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The effect of dichloromethane extract from *Humulus lupulus* L. on two human breast cancer cell lines

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Abstract

Breast cancer is known as the most frequently diagnosed cancer and second cause of cancer death in women. Nowadays standard treatments for cancer patients are surgery, radiation-, and chemotherapy which all have limited success. Statistics also indicate an urgent need to find more effective therapeutic methods for the patients. Today there is an increasing attention to botanical treatments worldwide. *Humulus lupulus L.* or Hops is a climber plant belonging to family cannabinaceae. It contains phytoestrogens with various therapeutic properties particularly in cancer research. There are controversial results regarding proliferative/anti-proliferative effects of phytoestrogens on the cancer cell lines. However, the effects of dichloromethane extract of Humulus lupulus on human breast cancer cell lines have never been reported. The aim of this study is to evaluate the effects of dichloromethane extract of Humulus lupulus on the two human breast cancer cell lines, MCF-7 and MDA-MB 231. Fresh hop extract was prepared by maceration method. MTT assay used to determine cell viability in two human breast cancer cell lines. The IC50 values of MCF-7 cells were 95.35±1.94, 19.58±0.21 and 19.10±0.76 µg/ml for 24, 48 and 72 h of incubation, respectively. MDA-MB 231 cells showed the IC50 values of 111.38± 2.56, 28.89± 1.08 and 19.70± 0.98 µg/ml for 24, 48 and 72 h, respectively. Our findings indicated that dichloromethane extract of hops has anti-proliferative effect on both MCF-7 and MDA-MB 231cells and its inhibitory effects are higher to MCF-7 cells than to MDA-MB-231 cells.

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Introduction

Breast cancer is the most common malignancy and second most lethal cancer among women worldwide that resulted from developed metastasis of primary stage in cell tumors (Hamedeyazdan *et al.* 2012; Ferlay *et al.* 2012; American Cancer Society 2014). It is estimated that about 2×10^5 new cases of invasive breast cancer and more than 6×10^4 new cases of carcinoma in situ (CIS), will be diagnosed in women in the United States in 2014 (American Cancer Society 2014). Today, there are many therapeutic strategies for breast cancer treatment, including surgery, chemotherapy, radiotherapy, hormonal therapy or targeted therapy which have been achieved limited success (Simstein *et al.* 2003; Bandala *et al.* 2013). Chemotherapy is the most common method used for breast cancer treatment. Nevertheless, it is not a selective and reliable method because it has detrimental effects on normal cells, particularly in combination with biologic and /or endocrine therapy (Lee & Nan 2012; Wang *et al.* 2013). In some patients who their breast tumor(s) overexpress estrogen receptor (estrogen receptor-positive breast tumor), hormonal therapy (HT) is used as a therapeutic strategy. Although selective estrogen receptor modulators (SERMs) such as tamoxifen and aromatase inhibitors are used for breast cancer hormonal therapy, resistance has been reduced efficacy of these agents (Thomas *et al.* 2013; Lage 2003). In recent years, there has been a tendency for the use of alternative medicine among cancer patients (Tsai *et al.* 2013). Despite applying hormonal therapy for alleviation of menopausal symptoms which seriously affect quality of life for women, many women avoid utilizing HT and have been turned to herbal medicine for the relief of menopausal symptoms, probably because they believe that plants are natural and safe (Overk *et al.* 2008; Hajirahimkhan *et al.* 2013). To date, many chemicals with estrogenic activity which occur in plants (phytochemicals) have been applied in treating menopausal symptoms and have potential therapeutic properties for prevention and treatment of many diseases such as breast cancer (Huang *et al.* 2013; Sunita & Pattanayak 2011). Phytoestrogens belong to a group of plant derivatives containing nonsteroidal and natural phenolic compounds which their main property is mimicking or modulating the function of the human endogenous estrogens (Oseni *et al.* 2008; Erkkola *et al.* 2010; Michel *et al.* 2013; Sunita & Pattanayak 2011). Biologic role of phytoestrogens is mainly due to their binding ability to estrogen receptors (ERα and ERβ) and this is a good reason why phytoestrogens have been proposed as natural SERMs (Michel *et al.* 2013; Oseni *et al.* 2008). Phytoestrogens are widely regarded as an alternative to traditional hormonal replacement therapy for alleviation of menopausal disorders (Sunita & Pattanayak 2011; Oseni *et al.* 2008). Although phytoestrogens have been shown to have controversial effects on hormone-dependent tumors, numerous recent studies have been focused on the potential roles of phytoestrogens on the prevention and treatment of hormone-dependent malignancies including breast cancer (Hajirahimkhan *et al.* 2013; Michel *et al.* 2013; Huang *et al.* 2013; Gaete *et al.* 2012). *Humulus lupulus L.* or Hops is a climber plant belonging to family cannabinaceae and contains phytoestrogens. The origin of Humulus lupulus L. is central Europe and this plant is widely cultivated in temperature regions of the world (Negri *et al.* 2010; Wang *et al.* 2008). Hops is commonly used as a flavoring ingredient in beer industry owing to its bitterness and aromatic properties (Negri *et al.* 2010; Chadwick *et al.* 2006; Hemachandra *et al.* 2012). Treatment of sleep disorders, restlessness, and nervousness are some medicinal applications of hops. Furthermore, alleviation of gynecological disorders with hop baths are highly recommended (Keiler *et al.* 2013; Hejazian *et al.* 2013; Chadwick *et al.* 2006). Recently, many *in vitro* and *in vivo* studies have been conducted to evaluate the cancer chemopreventive activities of Humulus lupulus (Chadwick *et al.* 2006; Hejazian *et al.* 2013; Negri *et al.* 2010). Due to the previous controversial effects of phytoestrogens on the hormone-dependent tumor cell lines we decided to evaluate the effect of dichloromethane extract of Humulus lupulus on the two human breast cancer cell lines, MCF-7 and MDA-MB 231.

