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Original article

Cytotoxic Effects of *Eupatorium cannabinum* on MCF-7 Human Breast Cancer Cell Line

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Abstract

Background and objectives: Cancer is known to be the second cause of death around the world. The most prevalent cancer among women is breast cancer. Use of plant-derived products in cancer treatment may reduce adverse side effects. Extensive research around the world has led to the discovery of herbal compounds that can be used to treat some types of cancer. According to previous studies, the methanol extract of *Eupatorium cannabinum* has shown cytotoxic effects in some cancer cell lines. In the present study, bioassay guided fractionation and isolation was conducted on *E. cannabinum* to evaluate the cytotoxic activity in MCF-7 breast cancer cell line. **Methods:** The extraction from the aerial parts was performed by maceration method. Isolation and purification of the extracts were performed by column chromatography. The cytotoxic activity of different extracts of *E. cannabinum* was evaluated against MCF-7 cell line by MTT assay and a compound was isolated according to bioassay guided fractionation. The cytotoxic activity and apoptotic property of the isolated compound was determined. **Results:** The chloroform extract was the most active one with IC_{50} of 21.39±3.24 µg/mL followed by the n-hexane and methanol extracts with IC_{50} values of 60.23±2.16 µg/mL and 81.74±3.41 µg/mL, respectively. IC_{50} of subfractions (1-6) from the chloroform extract were 60.83±2.56 µg/mL, 58.93±2.73 µg/mL, 37.5±3.65 µg/mL, 7.86±1.34 µg/mL, 10.61±2.34 µg/mL and 13.77±4.17 µg/mL, respectively. Eucannabinolide, a sesquiterpene lactone, was isolated from the chloroform extract according to bioassay guided fractionation. Its IC_{50} was found to be 13±2.45 µg/mL. Eucannabinolide induced 46.91% apoptosis at concentration of 13 µg/mL in MCF-7 cell line in Annexin V/PI assay. **Conclusion:** Eucannabinolide is a promising candidate for further breast cancer drug discovery studies.

Keywords: apoptosis; cytotoxicity; eucannabinolide; *Eupatorium cannabinum*; MCF-7

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Introduction

According to world health organization (WHO), cancer is the second cause of death around the world, while the most prevalent cancer among women is breast cancer. Medications of natural origin may reduce the adverse effects of cancer treatment [1]. *Eupatorium cannabinum* L. is a perennial plant which belongs to the Asteraceae family and is distributed in Europe,

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Asia, and Northwest Africa. Geographical distribution of this plant in Iran is in humid and shady forested areas of Gorgan, Mazandaran, Guilan and Alborz provinces [2].

More than 300 compounds including flavonoids [3,4], sesquiterpene lactones [3,5], pyrrolizidine alkaloids [6–8], phenylpropanoids [2], quinonoids [9] and essential oils [10] have been reported for *Eupatorium* species. The roots and leaves of the plant are used for medicinal purposes [11]. *Eupatorium cannabinum* has shown variety of biological activities such as cytotoxic [12-14], antifungal [15,16], anti-inflammatory [2], antibacterial, insecticidal [16] and antinociceptive [13] properties. Woerdenbag H. J and et al compared cytotoxic activity of 13 semi-synthetic derivatives from eupatoriopicrin , a compound isolated from *E. cannabinum*, against the tumor cell lines EAT, P388, FIO 26, L5178Y(s) (murine) and HeLa (human). They found that acetalization of both hydroxyl groups in the ester side chain of eupatoriopicrin with acetone enhanced cytotoxicity 2-7 fold. Also, introduction of bulky groups, such as alkyl groups with a longer carbon chain and (halogenated) acetophenone derivatives, via acetalization, reversed the enhancement and oxidation at the germacrance ring structure of eupatoriopicrin acetonide, yielding an alcohol or an epoxy derivative, affected cytotoxicity adversely [17]. Also, in a previous study, the methanol extract of *E. cannabinum* has shown cytotoxic activity in MCF-7 cell line [18]. The present study was designed to further evaluate the cytotoxic and apoptotic properties of *E. cannabinum* constituents in human breast adenocarcinoma cell line (MCF-7) by bioassay guided fractionation.

