular proteases known as caspases (Gudipaty et al., 2018). Nevertheless, deregulation of normal apoptosis pathways is associated with a variety of diseases, such as cancer.

There are two main types of apoptosis pathways: extrinsic (death receptor-mediated) and intrinsic (mitochondria-mediated). Both pathways converge on the same terminal or execution lane to activate caspases, resulting in morphological and biochemical cellular changes (Yu et al., 2020).

Caspases (aspartate-specific cysteine proteases) play a crucial role in the execution of mammalian apoptotic programs. In most cells, caspases are expressed in an inactive form (procaspases), and once activated, they can switch on other procaspases, allowing initiation of a protease cascade (Kesavardhana et al., 2020). This cascade amplifies the apoptotic signaling pathway leading to rapid cell death. Caspases have been categorized into initiators (caspase-2, -8, -9, -10), effectors or executioners (caspase-3, -6, -7) and inflammatory caspases (caspase-1, -4, -5). Initiator caspases activate executioner caspases that coordinate their activities to destroy key structural proteins and stimulate other enzymes (Seaman et al., 2016).

Both extrinsic and intrinsic stimuli can stimulate apoptosis. The extrinsic pathway through death receptors (such as tumor necrosis factor–α, or FAS-receptors) activates caspase-8. In comparison, intrinsic stimuli (such as expression of B-cell lymphoma 2 (BCL2) family BH3-only proteins BIM or puma) cause mitochondrial depolarization and activation of caspase-9 (D'Arcy, 2019; Carneiro and El-Deiry, 2020).

Apoptosis evasion is a hallmark of all cancer cells regardless of the type or the cause (Arbiser et al., 2017). Therefore, targeting apoptosis is a powerful method for all types of cancers. Therapeutic strategies targeting molecules involved in apoptotic resistance represent an efficient approach to restore cancer cells' sensitivity to apoptosis and overcome the classical treatments' ineffectiveness (Pfeffer and Singh, 2018).

Recently, many wild medicinal plants in Jordan have been screened for their cytotoxic activities using various cancer cell lines. *Ruta chalepensis* L. (*Rutaceae*) is an aromatic medicinal plant of the flora of Jordan and has important medicinal properties. It is used in herbal remedy medicine for the treatment of a variety of disorders (Althaher et al., 2020). In a previous investigation, the essential oil of *R. chalepensis* showed potent antioxidant capacity in addition to cytotoxic activity against breast cancer cell lines (MCF-7) without any toxic effects on the normal fibroblast cell line (Althaher et al., 2020). In Tunisia, the essential oil and methanolic extracts of the aerial parts of *R. chalepensis* demonstrated remarkable antioxidant and antimicrobial activity (Ghazghazi et al., 2015). On the other hand, both methanolic and ethanolic extracts of *R. chalepensis* demonstrated antiproliferative activity against various cancer cell lines (Khlifi et al., 2013; Terkmane et al., 2018).

However, little is known about its potential cytotoxic activity and mode of cell death. As a result, the current study aims to evaluate the antiproliferative and apoptotic activities of *R. chalepensis* essential oil against mammary gland carcinoma cells (MCF-7).

MATERIAL AND METHODS

Essential oil of *Ruta chalepensis*

Our research team previously published the chemical composition of the essential oil of *Ruta chalepensis* (Althaher et al., 2020). The oil was shown to be rich in non-terpenoid aromatic compounds (80.32%). The major identified compounds were 2-cyclohexen-1-one, 3-[(2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-1-yl) methyl] (45.97%), 2-nonanone (19.45%), methyl hexadecanoate (4.04%) and 4,5-dimethoxy-6-prop-2-

enyl-1,3-benzodioxole (1.87%). Moreover, the non-terpenoid non-aromatic compounds (10.31%) were higher than monoterpenes (2.24%). None of the sesquiterpenes or diterpenes were detected in the oil constituents.

The aerial parts from *R. chalepensis* were collected from the campus of the Faculty of Sciences, University of Jordan (UJ), Amman (32°00'48.5"N 35°52'26.4"E) during summer 2020. Prof. Sawsan Oran taxonomically identified the plant materials, Department of the Biological Sciences, University of Jordan taxonomically identified the plant materials. Specimen ID: UJ (GH-RC-12, for greenhouse) was deposited at the herbarium of the Department of the Biological Sciences, University of Jordan, Amman. Then the aerial parts of *R. chalepensis* were air-dried and ground into a fine powder. Hydrodistillation was performed to obtain the essential oil using a Clevenger-type apparatus (JSGW, India). Then, the chemical composition of essential was characterized using Varian Chrompack CP 3800 GC/MS/MS-200 (Satum, Netherlands).

Cell culture

Human mammary gland carcinoma MCF7 (ATCC HTB-22) cells were cultured in Dulbecco's modified eagle medium (DMEM, Caisson Laboratories Inc., USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA), 1% L-glutamine (2 mM) (Euro-clone S.p.A; Pero, Milan, Italy), and 50 IU/mL penicillin and 50 g/mL streptomycin (Euro-clone S.p.A; Pero, Milan, Italy). They were maintained at 37°C in a 5% CO_2 atmosphere with a humidity of 95%. After approaching 80% confluency, they were collected by trypsin-EDTA solution (Euro-clone S.p.A; Pero, Milan, Italy) and washed in phosphate buffer saline (PBS, Euro-clone S.p.A; Pero, Milan, Italy). The cells were then re-suspended in a growth medium to achieve a single-cell suspension. Moreover, the number of viable cells was counted via trypan blue dye exclusion assay (Bustanji et al., 2012).