Materials and methods

Chemicals

The human breast cancer cell lines including MCF-7 and MDA-MB-231 were obtained from National cell bank of Iran (Pasteur institute, Iran). Phenol red-free RPMI 1640 and fetal bovine serum (FBS) were purchased from GIBCOBRL, Invitrogen. 3(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), trypsin, streptomycin and penicillin G were all from Sigma Chemical Co (St. Louis, MO, USA). Hop flowers originated from Iran was purchased from a commercial source.

Cell culture and cell treatment

MCF-7 and MDA-MB-231 cells were grown in a monolayer culture in 25 cm^2 T flasks in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 µg/mL). Both cell lines were maintained in a humidified environment of 5% CO2 and 95% air at 37°C. Since normal FBS may contain estrogens, the cells were transferred to RPMI 1640 medium in which the FBS was treated with charcoal to remove the estrogens. Experiments were done in phenol red-free RPMI in order to eliminate the stimulatory effects of existing phenol red on the cells.

Plant extraction method

In this process, fresh hop extract was prepared by maceration method. Dry hop flowers were ground into powder using an electric coffee grinder. The resulting powder was subjected to serial extraction with n-hexane and dichloromethane at room temperature according to the Scheme 1. First of all, the extraction was performed using hexane (12 h). The mixture then was thoroughly air-dried and the procedure was followed by 12 h extraction with dichloromethane. Each two procedure was repeated three times. Finally, dichloromethane extract was filtered and solvent was evaporated under vacuum using a rotary evaporator at 45°C to obtain a dried powder and was kept at 4°C prior to the experiment. The dried and concentrated residue was used in experiment.

MTT assay

We evaluated cell viability using MTT assay. Both cell lines were detached with 0.05% trypsin/EDTA when they reached approximately 90% confluency. The cells were then seeded in 96-well microtiter plates at a density of 10×10^3 and 13×10^3 per well for MDA-MB-231 and MCF-7 cells, respectively. After 24 hours, the media were discarded and replaced with fresh phenol red-free media containing different concentrations of the dichloromethane extract of hops in triplicate for each concentration. (25, 50, 75, 100, 120, 140, 150 µg/ml). To prepare different concentrations of the extract, dichloromethane extract was weighted and dissolved in dimethyl sulfoxide (DMSO) to make a 2 mg/ml stock solution and final concentration of DMSO was 0.1%. This stock solution was then diluted with culture media to make a working solution. Control groups received the same amounts of DMSO (0.1%). In addition three wells remained as untreated controls. The plates were then incubated for 24, 48 and 72 h. Each time the media were discarded and were replaced with 150 µl fresh media and 50 µl MTT reagent (2 mg/mL in PBS). We applied cells without extract and the extract without cells as controls. The plates were then incubated for additional 4 h. Afterwards, MTT solution was replaced with 200 µl of DMSO and 25 µl of Sorenson's buffer (0.1M glycine, 0.1M NaCl optimized to pH: 10.5 with 1M NaOH). The plates were incubated for 15 min at 37°C while shaking. Ultimately, optical density was measured at 570 nm using a plate reader (Sunrise Tecan, Austria). The relative cell viability was calculated as (A_{570} of samples/A_{570} of controls) ×100. The whole experiment was done in triplicate.