Material and Methods

Ethical considerations

The Ethics Committee of Tehran University of Medical Sciences approved this research with the code of IR.TUMS.PSRC.REC.1396.3284.

Plant material

The aerial parts of *E. cannabinum* were collected in summer 2017 from Tangrah (Golestan Province, Iran). The plant samples were identified by Mr. Yousef Ajani and a voucher specimen (6598-TEH) was deposited at the Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences.

Extraction and isolation

The aerial parts of *E. cannabinum* were dried at room temperature in shade and ground (1kg). Extraction was performed by methanol in three stages, each stage for two days. The liquid extracts obtained from all three stages were thoroughly mixed and concentrated by vacuum rotary evaporator at 40 °C. The concentrated methanol extract was then mixed with silica gel (35-70 mesh) to get a fine dried green powder. Column chromatography was conducted with a column (20 ×10 cm); the powder was eluted several times with n-hexane, chloroform and methanol successively. The obtained liquid extracts were dried by vacuum rotary evaporator at 40 °C and kept in the refrigerator until the experiments.

Regarding the MTT results, the chloroform extract (10 g) was subjected to fractionation by column chromatography (45×3 cm) with silica gel 230–400 mesh (500 g) and eluted with petroleum ether–acetone (100/0 to 0/100). A total of 25 subfractions were collected. TLC was performed using pre-coated silica gel GF254 plates. The spots were visualized under UV light dipping in anisaldehyde-sulfuric acid reagent followed by heating at 105 °C. Based on TLC similarities, subfractions were combined to obtain six final subfractions. All were evaluated for cytotoxic activity with MTT assay. Among the six subfractions, subfraction 4 showed more cytotoxic effects; thus, further separation was performed on this subfraction (0.85 g) using vacuum liquid chromatography (20×3 cm) with silica gel 230–400 mesh (200 g) eluted with petroleum ether–acetone (100/0 to 0/100). The structure of the isolated compound from this subfraction was confirmed by NMR data. The cytotoxic and apoptotic activities were then evaluated against MCF-7 cells.

Cytotoxicity assay

The human adherent epithelial breast adenocarcinoma cell line (MCF-7) was obtained from Pasteur Institute, Tehran, Iran. The cytotoxicity of the extracts and the isolated compound was evaluated by MTT assay as one of the most common methods to monitor the viable cells in multi-well plates [19].

The cells were seeded at 8500 cells/well; after 24 h, they were exposed to different concentrations of the extracts (100-3.125 μg/mL) and the isolated compound (50-1.5 μg/mL) in two fold

dilutions. Passing 48 hours exposure of the cells to each sample at 37 °C, the medium was replaced with fresh medium containing MTT, with a final concentration of 0.5 mg/mL [20,21]. The cells were incubated for another 4 hours; then, the medium containing MTT was removed, and the remaining formazan crystals were dissolved in DMSO. The absorbance was recorded at 570 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Tecan Trading AG, Switzerland). DMSO 1% and tamoxifen were used as the negative and positive controls, respectively. The relative cell viability (%) related to negative control was calculated by the following formula: [A] samples/[A] control × 100, where [A] samples was the absorbance of the test sample and [A] control was the absorbance of wells containing cells, cell culture medium, and DMSO 1%. IC_{50} was calculated with Graph Pad Prism 7.0.

Annexin V/PI assay

Flow cytometry is a useful tool for the concurrent evaluation of necrosis and apoptosis in population of cells [22]. MCF-7 cells were cultured into the six-well plate at 1×10^5 cells/well. The cells were treated with eucannabinolide, (isolated compound from the most cytotoxic subfraction of *E. cannabinum* chloroform extract) at the concentration of the IC_{50} obtained in MTT assay. After 48h, Annexin V/PI assay was conducted according to previous studies [23]. DMSO 1% was used as the negative control.

Statistical analysis

Cytotoxic activity of the extracts and subfractions were evaluated by the concentration-response curve (Graph Pad Prism 7.0) and expressed as IC_{50} value ± SD. The significant difference between each group and the negative control group (DMSO) was analyzed by Student's t-test and ANOVA; p value <0.05 was considered as the level of significance.