Cytotoxicity test-MTT assay

The present study evaluated the cytotoxic potential of *R. chalepensis* essential oil by MTT assay against breast cancer (MCF-7) and non-cancerous (MRC-5) cell lines. According to the procedure reported by Bustanji et al. (2012), the cell viability was determined. For 24 h, each cell line was seeded at a density of 1×10^4 cells per well in 96-well plates. Furthermore, an essential oil stock solution (20 000 µg/mL) was created by dissolving 20 mg of essential oil in 1 mL of DMSO (Merck-Schuchardt, German). The stock solution was then serially diluted with a growth medium to achieve final concentrations (3.125-200 µg/mL) (the sample's final DMSO concentration of 0.01%). Doxorubicin (Ebewe Pharma GMBH Nfg. KG, Austria) (0.085-4.25 µg/mL) was used as a positive control (standard anticancer drug). The untreated cells were seeded with fresh growth media as an assay control, while the growth media-free cells served as a blank. After seeding, each cell line was treated with 100 µL of the essential oil (3.125-200 µg/mL) for 24, 48, and 72 h. The plate was then incubated for 3 h with 20 µL of MTT (Sigma-Aldrich, St. Louis, MO, USA) (5 mg/mL) added to each well. After carefully removing the supernatant from each well, 200 µL of DMSO was added to dissolve the resulting formazan crystals. A microplate reader (Bio-Tek Instrument, USA) was used to read absorbance at 570 nm using a reference wavelength of 630 nm. The difference between readings was used to analyze the results. The absorbance of control cells was assumed to be 100% viability, and the values of treated cells were calculated as a percentage of the control. The following equation [1] was used to calculate the percentage of viability of the cells.

$$\text{Cell viability} = \frac{OD_{\text{sample}}}{OD_{\text{control}}} \times 100\ \% \qquad [1]$$

Where OD: optical density.

The relation between cell viability% and essential oil concentration was plotted to get the cell lines' viability curve with the essential oil. The inhibitory concentration (IC_{50}) the concentration of the sample (essential oil, EO) showed 50% of cell growth inhibition.

The selectivity index (SI) was calculated by dividing the IC_{50} value into normal fibroblast cells (MRC-5) by the IC_{50} value on cancerous breast cells (MCF-7) [2].

$$\text{Selectivity index (SI)} = \frac{IC_{50} \text{ of EO on normal cells (MRC-5)}}{IC_{50} \text{ of EO on cancerous cells (MCF-7)}} \qquad [2]$$

The SI value indicates the sample's selectivity to the cell lines tested. Samples with a SI>3 was considered to have a high selectivity for cancerous cells (Chothiphirat et al., 2019).

Determination of apoptosis by flow cytometry

Apoptosis detection was performed using an annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kit (ab14085; Abcam, USA). Each cell line was seeded at a density of 2×10^5 cells/ 2 mL/well onto a six-well plate. After 24 h, the cells were treated with 2 mL of *R. chalepensis* essential oil at 40, 80, and 160 µg/mL concentrations, respectively. The cells were grown in 5% dimethyl sulfoxide (DMSO) as a control. Doxorubicin (0.2 µg/mL) was used as a standard. After 72 h, the cells were stained with an annexin V-FITC conjugated and PI, and the percentage of apoptotic, necrotic, and via-

ble cells was determined using the apoptosis kit's protocol. Moreover, flow cytometry (BD FACSCanto™ II Flow Cytometer, San Jose, USA) was used to analyze the data (BD FACS DivaTM software, San Jose, USA). The experiments were carried out twice.

Cell morphology analysis

To investigate the morphological changes in the MCF-7 cells after treatment with *R. chalepensis* essential oil. The cells (2×10^5) were seeded in a 6-well plate for 24 h. After incubation and attachment, the cells were treated with *R. chalepensis* essential oil using the ½ IC_{50}, IC_{50}, and $2IC_{50}$ concentrations (40, 80, 160 µg/mL) for 72 h. Doxorubicin (0.2 µg/mL) was used as a positive control. Cells were observed under the ZEISS Axio Vert.A1 Inverted Microscope (Jena, Germany) connected to ZEISS ZEN LITE 2.6 software (Germany) (Magnification 100×).

Morphological observation of nuclear change by DAPI staining assay

The apoptotic cells can be observed according to the strength of fluorescence and the conformation of the nucleus. DAPI (4, 6-diamidino-2-phenylindole) is a fluorescent blue dye that binds strongly to the minor groove of a double-strand of DNA in a high affinity to adenine–thymine rich regions. It can pass through intact, living cell membranes, but apoptosis increases cell membrane permeability and improve uptake of DAPI so that the apoptotic cells will produce stronger blue fluorescence. Also, the nuclear morphology of normal cells is round, clear-edged, uniformly stained. The nucleus's margin is irregular in the apoptotic cells, and the condensed chromosome is easily stained with heavier coloring (Atale et al., 2014).

