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Dichloromethane EXTRACT of *Cuscuta epithymum* Inhibits Triple-Negative Breast Cancer Development Via Inducing Apoptosis and Suppression of Migration

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Abstract

Background: A major challenge involved in breast cancer management is the therapeutic agents. Herbal medicine tries to find the therapeutic agent for this type of cancer from nature. *Cuscuta epithymum*, as a member of the Convolvulaceae family, has demonstrated that different extracts of this medical plant have antibacterial and antitumor effects in different types of cancer. The aim of this study is to illustrate the effect of different alcoholic extract of *Cuscuta epithymum* on breast cancer cells. **Methods:** The cytotoxic assay was performed on human and mouse triple-negative cell lines by MTT assay. The type of cell death was investigated by annexinV/PI method via flow cytometry analyzer. The Caspase-3 and -9 and Bcl-2 mRNA expression level were assessed by qRT-PCR. The cell migration was performed by scratch wound healing assay. **Results:** The MTT assay showed that methanol, dichloromethane, and N-hexane extracts of *Cuscuta epithymum* inhibited breast cancer cell growth by inducing cell toxicity. Following the results of the apoptosis assay showed that dichloromethane extract induced apoptosis more significantly than two others. The molecular analysis showed that this extract induced Caspase-3 and -9 mRNA expressions and reduced the Bcl-2 mRNA expression. The results of the migration assay also showed that it could suppress the mobility feature of invasive triple-negative breast cancer cells. **Conclusion:** The present study verified that the dichloromethane extract of *Cuscuta epithymum* could inhibit triple-negative breast cancer cells growth via inducing apoptosis through the intrinsic apoptosis pathway. The results encourage us to extend the use of this extract in animal and clinical experiments.

Key words: Cuscuta epithymum extract, Triple-negative Breast cancer, Apoptosis, Migration.

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Introduction

Breast cancer has been reported as the most common malignancy and as one of the life-threatening diseases among the females of worldwide. (Smith and DeSantis. 2018) The incidence rate of this malignancy is increasing in developing countries such as Iran. The main reason for this increasing number is the late diagnosis and lack of therapeutic application in developing countries. Based on the receptors type, breast cancer is divided into three types including estrogen receptor positive (ER+), progesterone receptor positive (PR+), and triple-negative. (Nguyen et al., 2008)

Anticancer agents are widely used in cancer therapy. Common cancer treatment strategies including surgery, chemotherapy, and radiotherapy are not useful always and the clinical results are not acceptable. One of the most important features that anticancer agents should have, is the slight side effects on normal cells that possibly occurs by inducing apoptosis cell death. (Safarzadeh et al., 2014) Apoptosis known as programmed cell death is involved in human body homeostasis. (Goldar et al., 2015) Inducing apoptosis in tumors could be the best strategy for cancer treatment. The history of herbal medicine use roots back to thousand years ago. (Safarzadeh et al., 2014) Some natural plants induce apoptosis in cancer cells via multiple mechanisms. (Clément et al., 1998; Mohammadi et al., 2016; Mohammadi et al., 2016; Mansoori et al., 2016) It has been demonstrated that apoptosis induction is related to the active components existing in medical plants such as phenolic, alkaloids, lectines, and terpenoids. (Safarzadeh et al., 2014)

Cuscuta epithymum is a herb belonging to the Convolvulaceae family. (Costea et al., 2011) There are 150 species of this plant around the world. *Cuscuta epithymum* is the most common species of this herb, which is widely used in various treatments of traditional medicine. (Costea et al., 2011) Previous studies have shown that some species of Convolvulaceae have anti-cancer properties. (Abdel Khalik, 2006; Sepehr et al., 2011; Ghazanfari et al., 2013) *Cuscuta Chinensis* and *Cuscuta kotschyana* extracts have shown a cytotoxic effect on HLA-60, MCF-7, T47D, and Jurkat cell lines. (Sepehr et al., 2011; Zeraati et al., 2010) In another study, it was demonstrated that the methanolic extract of Cuscuta epithymum has anti-bacterial and anti-cancer effects. (Biswas et al., 2012) A recent study showed that the chloroform and hydroalcoholic extracts of air limbs of *Cuscuta Chinensis* and *Cuscuta epithymum* have a cytotoxic effect on MDA-MB-468, HT-29, and Hela cell lines. (Jafarian et al., 2014)