Results and Discussion

Extraction yielded 60, 10 and 30 g of dried n-hexane, chloroform and methanol extracts, respectively. According to Table 1, the MTT assay revealed that the chloroform extract with IC_{50} value of 21.39±3.24 µg/mL showed more considerable cytotoxicity compared to the n-hexane and methanol extracts (IC_{50} 60.23±2.16 µg/mL and 81.74±3.41 µg/mL, respectively). Thus, further separation and purification was performed on the chloroform extract. Chromatographic separations led to the isolation of eucannabinolide (Figure 1) from subfraction 4 as confirmed by NMR data (Table 2). The cytotoxic and apoptotic activities of eucannabinolide were then evaluated against MCF-7 cells. The cytotoxic effect of the *n*-hexane, chloroform and methanol extracts of *E. cannabinum*, subfractions of the chloroform extract and the isolated compound (eucannabinolide) were determined using the MTT assay. The results are summarized in Table 1 and Figure 2. The cytotoxic activity of the chloroform extract was more considerable than the *n*-hexane and methanol extracts and subfraction 4 (IC_{50} 7.86±1.34 µg/mL) demonstrated considerable cytotoxic activity.

The genus *Eupatorium* has been used for various therapeutic purposes [11].

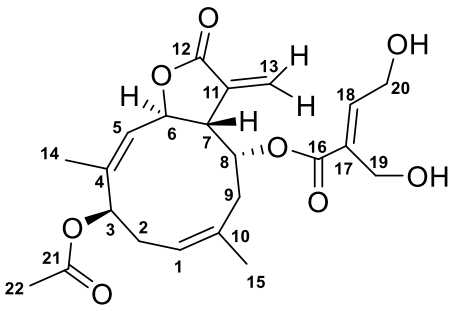

Figure 1. Eucannabinolide structure

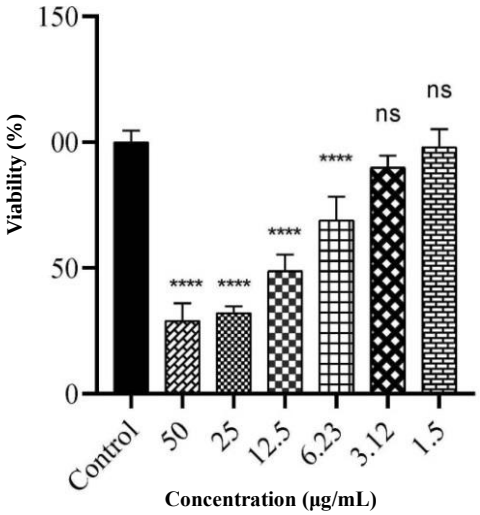

Figure 2. Effect of eucannabinolide on MCF-7 cell line; Experiments were performed in triplicate. Significant difference with control group: ****p< 0.0001; ns: not significant p> 0.05

Table 1. Cytotoxicity of *Eupatorium cannabinum* extracts, chloroform subfractions and eucannabinolide against MCF-7 cell line

Samples	**MCF-7** (IC_{50}*, µg/mL)
n-Hexane extract	60.23 ± 2.16
Chloroform extract	21.39 ± 3.24
Methanol extract	81.74 ± 3.41
Subfraction 1	60.83 ± 2.56
Subfraction 2	58.93± 2.73
Subfraction 3	37.50± 3.65
Subfraction 4	7.86 ± 1.34
Subfraction 5	10.61 ± 2.34
Subfraction 6	13.77 ± 4.17
Eucannabinolide	13.00 ± 2.45
Tamoxifen (positive control)	5.30 ±1.78