DAPI staining assay was carried according to the procedure described by Ferro et al. (2017). A 1×10^5 of MCF-7 cells were seeded/1000 µL of growth medium in each well of the 12-well plate, then kept in 5% CO_2, 37°C humidified incubator for 24 h. The cells were then treated with 1000 µL of 40, 80, 160 µg/mL concentration of essential oil of *R. chalepensis*. Doxorubicin (0.2 µg/mL) was used as a positive control. Untreated cells were used as a control. After 72 h, the cells were washed twice with 1× phosphate buffer saline (PBS) for 5 min each and then fixed with 4% formaldehyde/PBS (freshly prepared). Cells were then rinsed three times in PBS 1× for 5 min to eliminate any residual and reduce any background before mounting and imaging. The nuclei were then stained with 5 µM DAPI staining solution (ab228549; Abcam, USA) (it is prepared by appropriately diluting the DAPI stock solution in PBS) coverslips were then mounted on slides. All analyses were conducted in duplicate at room temperature. Mounted slides were kept in the dark at ≤ -20°C before image acquisition by Zeiss Axio Imager; the cells were observed (Zeiss Imager.Z2 upright fluorescence microscope, Oberkochen, Germany) connected to (ZEISS ZEN 2.6 LITE software, Oberkochen, Germany) to estimate fluorescents intensity.

Effect of *R. chalepensis* essential oil on the activity of caspase-3/7, -8 and -9

Briefly, each cell line was cultured at a density of 10 000 cells/well in 96-well plates in triplicate for 24 h and treated with *R. chalepensis* essential oil (40, 80, and160 µg/mL). The blank wells were a cell culture medium without cells. Negative control (untreated cells) was treated with 0.01% DMSO. Then incubated all plates for 72 h.

Caspase-3/7, -8, and-9 activities were investigated using a luminescent caspase-Glo 3/7(G8090), -8 (G8200), and -9 (G8210) assay kits (Promega, USA) following the manufacturer's instructions. Briefly, caspase reagents were added to blank, control, and treated cells. The plate was mixed well on an orbital shaker and incubated for 2.5 h in the dark at room temperature. Then, GloMax Multi-detection System (Promega, USA) was used to measure the luminescent signal.

The blank wells were used to measure background luminescence associated with the culture medium and Caspase-Glo® 3/7, 8, or 9 reagents. The luminescence value for the blank reaction was subtracted from the experimental values.

Statistical analysis

The data were presented as mean± SEM. One-way analysis of variance (ANOVA) was used for statistical analysis using GraphPad Prism 9.0.2 (GraphPad Software, San Diego, CA, USA). The results were considered to be statistically significant at $p<0.05$.

RESULTS

Effects of *R. chalepensis* essential oil on MCF-7 cell proliferation

The cytotoxic activity of *R. chalepensis* essential oil was evaluated by MTT assay against MCF-7 cancer cells and MRC-5 non-cancerous cells for 24, 48, and 72 h. Overall, dose-dependent inhibition of MCF-7 cancer cell viability by *R. chalepensis* essential oil was observed compared to untreated cells (control). Also, at maximum concentration tested (200 µg/mL) was cytotoxic at all incubation times. Moreover, the essential oil of *R. chalepensis* significantly inhibited the proliferation of MCF-7 cells at 72 h (6.25-200 µg/mL)

(Fig. 1C). After 48 h (Fig. 1B), the essential oil (25-200 µg/mL) significantly inhibited the proliferation of MCF-7 cells compared to the incubation period (24 h) **to** the effective concentrations (100-200 µg/mL) (Fig. 1A). On the other hand, the results showed that the tested essential oil had no cytotoxic activity against normal fibroblast cell line (MRC-5) (Fig 1A-C and IC_{50} in Table 1). Additionally, doxorubicin showed significant inhibition in the proliferation of both cancerous and non-cancerous cells (Fig. 1D-E). Moreover, the selectivity index (SI) indicates the selective effects of the *R. chalepensis* essential oil against cancer cells *versus* non-cancerous cells (Chothiphirat et al., 2019). Also, *R. chalepensis* essential oil showed high selectivity (SI > 3) against MCF-7 cancerous cells at different incubation times in comparison to the doxorubicin (Table 1).