Statistical analysis

Statistical comparisons were made by Student's *t* test. All statistical analyses were performed using SPSS software version 13.0. Data are shown as mean ± SD and $p < 0.05$ was considered statistically significant. All graphs were drawn applying Microsoft Excel version 2010.

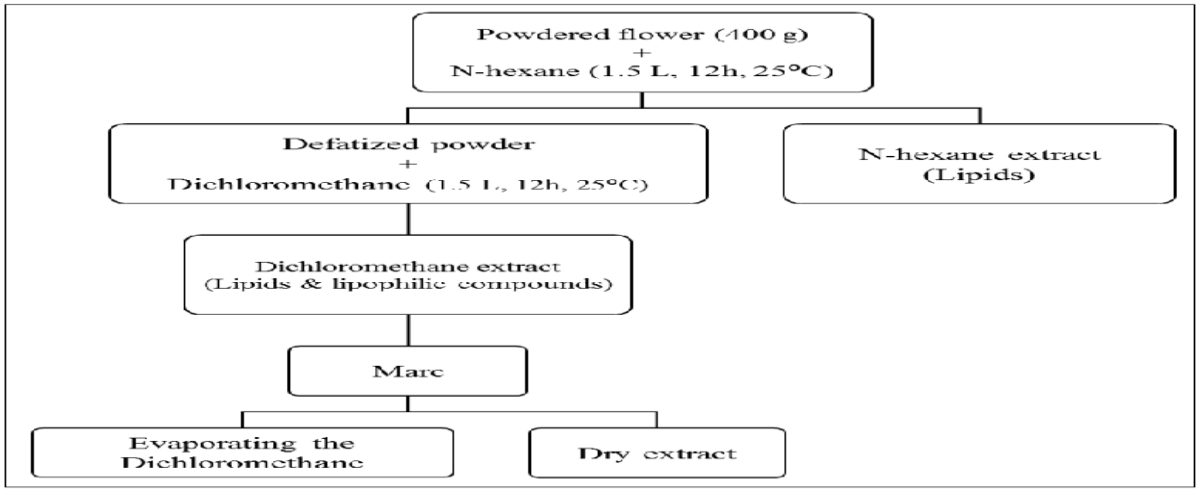
Results

In present study maceration method was used for

preparation of dichloromethane extract from hop flowers as shown in Scheme 1. The anti-proliferative activity of dichloromethane extract of hops on human breast cancer cell lines was evaluated by standard MTT test. Results of MTT assay were presented in Table 1 which were obtained from dose–response curves of each cell line. According to our results, dichloromethane extract of hops exhibited anti-proliferative activity towards MCF-7 and MDA-MB-231 cells in a time and dose-dependent manner (Figure 1 and 2). Cell proliferation was analyzed after 24, 48 and 72 hours. Based on this results, IC50 values of the extract on cell lines after 24 hours of incubation were 95.35±1.94 µg/ml for MCF-7 and 111.38±2.65 µg/ml for MDA-MB-231 cells ($p < 0.05$) (Figure 3). Additional incubation of the cells with the extract for 48 hours yielded IC50 values of 19.58±0.21 µg/ml and 28.89±1.08 µg/ml for MCF-7 and MDA-MB-231 cells, respectively that percentage of cell death was 79.46% for MCF-7 and 74.06% for MDA-MB-231 cells ($p < 0.05$) (Figure 4). The minimum difference in IC50 values were seen after 72 hours of incubation where the IC50 values were 19.10±0.76 µg/ml for MCF-7 and 19.70±0.98 µg/ml for MDA-MB-231 cell lines. (Figure 5).There was no significant difference in IC50 values after 72 hours of incubation ($p > 0.05$). Our data showed that dichloromethane extract of hops has anti-proliferative effects on these two human breast cancer cells and the cytotoxicity of dichloromethane extract of hops is higher to MCF-7 cells than to MDA-MB-231 cells ($P < 0.05$).

Table 1. IC50 values of hops dichloromethane extract at different times of incubation.

Incubation time	IC_{50} value (µg/ml) Mean ± SD	
	MCF-7	MDA-MB-231
24 h	95.35±1.94	111.38± 2.56
48 h	19.58±0.21	28.89± 1.08
72 h	19.10±0.76	19.70± 0.98



Scheme 1. Extraction protocol with different solvents.