Various studies have shown that the pharmacological effects of different species of Convolvulaceae are attributed to their active compounds, including flavonoids, polysaccharides, and lignans. (Ghazanfari et al., 2013; Suresh et al., 2011)

The aim of this study is to evaluate the methanol, dichloromethane, and N-hexane extracts of *Cuscuta epithymum* in triple-negative breast cancer cytotoxicity, apoptosis, and the molecular mechanism involved in the apoptosis pathway. In addition, the effect of *Cuscuta epithymum* extract on invasive and noninvasive breast cancer cells migration is evaluated.

Materials and methods

Cell lines and cell culture

Human breast cancer cell lines 4T1 (mouse) and MDA-MB-231 (human) were obtained from the cell bank of Pasture institute (Iran, Tehran). The mice and human cancerous cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotics (Penicillin-Streptomycin) (Gibco) at 37℃ in a 95% humidity and 5% CO2 condition. All the experiments were performed on the logarithmic phase of cells.

Cell Toxicity Assay

The cytotoxicity effect of methanol, dichloromethane, and N-hexane of *Cuscuta epithymum was* evaluated by MTT assay. Briefly, 15×10^3 cells were seeded in 96-well cell culture plates. The cells were treated with a range of 25-2000 µg/ml of each extract for 24 and 48 h. Then, the cells were incubated with 150 µl media contained 50 µl of MTT (2 mg/ml of MTT in PBS). The plates were incubated in cell culture incubator in dark for 4 h. In this period, the yellow tetrazolium salts were converted to purple crystals called formazans. About 200 µl DMSO and 25 µl Sorenson's buffer and shaking in 1000 rpm for 1 min were used to solubilize the crystals. The optical density of plates was measured in 570 nm by a microplate reader (Tecan, Sunrise, Australia).

Annexin V/PI apoptosis assay

The apoptosis was evaluated by annexin V/PI staining method via the flow cytometer instrument (MACS-Quant 10, Miltenyi Biotec, Germany). A total of 2×10^5 cells were seeded in a 6-well cell culture plate. After treatment of the cells with a proper concentration of the extract, the cells were harvested and stained with annexin V-FITC and PI staining kit for 20 min in dark (Exbio, Prague, Czech Republic). The apoptosis rate was analyzed by FlowJo software (Tree Star, San Carlos, Ca).

Gene expression assay

- *RNA extraction*

Total RNA was extracted via RiboEx reagent (GenAll, Korea). As mentioned, these 2×10^5 cells were seeded in a 6-well cell culture plate, followed by lysing them with 1 ml RiboEx reagent and 250 µl chloroform. Next, the lysate was centrifuged in 12000 rpm at 4℃ for 20 min. The aqueous phase was transferred to another microtube and then an equal amount of isopropanol was added and incubated on ice for 30 min. After that, the reactions were centrifuged in 12000 rpm at 4℃ for 20 min. Finally, the tubes were washed with 75% ethanol and resuspend the RNA pellet in DEPC and the concentration was measured by NanoDrop2000 (Thermo Scientific, USA).

- *cDNA synthesis*

The cDNA synthesis was done by using 1 µg of total RNA with Biofact cDNA synthesis kit (Daejeon, South Korea). Briefly, 10 µl of premix, 0.5 µl random hexamer, and 0.5 µl oligo dt primer were added up to 20 µl with DEPC water. The thermocycler protocol was 5 min RT, 30 min at 50℃ and finally 5 min at 85℃.

- *qRT-PCR*

The mRNA expression levels of Caspase-3, Caspase-9, and Bcl-2 were measured by the qRT-PCR assay. According to Sybr green qRT-PCR protocol, 5 µl of SYBR® Green Master Mix, 0.5 µl of 4 pmol specific primers (Table 1), 0.5 µl of cDNA template, and 4 µl H202 were used. The thermocycler protocol was 15 min at 95℃, followed by 45 cycles at 95℃ for 10 sec, 60℃ for 35 sec, and 70℃ for 20 sec.