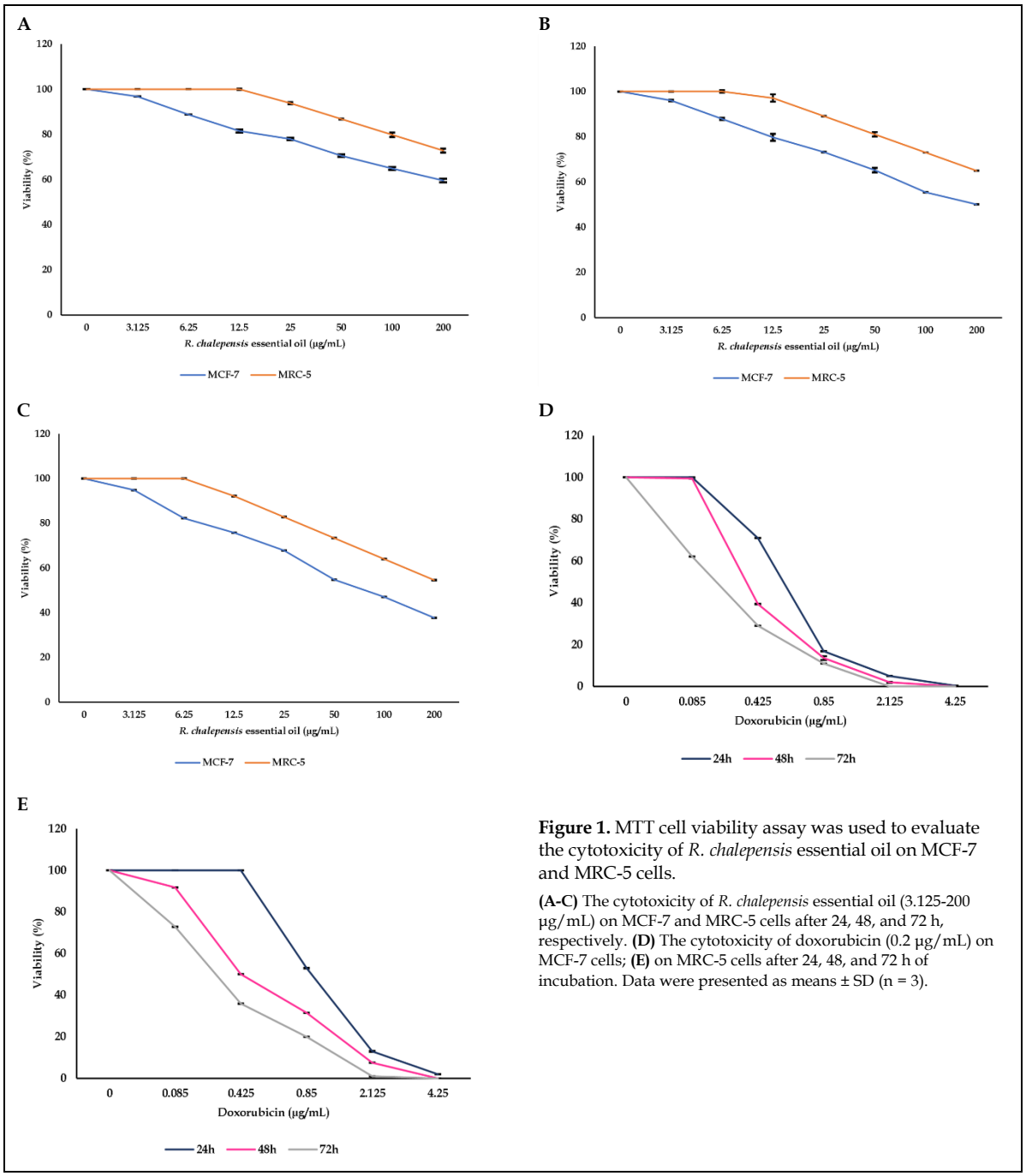

Figure 1. MTT cell viability assay was used to evaluate the cytotoxicity of *R. chalepensis* essential oil on MCF-7 and MRC-5 cells.

(A-C) The cytotoxicity of *R. chalepensis* essential oil (3.125-200 µg/mL) on MCF-7 and MRC-5 cells after 24, 48, and 72 h, respectively. **(D)** The cytotoxicity of doxorubicin (0.2 µg/mL) on MCF-7 cells; **(E)** on MRC-5 cells after 24, 48, and 72 h of incubation. Data were presented as means ± SD (n = 3).

Table 1. Cytotoxicity and selectivity index (SI) of essential oil of *R. chalepensis* and doxorubicin on MCF-7 and MRC-5 cell lines at 24, 48, and 72 h of treatment.

Incubation time (h)	*R. chalepensis* essential oil IC_{50} (µg/mL)			Doxorubicin IC_{50} (µg/mL)		
	MCF-7	MRC-5	SI	MCF-7	MRC-5	SI
24	551.28 ± 0.05	1870.55 ± 0.02	3.39[a]	0.556 ± 0.08	0.775 ± 0.08	1.39
48	197.28 ± 0.20	662.40 ± 0.01	3.36[a]	0.320 ± 0.01*	0.425 ± 0.06*	1.33
72	80.03 ± 0.04*	280.38 ± 0.01	3.50[a]	0.151 ± 0.06*	0.230 ± 0.03*	1.52

Data represented as mean ± SD of three independent readings. *$p<0.05$. [a]SI value > 3 indicates high selectivity (Chothiphirat et al., 2019).

Table 2. Flow cytometric analysis of apoptosis in MCF-7 and MRC-5 cells.

Treatment	Q1 (Necrotic %)		Q2 (Late apoptosis%)		Q3 (Viable %)		Q4 (Early apoptosis %)	
	MCF-7	MRC-5	MCF-7	MRC-5	MCF-7	MRC-5	MCF-7	MRC-5
Control	9.6 ± 0.5	0	0	0	90.4 ± 0.1	99.0 ± 1.41	0	0
***R. chalepensis* essential oil**								
40 µg/mL	1.8 ± 0.2	0.2 ± 0.08	25.3 ± 0.9	11.9 ± 0.01	54.7 ± 0.7	73.2 ± 0.45	9.1 ± 0.5	14.6 ± 0.13
80 µg/mL	1.0 ± 0.08	0	20.1 ± 0.22	0.1 ± 0.03	47.3 ± 0.9	62.7 ± 0.31	31.7 ± 0.1	37.2 ± 0.14
160 µg/mL	4.7 ± 0.18	0	38.4 ± 0.5	11.4 ± 0.08	15.2 ± 0.33	56.9 ± 0.18	41.7 ± 0.62	31.7 ± 1.2
Doxorubicin								
0.2 µg/mL	33.3 ± 0.05	3.7 ± 0.07	0	37.4 ± 0.13	66.2 ± 0.25	30.4 ± 0.21	0	28.5 ± 1.5

Data represented as mean ± SD of two independent readings.