Discussion

As a result of general descriptions of phytoestrogens, these phytochemicals may have therapeutic effects on estrogen-related conditions and diseases including menopausal symptoms, osteoporosis, cardiovascular diseases and prostate and breast cancers (Erkkola *et al.* 2010). Other tumor growth prevention activities of phytoestrogens are inhibition of angiogenesis, tyrosine kinase, topoisomerase II and cell proliferation. Flavonoids, lignans, coumestans and stilbenes are classified as main phytoestrogen groups which can be found in fruits, vegetables, grains and soybeans (Oseni *et al.* 2008). Phytoestrogenic compounds have different chemical structures and mechanisms of action (Hejazian *et al.* 2013; Sunita & Pattanayak 2011). Recently, much attention has been

given to biological properties and health benefits of hops such as potential cancer chemopreventive and anti-proliferative activities (Deeb *et al.* 2010; Chadwick *et al.* 2006). Xanthohumol has been introduced as an anti-cancer compound in hops with a broad-spectrum chemopreventive activities (Overk *et al.* 2008; Chadwick *et al.* 2006; Anioł *et al.* 2012).

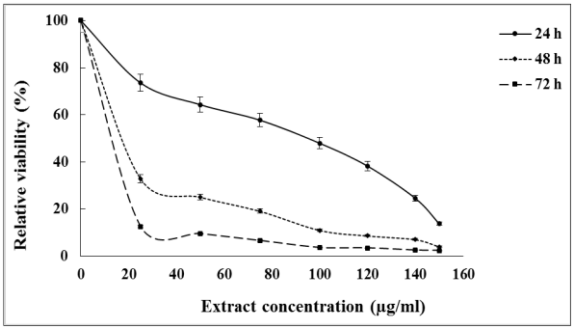


Fig. 1. IC50 values of the hops dichloromethane extract in MCF-7 cells at different times of incubation.

In recent years menopausal symptoms also have been treated with use of some prenylated flavonoids derived from hops and one of these compounds is 8-prenylnaringenin (8-PN) that has a strong estrogenic activity and is one of the most known potent phytoestrogens (Erkkola *et al.* 2010; Oseni *et al.* 2008). One study showed that 8-PN in hop extract is responsible for the inhibition of estrogen oxidative metabolism and estrogen-induced malignant transformation in the MCF-10A model of mammary carcinogenesis (Hemachandra *et al.* 2012). Results obtained from one randomized, double blind, placebo-controlled, cross-over study showed that hop extract standardized on 8-PN can reduces discomforts and complaints associated to the menopauses in post-menopausal women (Erkkola *et al.* 2010). Liu *et al* in their study demonstrated that methanol hop extract has estrogenic activity with binding capacity to both estrogen receptors α and β, the induction of alkaline phosphatase activity in Ishikawa cells (human endometrial adenocarcinoma epithelial cell line), the up-regulation of progesterone receptor mRNA in Ishikawa cells and up-regulation of presenelin-2, an estrogen-inducible gene in S30 cells (breast cancer cell line transfected with ER). Moreover, these results were confirmed by Overk *et al* (Chadwick *et al.* 2006). In a crossover pilot study, the effectiveness of hop extract was evaluated as a drug to relieve menopausal complaints and showed an improvement of menopause-related symptoms (Keiler *et al.* 2013). In spite of these results, there is a little evidence for effectiveness of hop extracts especially for in vitro cytotoxicity against different human cancer cell lines (Keiler *et al.* 2013). In the other hand, investigations have been shown that phytoestrogens act as agonist or antagonist of estrogen (Oseni *et al.* 2008; Michel *et al.* 2013). In other words, effect of phytoestrogens on breast cancer cells may be dose-dependent because findings demonstrate that phytoestrogens at low concentrations may stimulate growth, and at high concentrations inhibit cell growth (Oseni *et al.* 2008).

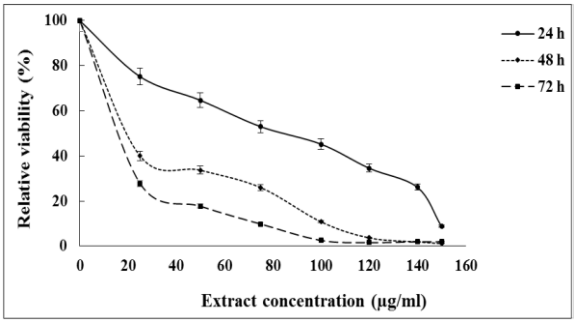


Fig. 2. IC50 values of the hops dichloromethane extract in MDA-MB-231 cells at different times of incubation.