Table 1. Primers sequences

Primer		**Sequences(5' to 3')**	
Human	**18s RNA**	**F**	GATCAGATACCGTCGTAGTTCC
		R	CTGTCAATCCTGTCCGTGTC
	Caspase-3	**F**	TGTCATCTCGCTCTGGTACG
		R	AAATGACCCCTTCATCACCA
	Caspase-9	**F**	GCAGGCTCTGGATCTCGGC
		R	GCTGCTTGCCTGTTAGTTCGC
	Bcl-2	**F**	CCTGTGGATGACTGAGTACC
		R	GAGACAGCCAGGAGAAATCA
Mouse	**18s RNA**	**F**	GATCAGATACCGTCGTAGTTCC
		R	CTGTCAATCCTGTCCGTGTC
	Caspase-3	**F**	GGGGAGCTTGGAACGCTAA
		R	CACATCCGTACCAGAGCGAG
	Caspase-9	**F**	CCTTCCTCTCTTCATCTCCTGCT
		R	TTGCTGTGAGTCCCATTGGT
	Bcl-2	**F**	GAGTTCGGTGGGGTCATGTG
		R	CACCTACCCAGCCTCCGTTA

Migration assay

Breast cancer migration was assessed by wound healing assay. According to our previous protocol, 2×10^5 cells were seeded in a 6-well cell culture plate for gap generation in cell monolayer in 90% confluence. Subsequently, the cells were treated with different *Cuscuta epithymum* extract. The gap area was evaluated and imaged in 0 and 48 h after treatment.

Statistical analysis

Each experiment was performed in triplicate by reporting mean±SD. The student T-test and one way ANOVA and Tuckey post-test statistical analysis were performed for each experiment by GraphPad Prism statistical software (GraphPad Software, Inc., San Diego, CA).

Results

Different types of Cuscuta epithymum extract cause breast cancer toxicity

The cytotoxicity of methanol, dichloromethane, and N-hexane extract of ***Cuscuta epithymum*** on the mouse and human breast cancer cells was performed by MTT assay. The N-hexane, dichloromethane, and methanol extracts showed a significant reduction in breast

cancer cell viability in a dose and time-dependent manner (Fig. 1). Among the extracts, the best results in breast cancer cytotoxicity were obtained with methanol and dichloromethane extracts of *Cuscuta epithymum* as displayed for by the lowest IC_{50} values for 4T1; which was 72.83± 0.87 µg/ml, 24 h after treatment and for MDA-MB-231 was 53.24 ± 0.73 µg/ml after 48 h (Table 2). For control, MTT assay was carried out by treating 4T1 and MDA-MB-231 cells with 1% DMSO; no significant decrease in cell viability was observed.

Table 2. IC_{50} of different extracts of *Cuscuta epithymum*.

Cell lines	Extract/Time	N-h	Di	M
		µg/ml		
4T1	**C.E 24h**	-	132.8	151.1
	C.E 48h	525.3	118.9	72.83
MDA-MB-231	**C.E 24h**	-	90.45	263.3
	C.E 48h	-	53.24	137.8

C.E: *Cuscuta epithymum*, N-h: N-hexane extracts; Di: dichloromethane extracts; M: methanol extracts.