Determination of apoptosis by flow cytometry

The detection of apoptosis is based on the sequential changes in the plasma membrane that occur during the early events of apoptosis when cells translocate membrane phosphatidylserine molecules from the inner leaflet of the plasma membrane to the cell surface. Phosphatidylserine on the cell surface can be recognized by annexin V, which has been fluorescently labeled with fluorescein isothiocyanate (FITC) and can be used as a probe to identify apoptotic cells by displaying green fluorescence (Kupcho et al., 2019). Furthermore, necrosis is detected by measuring the permeability of the plasma membrane to propidium iodide, which cannot stain viable or early apoptotic cells due to the presence of an entire plasma membrane. In the late apoptotic and necrotic cells, the cell membrane's integrity and nuclear membrane decrease, allowing PI to pass, then bind to DNA ad display red fluorescence (Vossenkamper and Warnes, 2018).

According to the results obtained by the MTT assay, the reduced cell viability of MCF-7 and MRC-5 was further investigated to assess cell death and detect if it was related to the apoptotic process. MCF-7 and MRC-5 cells were prepared for flow cytometry according to the protocol described by the apoptosis kit. The cell viability (Q3) of MCF-7 and MRC-5 cell lines of control cells was higher than treated cells with *R. chalepensis* essential oil and doxorubicin. However, the viability of the treated cells with *R. chalepensis* essential oil (40, 80, and 160 µg/mL) was significantly reduced in a dose-dependent manner (Table 2). Furthermore, the number of apoptotic cells (early Q4 and late Q2 stage of apoptosis) was significantly increased in the groups treated with *R. chalepensis* essential oil than in control cells, as illustrated in Fig. 2. On the other hand, the apoptotic cells in the MCF-7 cells treated with doxorubicin (0.2 µg/mL) were not observed compared to MRC-5.

The percentage of necrosis (Q1) in MCF-7 and MRC-5 cells was lower in the group treated with essential oil than doxorubicin. The aforementioned indicates that *R. chalepensis* essential oil induces apoptosis (preferred mode of cell death).

Cell morphology analysis

Light microscopy observation of *R. chalepensis* essential oil-treated MCF-7 cells revealed a decrease in the number of viable cells with increasing *R. chalepensis* essential oil concentration. Furthermore, as shown in Fig. 3, MCF-7 treated cells underwent morphological changes characterized by the smaller size and the

roundness of the cells, which are characteristics of apoptotic cells accompanied by an abundance of de-attached and floating dead cells.

Morphological observation of nuclear change by DAPI staining assay

The apoptosis-inducing potential of *R. chalepensis* essential oil on MCF-7was assessed by DAPI staining Fig. 4. Untreated cells (A) exhibited normal morphology. In contrast, the treated cells with 40 and 80 µg/mL of essential oil showed apparent nuclear morphology changes (chromatin condensation and nuclear fragmentation) (Fig. 4C and D). None of the cells were observed after treatment with 160 µg/mL of essential oil and 0.2 µg/mL of doxorubicin (**Fig. 4**B and E). The main morphological hallmarks of apoptosis in the nucleus were chromatin condensation and nuclear fragmentation. The condensation was started peripherally along the nuclear membrane, formed a crescent or ring-like structure. During later stages of apoptosis, the nucleus is further condensed, and finally, it has broken up inside a cell with an intact cell membrane. Necrotic cell death was characterized morphologically by swelling of cell membranes, often were accompanied by intense chromatin condensation. The cytoplasmic membranes dilated and released the cytoplasmic contents into the extracellular space.

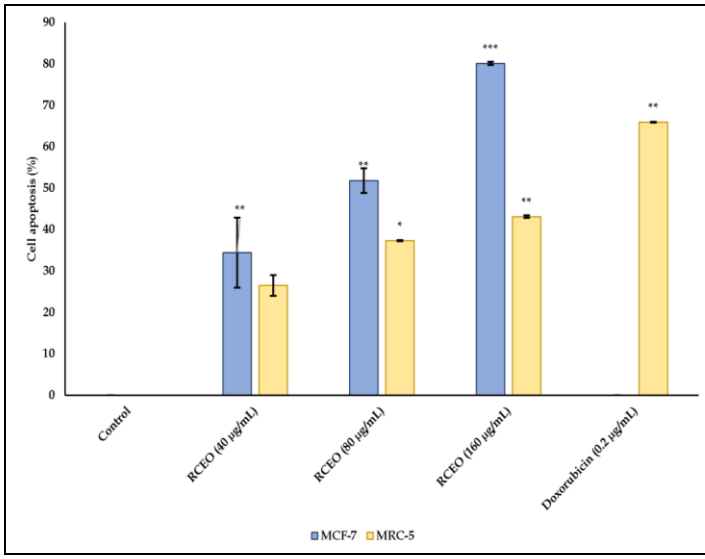

Figure 2. The apoptosis rate (early and late) of MCF-7 and MRC-5 cells treated with *R. chalepensis* essential oil (RCEO) using flow cytometry.

Apoptotic cells of two independent experiments were shown in column statistics. Doxorubicin (0.2 µg/mL) was used as a reference anticancer drug. All data were expressed as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 compared to untreated group (control).