*average of three tests

Considering that isolation of effective compounds from plants with cytotoxic activity can help in the discovery of anti-cancer drugs, in the present study, the cytotoxic activity of *E. cannabinum* against MCF-7 breast cancer cell line was evaluated. According to the literature, the cytotoxic properties of *Eupatorium* genus have been already evaluated in different studies. Li-Chai Chen and et al. isolated 8 compounds from root of *Eupatorium cannabinum* subsp. *asiaticum*. Among the isolates, 9-acetoxy-8,10-epoxythymol 3-O-tiglate was the most cytotoxic compound with IC_{50} values of 0.02±0.01, 1.02±0.07, and 1.36±0.12 µg/mL against DLD-1, CCRF-CEM, and HL-60 cell lines, respectively. In addition, 10-acetoxy-9-O-angeloyl-8-hydroxythymol and eupatobenzofuran exhibited cytotoxicity, with IC_{50} values of 1.14±0.16 and 2.63±0.22 and 7.63± 0.94 and 2.31±0.14 µg/mL, against DLD-1 and CCRF-CEM cell lines respectively [24]. Solomon Habtemariam et al. investigated the cytotoxic activity of *Eupatorium perfoliatum* leaves. They found the IC_{50} values of 13±2 and 12±2 and 14±3 µg/mL against EAhy 926 and L929 and HeLa cell lines, respectively [25]. Also, Shahtaghi et al. studied the cytotoxicity of *E. cannabinum* methanol extract against MCF-7 breast cancer cell line. The extract demonstrated cytotoxic effects with IC_{50} value of 69.5 µg/mL [18], which is closely similar to the results obtained in this study. Ribeiro-Varandas et al. investigated cytotoxic activity of *E. cannabinum* aerial parts against colon cancer cell line (HT29). Their findings showed that severe loss of HT29 cell viability was detected for 50 µg/mL after 24 hours [26]. In another study, Grigore et al. evaluated antiproliferative effect of *E. cannabinum* extracts against BT-20, HepG2, Caco-2, and Jurkat cancer cell lines. Their findings showed that Jurkat cells were more sensitive to both chloroform and distilled water extracts (IC_{50} of 7.35±0.35 µg/mL for chloroform extract and IC_{50} of 13.77±2.16 µg/mL for distilled water extract) [27].

Table 2. NMR data of eucannabinolide

Position	**δ_H (*J* in Hz)**	**δ_C**	**COSY**	**HMBC**
1	5.10, t (7.9)	124.4	2	2, 9, 15
2	2.73, d (3.3) 2.43, dd (14.1, 3.3)	43.2	1, 3	1, 4, 6, 7, 8, 10, 15 1, 10, 15
3	5.30, m	74.5	2	21
4	-	136.2		6, 3
5	5.23, m	125.2		4, 6, 7, 14
6	5.61, dd (11.8, 5.1)	70.7	7	4,5,9, 12, 14
7	3.00, m	48.8	8, 6	-
8	5.28, m	79.8	7	7,10, 16
9	2.75, d(3.3) 2.10, m	30.7	-	1, 10
10	-	135.9	-	-
11	-	137.1	-	7, 13
12	-	170.0	-	-
13	6.39,d (2.2) 5.81, d(2.2)	125.2	-	7, 8, 11, 12 7,8, 11, 12
14	1.81, d (1.3)	18.1	-	4
15	1.92, s	18.7	-	10
16	-	165.9	-	-
17	-	131.5	-	-
18	6.95, t (5.8)	144.9	20	8, 16, 17, 19
19	4.38, s	57.1	-	16, 17, 18
20	4.45, d (5.9)	59.2	18	-
21	-	169.7	-	-
22	2.11, s	21.12b	-	-

Spectra recorded in CDCl3, J in Hz at 500 MHz; assignments aided by COSY, HSQC, and HMBC experiments