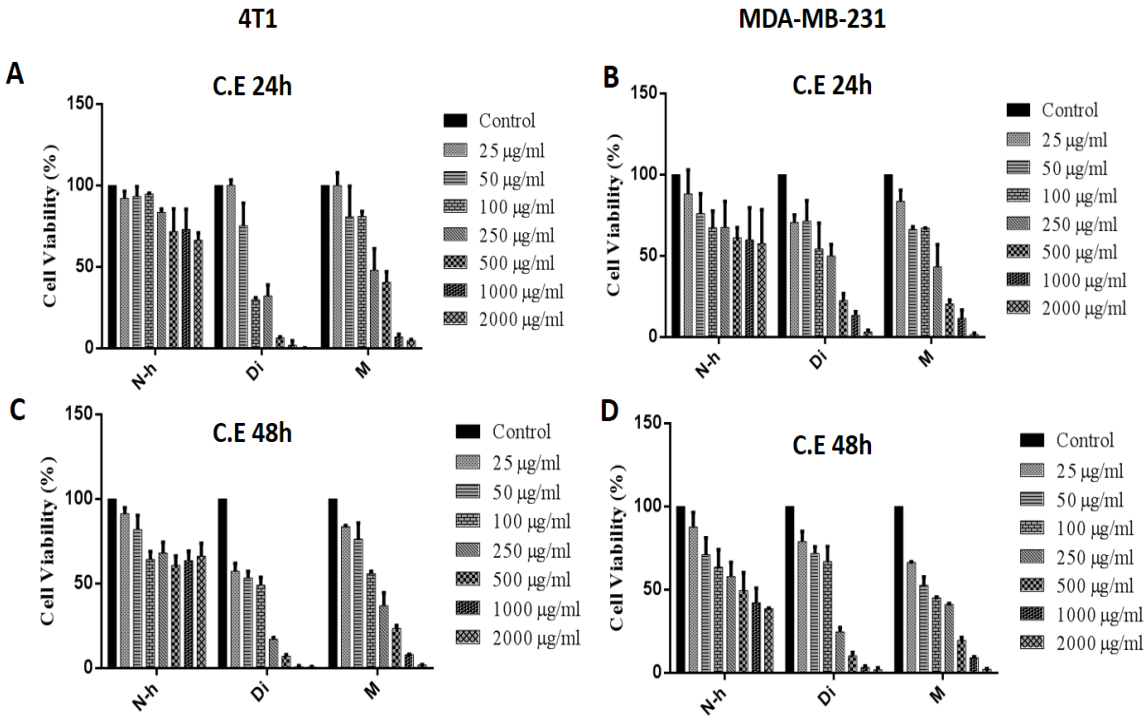

Fig. 1. Cytotoxic effect of *Cuscuta epithymum* extracts in 24 and 48 h post-treatments: (A) Different concentrations of C.E extract after 24 h of treatment in 4T1 cells; (B) Different concentrations of C.E extract after 24 h of treatment in MDA-MB-231 cells; (C) Different concentrations of C.E extract after 48 h of treatment in 4T1 cells; and (F) Different concentrations of C.E extract after 48 h of treatment in MDA-MB-231 cells (C.E: *Cuscuta epithymum*. N-H: N-hexane extracts; Di: dichloromethane extracts; M: methanol extracts).

The major cell toxicity of Dichloromethane extract of Cuscuta epithymum is the induction of apoptosis

Apoptosis process was evaluated with Annexin V/PI assay following the MTT assay. The results showed treatment with IC_{50} concentration of methanol, dichloromethane, and N-hexane could induce apoptosis after 24 h. Among the extracts, the dichloromethane extract showed the highest apoptosis induction of 9.2%±0.83% and 24.5%±1.08% in 4T1 and MDA-MB-231 cell lines, respectively (Fig. 3).