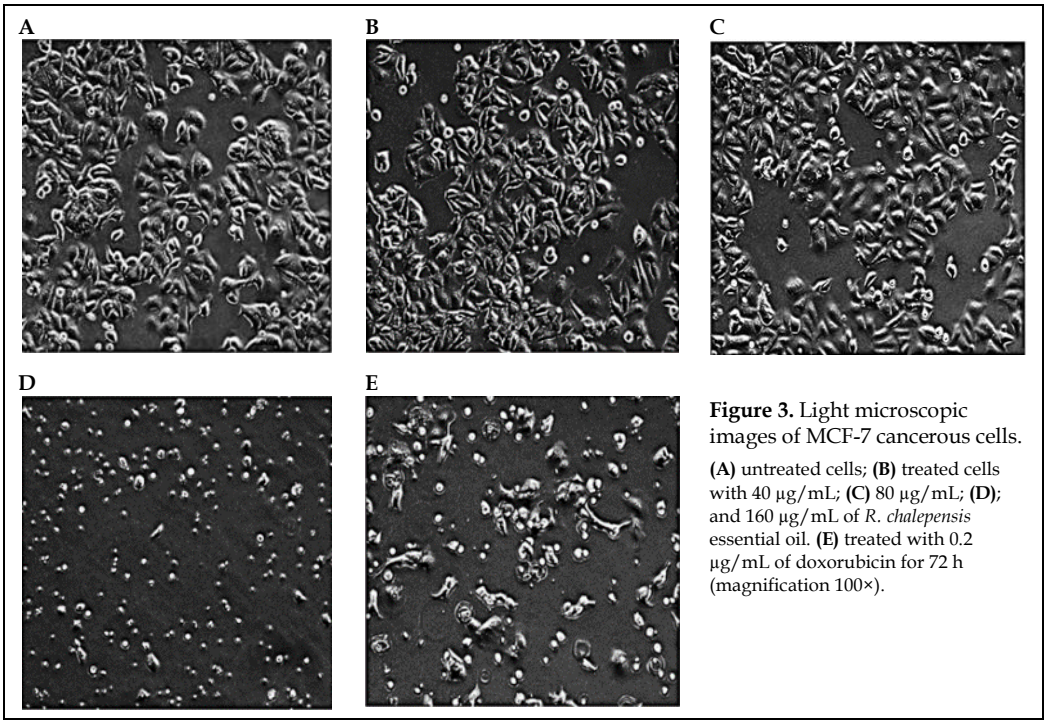

Figure 3. Light microscopic images of MCF-7 cancerous cells.

(A) untreated cells; **(B)** treated cells with 40 µg/mL; **(C)** 80 µg/mL; **(D)**; and 160 µg/mL of *R. chalepensis* essential oil. **(E)** treated with 0.2 µg/mL of doxorubicin for 72 h (magnification 100×).

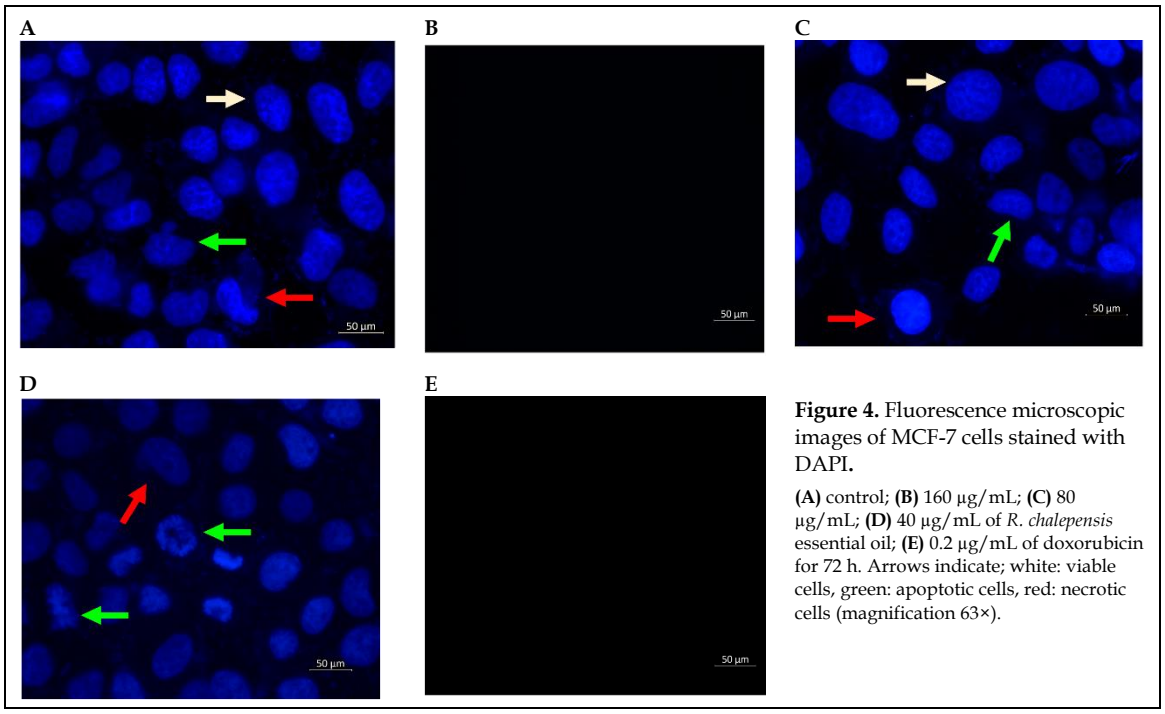

Figure 4. Fluorescence microscopic images of MCF-7 cells stained with DAPI.

(A) control; **(B)** 160 µg/mL; **(C)** 80 µg/mL; **(D)** 40 µg/mL of *R. chalepensis* essential oil; **(E)** 0.2 µg/mL of doxorubicin for 72 h. Arrows indicate; white: viable cells, green: apoptotic cells, red: necrotic cells (magnification 63×).