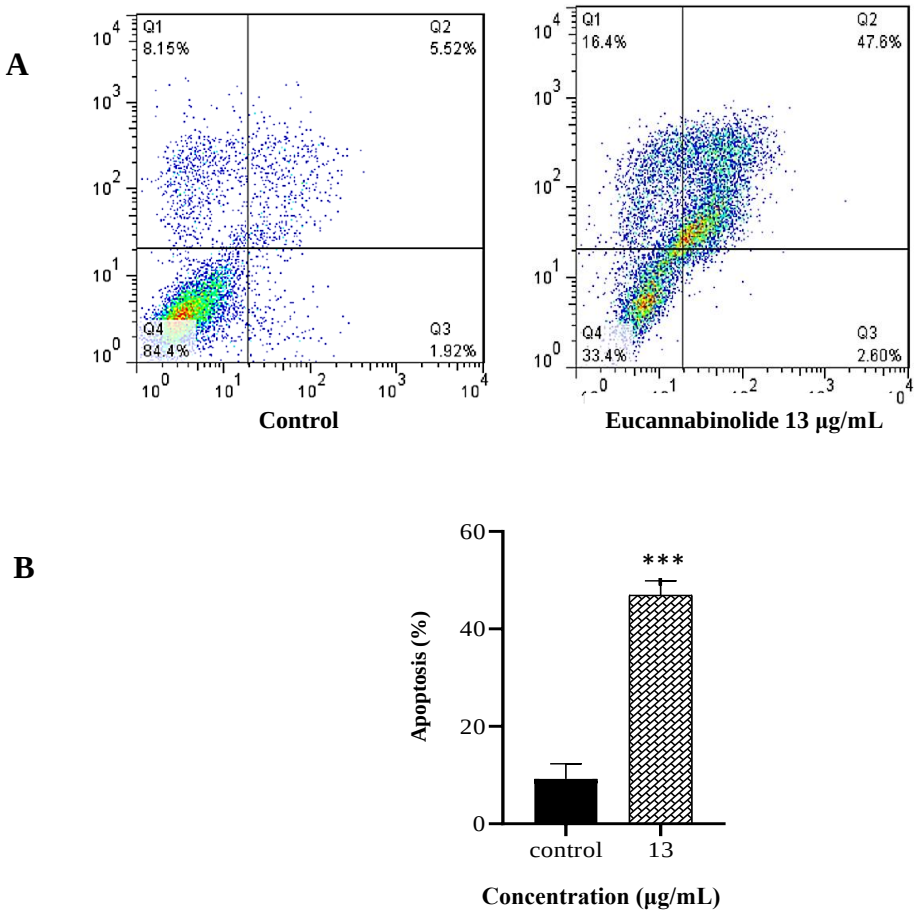

Figure 3. A) Flow cytometry results representing the apoptosis-inducing effect of eucannabinolide at 13 µg/mL on MCF-7 cell line. DMSO 1% was used as the negative control; B) comparison of apoptosis at 13 µg/mL concentration with negative control; Experiments were performed in triplicate; significant difference between eucannabinolide and control group: (***p<0.001).

In line with previous studies, this study confirmed the potential cytotoxicity of *E. cannabinum* extracts. Eucannabinolide, a germacronolide sesquiterpene lactone, was isolated previously from *E. formosanum* [28] and *E. glehnii* [29] and *E. cannabinum* [30]; however, in the present study we isolated eucannabinolide through a bioassay guided fractionation for cytotoxic activity against human breast cell line. As shown in Figure 3, eucannabinolide induced 46.91% apoptosis in MCF-7 cells at the concentration of 13µg/mL. Also, there was a significant difference between eucannabinolide and the control group in apoptosis induction which suggests that the compound is a promising agent for induction of apoptosis in cancer cells.

Conclusion

The bioassay guided fractionation of *E. cannabinum*, led to the isolation of eucannabinolide. Eucannabinolide showed cytotoxicity and ability to induce apoptosis in MCF-7 cells as confirmed by annexin V/PI assay. Considering the results of this study eucannabinolide is regarded as one of the constituents responsible for the cytotoxic properties of *E. cannabinum* and can be a promising candidate for developing and producing new anticancer treatments for human breast cancer studies.

Acknowledgments

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Author contributions

Mohammad Reza Shams Ardekani designed and supervised the study; Morteza Abuali performed

the experimental parts; Mahdi Vazirian was involved in data collection and analysis; Majid Balaei-Kahnamoei and Hassan Rezadoost were involved in structural elucidation; Maryam Hamzeloo-Moghadam supervised the cytotoxicity and apoptosis experiments. All authors approved the final version of the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; MCF-7: Michigan Cancer Foundation-7 (a breast cancer cell line); Annexin V/PI: Annexin V/Propidium Iodide; ELISA: enzyme-linked immunosorbent assay; DMSO: dimethyl sulfoxide; WHO: world health organization