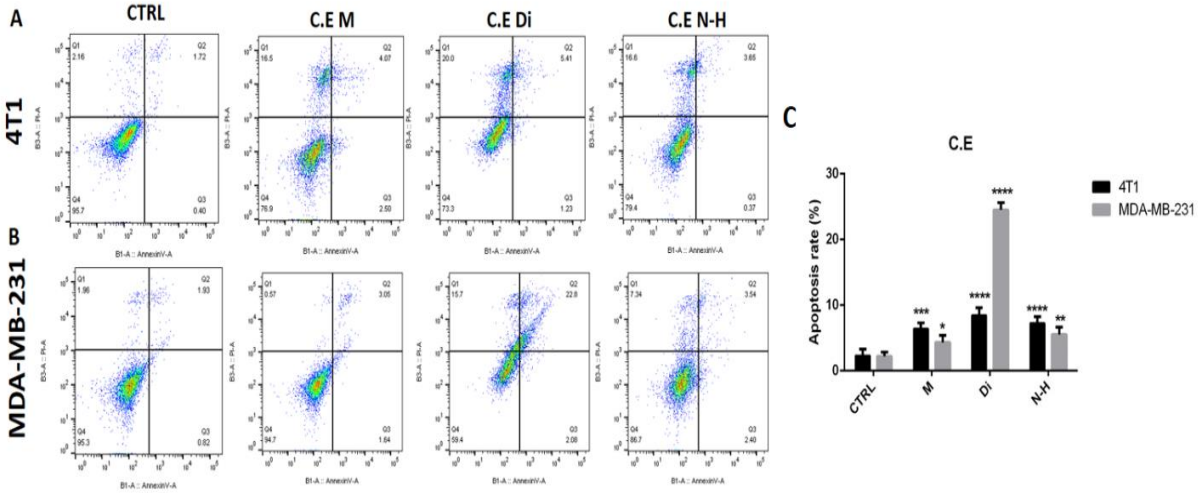

Fig. 2. Apoptosis induction in treated 4T1 and MDA-MB-231 cells; Cells were stained with Annexin V and PI kit. Annexin V positive cells correspond to apoptosis induced cells: A) and B) Dot plot of Annexin V/PI on 4T1 and MDA-MB-231 cells treated with different types of extract of E.A; and C) Bar chart of apoptosis rate in 4T1 and MDA-MB-231 cells treated with different types of extracts, ***<0.01, ****<0.001 (C.E.: *Cuscuta epithymum.* N-H: N-hexane extracts; Di: dichloromethane extracts; M: methanol extracts)

Cuscuta epithymum extract induces apoptosis via the intrinsic pathway

The results of qRT-PCR showed that the expression level of proapoptosis related genes including caspase-3 and caspase-9 mRNA following treatment with IC_{50} concentration of *Cuscuta epithymum* extract after 24 h. Apoptosis was significantly increased in dichloromethane extract compared to the control group. In addition, the antiapoptotic Bcl-2 mRNA expression was decreased in dichloromethane extract treated group (Fig. 4).

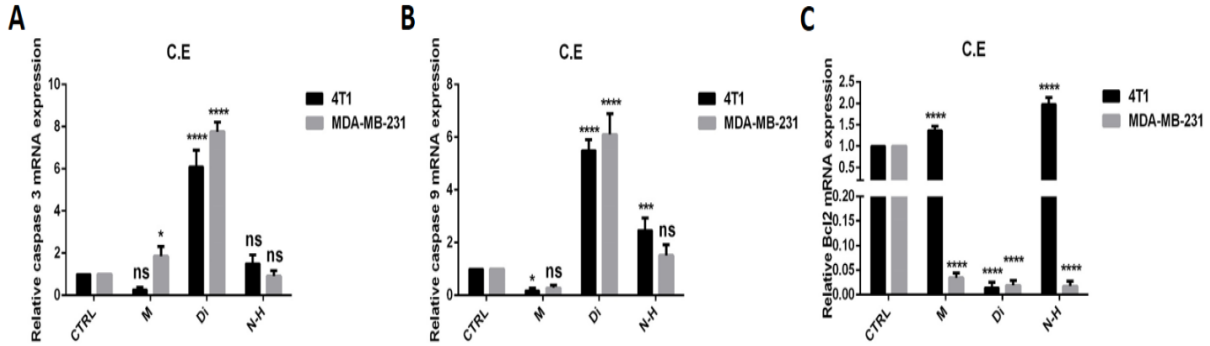

Fig. 3. Changes in caspase-3 (A), caspase-9 (B), and Bcl-2 (C) expressions in 4T1 and MDA-MB-231 cell lines treated with the IC_{50} concentration of the methanol, dichloromethane, and N-hexane extracts of *C.E* compared to control group (CTRL). *p< 0.05, ***p < 0.001, and ****p < 0.0001 versus control group (untreated cells); C.E.: *Cuscuta epithymum.* N-H: N-hexane extracts; Di: dichloromethane extracts; M: methanol extracts

Dichloromethane extract of Cuscuta epithymum suppresses breast cancer migration

Scratch assay was performed to survey the anti-migration potential of *Cuscuta epithymum* dichloromethane extract on 4T1 and MDA-MB-231 breast cancer cell lines. Treatment with IC_{50} concentration of dichloromethane extracts reduced the number of mobilized cells into the gap area compared to untreated cells (Fig. 4).