In this study we investigated the effects of dichloromethane extract of Humulus lupulus l. flowers on two human breast cancer cells, MCF-7 and MDA-MB 231. The effect of the dichloromethane extract of hops was assessed through MTT colorimetric assay. MTT assay is an appropriate method for fast evaluation of new material for cytotoxicity on cancer cells. In this test cell viability is evaluated through reduction of tetrazolium salts to formazan by mitochondrial enzymes where MTT is reduced to the water insoluble purple formazan (Jafari *et al.* 2013; Hamzeloomoghadam *et al.* 2013). Since in previous studies various prenylated chalcones and flavanones have been isolated from hops and reported to have anti-proliferative activity against the human cancer cell lines (Overk *et al.* 2008; Anioł *et al.* 2012), our results confirm that anti-proliferative effects of dichloromethane extract of hops on MCF-7 and MDA-MB 231 cell lines could be

due to the existence of these compounds in this extract.

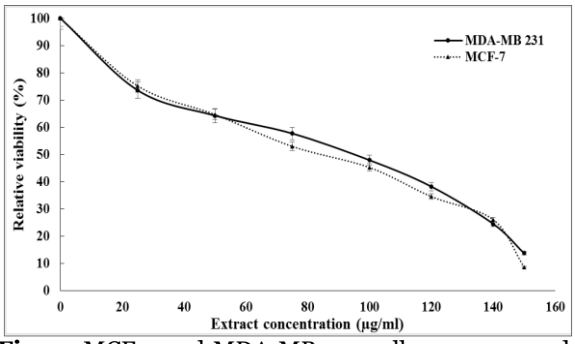


Fig. 3. MCF-7 and MDA-MB-231 cells were exposed to different concentrations of hops dichloromethane extract. The IC50 values after 24 h of incubation were 95.35±1.94 and 111.38± 2.56 µg/ml in MCF-7 and MDA-MB-231 cell lines, respectively ($p < 0.05$).

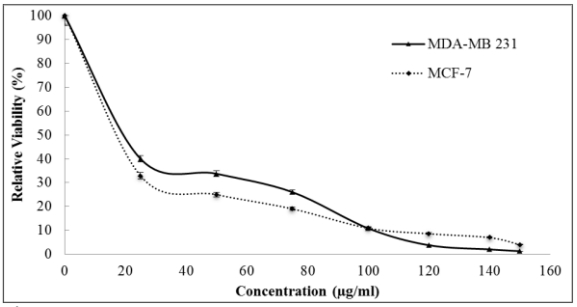


Fig. 4. MCF-7 and MDA-MB-231 cells were exposed to different concentrations of hops dichloromethane extract. The IC50 values after 48 h of incubation were 19.58±0.21 and 28.89± 1.08 µg/ml in MCF-7 and MDA-MB-231 cell lines, respectively ($p < 0.05$).

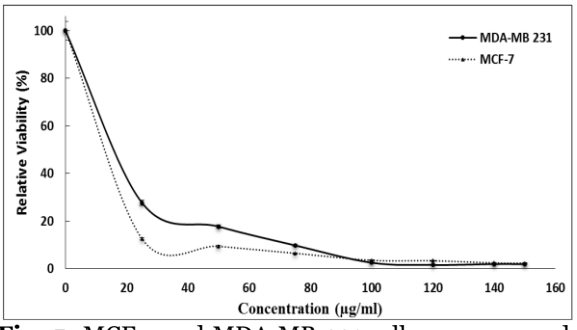


Fig. 5. MCF-7 and MDA-MB-231 cells were exposed to different concentrations of hops dichloromethane extract. The IC50 values after 72 h of incubation were 19.10±0.76 and 19.70± 0.98 µg/ml in MCF-7 and MDA-MB-231 cell lines, respectively ($p > 0.05$).

Conclusion

Our findings indicated that dichloromethane extract of hops has anti-proliferative effects on MCF-7 and MDA-MB 231cells and its inhibitory effects are higher to MCF-7 cells than to MDA-MB-231 cells. It should be noted that, since we used the whole extract of the hop flowers, these cytotoxic effects on tumor cells may be associated with other compounds in the extract. Therefore, performing further research is required to confirm the specific bioactive compounds responsible for the anticancer activity of the extract and the mechanism through which they eliminate cancerous cells. Moreover, we suggest further studies to evaluate the effects of hops dichloromethane extract on the other cancer cell lines in order to accumulate more evidence for therapeutic capacity of hops and other medicinal plants in treatment of different cancers as an adjuvant treatment.

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Conflict of Interest

The authors sincerely declare that they have no conflicts of interest.

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