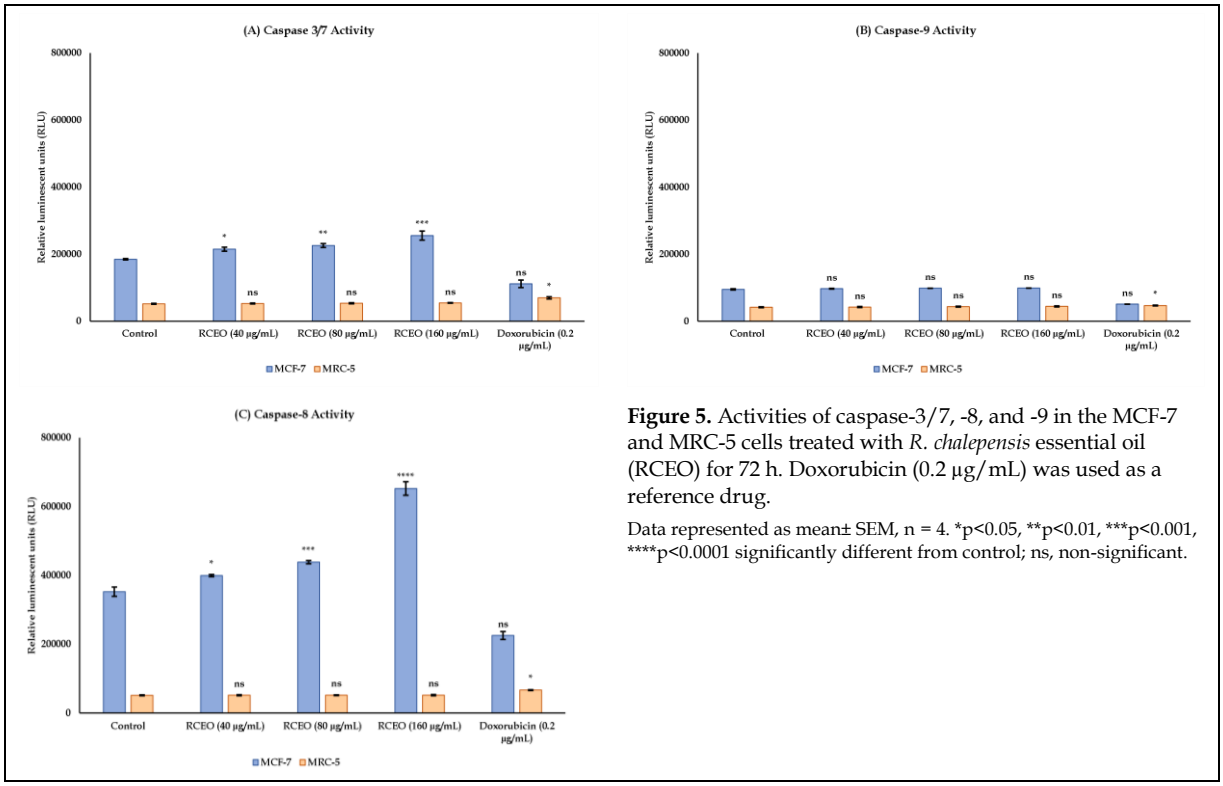

Figure 5. Activities of caspase-3/7, -8, and -9 in the MCF-7 and MRC-5 cells treated with *R. chalepensis* essential oil (RCEO) for 72 h. Doxorubicin (0.2 µg/mL) was used as a reference drug.

Data represented as mean± SEM, n = 4. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 significantly different from control; ns, non-significant.

Effect of *R. chalepensis* essential oil on the activity of Caspase-3/7, -8 and -9

The effect of *R. chalepensis* essential oil (40, 80, and 160 µg/mL) on the effector caspase-3/7 and in the initiator caspase-8 and -9 in the MCF-7 and MRC-5 cells was evaluated using caspase activity assay kits at 72 h post-treatment. The relative luminescence changes of caspase-3/7, caspase-8, and caspase-9 in the treated and untreated cells are shown Fig. 5A-C.

The results exhibited that after treating MCF-7 cells with *R. chalepensis* essential oil for 72 h, the cellular caspase-3/7 and -8 activities increased in a dose-dependent manner. At 40, 80, and 160 µg/mL of *R. chalepensis* essential oil, the caspase-3/7 activity was increased notably compared to the untreated cells

(Fig. 5A). There was no significant increase in the activity of caspase-9 is shown in Fig. 5B. Also, caspase-8 activity was significantly increased in response to 40, 80, and 160 µg/mL of essential oil (Fig. 5C). Furthermore, an apparent activation in the caspase-3/7 and caspase-8 occurred, but no activation of caspase-9. Based on the findings, *R. chalepensis* essential oil could has an apoptotic effect on MCF-7 cells via caspase-8 activity, associated with the extrinsic cell death pathway. On the other hand, the activity of caspases -3/7, -9, and -8 did not increase significantly in MRC-5 cells after treating with *R. chalepensis* essential oil compared to untreated cells (control). Nonetheless, doxorubicin (0.2 µg/mL) did not significantly increase the activity of the mentioned caspases in MCF-7 cells compared to its effect on MRC-5 cells ($p<0.05$) (Fig. 5A-C).

DISCUSSION

Cancer is a complex multifactorial disorder defined by uncontrolled cell division, invading those cells, and spreading from the initial site to different locations (Coltri et al., 2019). Cancer was a significant public health problem globally and in Jordan (Khader et al., 2018). The modern therapeutic procedures of cancer are correlated with multidrug resistance, severe side effects, and high cost. Therefore, an urgent need for more effective, less toxic interventions (Dehelean et al., 2021).