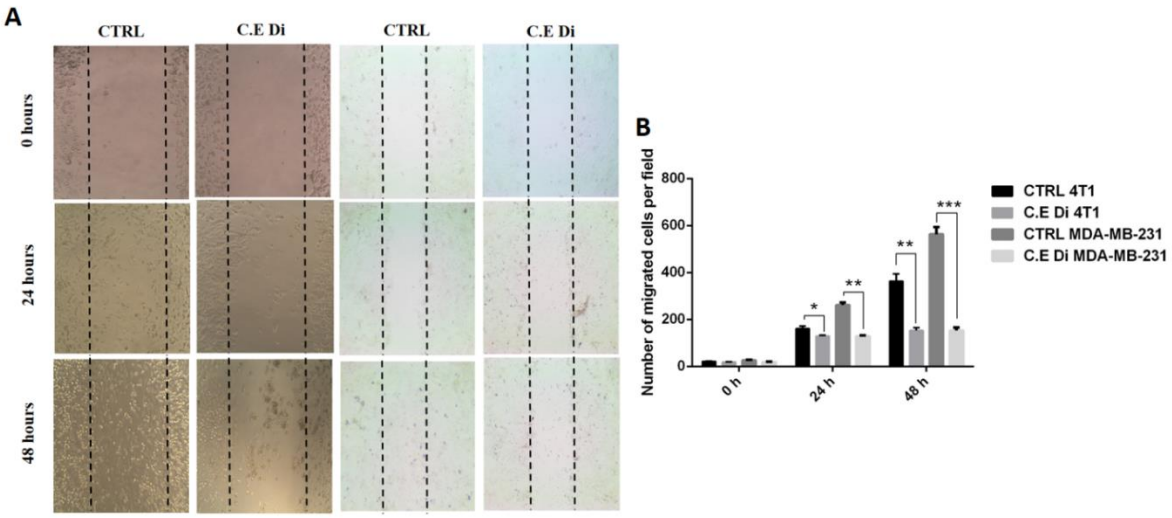

Fig. 4. Breast cancer cells treated with and/or without dichloromethane extracts were subjected to scratch assays: (A) 4T1 and MDA-MB-231 cells treated and untreated cells with C.E extract imaged at the time of 0, 24 and 48 h after scratching; (B) The number of 4T1 and MDA-MB-231 cells migrated into the gap area; Error bars indicate the mean±SD of three independent experiments (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001); C.E.: *Cuscuta epithymum.* N-H: N-hexane extracts; Di: dichloromethane extracts; M: methanol extracts;

Discussion

Breast cancer is one of the most life-threatening cancers among women. The most important issue about this disease is that the response of this cancer to common therapeutic strategy is not efficient. Therefore, researchers all over the world are trying to find novel therapeutic agents and discover their therapeutic mechanism. Herbal medicine as a therapeutic agent in cancer therapy was introduced 1000 years ago in Iranian and Chinese traditional medicine. Many studies have shown that herbal medicine could suppress cancer cell growth. (Safarzadeh et al., 2014; Shahneh et al., 2013)

Cytotoxicity assay showed that all three extracts of *Cuscuta epithymum* have cytotoxic effect in both triple-negative breast cancer cell lines. Among them, dichloromethane extract showed a strong breast cancer cell toxicity. Most of our studies on herbal medicine showed a different type of extracts could have different cancer cell toxicity. Inconsistent with the current study, our previous studies showed the dichloromethane extract of *Urtica Dioica* had a cytotoxic effect on breast and prostate cancer cells. (Mohammadi et al., 2016; Mohammadi et al., 2016; Aletaha et al., 2017; Mohammadi et al., 2016) Similar to our study Jafarian et al. (2014) demonstrated the chloroform extracts of *Cuscuta epithymum* strongly decreased Hela, HT-29, and MDA-MB-468 cells viability. Biswas et al. (2012) showed the methanolic extract of *Cuscuta epithymum* has a cytotoxic effect on several bacteria such as Bacillus megaterium, Pseudomonas aeroginose, and Escherichia coli. Ghazanfari et al. (2013) showed that *Cuscuta* extract could inhibit the growth of Raji cells; following their results, this extract was introduced as a potential anti-cancer agent against lymphoma and melanoma cancers. The studies have verified that the *Cuscuta epithymum* pharmacological effects could be associated with their active components including alkaloids, saccharides, flavonoids, saponins, lignans, and resin glycosides. (Biswas et al., 2012; Ghazanfari et al., 2013) In addition, it has been demonstrated that flavonoids significantly reduce cell viability via caspase activation. (Zeraati et al., 2010) These alcoholic extracts of *Cuscuta epithymum* with extra poly-phenolic components displayed lower cytotoxic effect related to extraction solutions, and we could conclude the cytotoxic effect is related to poly-phenolic components.

Following the MTT assay, in order to understand whether apoptosis is involved in the cell toxicity, annexin-V/PI apoptosis assay was performed. According to the results, all extracts induced apoptosis in triple-negative breast cancer cell lines. Among them, dichloromethane extract showed a strong apoptosis induction especially in human breast cancer cell line (MDA-MB-231).

According to the gene expression results, the major mechanism of apoptosis was occurred by inducing the intrinsic pathway. The mRNA expression levels of pro-apoptotic caspase-3 and -9 were increased in the mouse and human triple-negative cell lines treated by dichloromethane extract of *Cuscuta epithymum*. In addition, the mRNA expression level of the anti-apoptotic Bcl-2 was decreased after treatment with dichloromethane extract.

Similar to our study, Roshanravan et al. showed that dichloromethane and N-hexane extracts of Eryngium Billardieri induced apoptosis in the pancreatic cancer cell line. They also showed that dichloromethane extract induced apoptosis in a dose-dependent manner. (Roshanravan et al., 2018) They demonstrated the extract induced apoptosis via elevated Bax mRNA expression. Moradzadeh et al.

(2018) showed that *Cuscuta Campestris* activated apoptosis by inducing ROS production on leukemic cells. In our previous study, dichloromethane extract of Urtica Dioica could induce apoptosis in colorectal cancer cells. Later, we demonstrated this extract could induce apoptosis by increasing Caspase-3 and caspase-9 and reducing Bcl-2 mRNA expression. (Mansoori et al., 2016) It was demonstrated that *Cuscuta* family have flavonoid and this active component could active the caspase cascade. (Zeraati et al., 2010)

Totally, the dichloromethane extract results showed that this extract has an anti-proliferative effect compared to the two others. Therefore, we investigated the effect of this extract in the migration of noninvasive and invasive breast cancer cells. The results showed that dichloromethane extract could inhibit cell migration in both breast cancer cell lines. Our previous studies on dichloromethane extract of Urtica Dioica showed anti-migratory and anti-metastasis effect by decreasing miR-21, metastasis related mRNAs including MMP3, CXCR4, MMP9, Vimentin and increasing E-cadherin mRNA expression of this extract on breast cancer cell lines. (Mohammadi et al., 2016; Mansoori et al., 2017) Our another study on an alcoholic extract of Anacyclus Pyrethrum showed anti-migratory effect via reducing MMP1 and vimentin mRNA expression on colorectal cancer cells. (Mohammadi et al., 2017)

Although these results showed the therapeutic effect of dichloromethane *Cuscuta epithymum* extract in triple negative breast cancer cell lines, more research is still needed at pre-clinical and clinical levels for setting it as a potential agent for cancer therapy.

Taken together, our study found that *Cuscuta epithymum* dichloromethane extract induced cell death of triple-negative breast cancer cells by inducing apoptosis. Moreover, this study provided the mechanism of apoptosis induction via the intrinsic pathway. In addition, this extract suppressed breast cancer cells migration. Therefore, the results showed that dichloromethane extract of *Cuscuta epithymum* might be a good candidate as a therapeutic agent for triple-negative breast cancer from a natural source.

Conflict of Interest:

The authors declare that they have no conflict of interest.

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