Recently there is a great interest in screening for plants to be used in cancer prevention and treatment. For this reason, essential oils from different medicinal, aromatic plants have been extensively studied. The anticancerous effect and other biological activities of essential oils are related to their phytochemical constituents. The phytochemical profile of *R. chalepensis* essential oil was characterized in the previous study (Althaher et al., 2020). Twenty-nine compounds were identified, which represented 92.87% of the total composition. where the main components of the were 2-cyclohexen-1-one,3-[(2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-1-yl) methyl] (45.97%), 2-nonanone (19.45%), methyl hexadecanoate (4.04%) and 4,5-dimethoxy-6- prop-2-enyl-1,3-benzodioxole (1.87%). While the non-terpenoid non-aromatic compounds and monoterpenes accounted for 10.31%, 2.24%, respectively. However, sesquiterpenes and diterpenes were not detected in the oil constituents.

The cytotoxic activity of *R. chalepensis* essential oil against MCF-7, T47D, Caco-2, and normal fibroblast cell lines were previously identified (Althaher et al., 2020). In the current study, the cytotoxicity of essential oil was investigated against the MCF-7 cell line after 24, 48, and 72 h. The results revealed that *R. chalepensis* essential oil significantly inhibited the proliferation of MCF-7 cells after 72 h with IC_{50} 80.03 ± 0.04 µg/mL. In comparison, the ethanolic extract of *R. chalepensis* exhibited potent cytotoxicity against T-leukemic cells (CEM, H9, Jurkat, and CEM-IRCs) and B lymphoblast cells (SKW 6.4) without affecting normal blood cells (PBL and PBLs) (Terkmane et al., 2018). Moreover, the methanolic extract of *R. chalepensis* exhibited antiproliferative activity against human bladder carcinoma-RT112, human laryngeal carcinoma-Hep2, human myelogenous leukemia-K562, and murine macrophage cell line-RAW 264.7, without affecting normal peripheral blood mononuclear cells (Khlifi et al., 2013).

One of the cancer hallmarks is the resistance to cell death by apoptosis (Arbiser et al., 2017). Therefore, apoptosis induction is a powerful way for all types of cancer (Pfeffer and Singh, 2018). Several reports indicate that medicinal plants' anticancer activity is due to the presence of different bioactive compounds, such as essential oils that possess biological impacts via apoptosis induction in various cancer cell lines and are promising for developing novel anticancer agents. Therefore, studies on essential oils' biological activities have become increasingly crucial in searching for natural and harmless alternative medicines in recent times.

Apoptosis can be determined by measuring annexin V-FITC binding to membrane-bound phosphatidylserine, typically located in the inner leaflet of the cell membrane of normal cells and externalized to the outer leaflet of the plasma membrane of apoptotic cells.

The annexin V-positive/PI-negative staining and double-positive cell staining defined early and late apoptosis, respectively, with a total percentage of the apoptotic cell population of 48.8 ± 4.24% after 72 h of treatment MCF-7 cells with IC_{50} of *R. chalepensis* essential oil. Flow cytometry results were confirmed by an apoptotic assay using a DAPI staining assay in a fluorescence microscopy analysis. In this search, many cells changed their typical morphologies after *R. chalepensis* essential oil treatment, showing chromatin condensation, cell membrane blebbing, and DNA fragmentation. The light microscopy images of MCF-7 cells treated with *R. chalepensis* essential oil showed morphological changes such as small size and roundness cells.

Concerning caspase activity, *R. chalepensis* essential oil was induced apoptosis in MCF-7 cells via an extrinsic pathway, including activation of caspase-8. Compared to our data, an aqueous extract from *Ruta graveolens* has a potent antitumoral activity by inducing caspase 3-dependent apoptotic cell death in hu-

man glioma cells, and undifferentiated cells originated from mouse embryonic brain. The extract also showed the capability to discriminate between proliferating (tumor cells) and differentiated, non-proliferating neural cells (Gentile et al., 2015).

Chalepin is a compound identified among 12 compounds in *Ruta angustifolia*. Chalepin induced apoptotic cell death in the lung carcinoma cell line (A549) that involved the intrinsic mitochondrial pathway (Richardson et al., 2016). On the other hand, rutamarin is one of the compounds isolated from the chloroform extract of *Ruta angustifolia* Pers. Its cytotoxic activity was evaluated against colon carcinoma cell line (HT29) and normal colon fibroblast cells (CCD-18 Co). Rutamarin displayed remarkable cytotoxic activity against HT29 cells (IC_{50} value of 5.6 µM), with no toxic effect on CCD-18Co cells. Rutamarin induced apoptosis, including activation of caspases 3, 8, and 9, were observed in rutamarin-treated HT29 cells (Suhaimi et al., 2017).

CONCLUSION

The present study results revealed that *R. chalepensis* essential oil has cytotoxic activity against MCF-7 and induces apoptosis in a dose and time-dependent manner via an extrinsic caspase-8 dependent pathway. Therefore, it could be introduced as a potential therapeutic agent for breast cancer treatment after additional efforts to isolate the active chemical compounds from this oil and test their pharmacological impacts.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Contribution	Althaher AR	Oran SA	Bustanji YK
Concepts or ideas	x	x	x
Design	x	x	x
Definition of intellectual content	x	x	x
Literature search	x	x	
Experimental studies	x		x
Data acquisition	x	x	x
Data analysis	x	x	x
Statistical analysis	x		
Manuscript preparation	x		
Manuscript editing	x	x	x
Manuscript review	x	x	x

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