Original Article

# Growth inhibition of human breast cancer cells and down-regulation of ODC1 and ADA genes by *Nepeta binaloudensis*

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**A B S T R A C T**

*Nepeta binaloudensis* Jamzad, Lamiaceae, is a rare medicinal plant endemic to Iran. In spite of many studies about the chemical constituents and antibacterial effects of this species, no report has been provided about its cytotoxic and anticancer activities. In this study we have evaluated the effects of EtOH 70%, hexane and aqueous extracts of *N. binaloudensis* on the cell proliferation and *n*-hexane extract on the expression of adenosine deaminase and ornithine decarboxylase 1 genes in breast cancer cell lines (MCF-7, MDA-MB-231) compared to non-cancer line (MCF-10A). The cell lines were subjected to increasing doses of the extracts ranging from 10 to 320 µg/ml. Cell viability was quantified by MTS assay. Expression of adenosine deaminase and ornithine decarboxylase 1 genes was analyzed by real time PCR. *N. binaloudensis* inhibited the growth of malignant cells in a time and dose-dependent manner. Among extracts of *N. binaloudensis*, the hexane extract was found to be more toxic compared to other extracts. Results showed a marked decrease in the expression of ornithine decarboxylase 1 and adenosine deaminase genes in cancer cell lines. At 60 µg/ml concentration of *N. binaloudensis* hexane extract ornithine decarboxylase 1 and adenosine deaminase mRNA expression were reduced 4.9 fold and 3.5 fold in MCF-7 cell line and 3.6 fold and 2.6 fold in MDA-MB-231 cell line compared to control, respectively. The result of our study highlights the potential influences of *N. binaloudensis* hexane extract on ornithine decarboxylase 1 and adenosine deaminase genes expression in breast cancer cells and its relation to inhibition of cancer cell growth.

## Introduction

Cancer is the most common cause of death in the world. The majority of deaths from cancer occur in women with breast cancer. Statistics indicate that in 2014 about 29% of cancers were breast cancers among American women (Siegel et al., 2014). In Iran also breast cancer rates have increased in females (Mousavi et al., 2009). Many investigations are performed to detect the genes which are involved in breast cancer. One of the most important genes is ornithine decarboxylase (ODC) which encodes a key enzyme in polyamines synthesis (Zhu et al., 2012; Xu et al., 2015). Several studies indicate that there are close correlation between ODC activity in the cells and regulation of cell proliferation (Deng et al., 2008; Elmets and Athar, 2010; Hayes et al., 2011). Consequently, any changes in ODC activity and polyamines level have effects on cell differentiation and proliferation. In breast cancer cells, estrogens induce ODC activity. So, the level of ODC and estrogen is increased in these cells (Zhu et al., 2012). In addition, Adenosine Deaminase gene (ADA) encodes the enzyme that catalyzes hydrolyzation of adenosine to inosin and participates in purines metabolism, which has a crucial role in development of immune system and maturation of mammalian cells (Ri et al., 2010; Roberts and Roberts, 2012). Recent studies suggest that the level of ADA enzyme is increased in breast cancer cells in contrast with normal cells (Aghaei et al., 2010; Mahajan et al., 2013). Therefore, decreasing of ODC and ADA gene expression and respective enzyme activities can be a good strategy for breast cancer therapy.

Using medicinal plant extracts as anticancer drugs is a more effective way for cancer therapy. About 50% of prescribed drugs in the world originate from plants (Van Slambrouck et al., 2007). *Nepeta binaloudensis* Jamzad, Lamiaceae, is an endemic and rare medicinal plant which grows exclusively in the Binalud Mountains in Khorasan Razavi province in Northeast of Iran (Tundis et al., 2013). This plant has been applied widely in traditional medicine for the treatment of respiratory disease, digestive problems and osteoarthritis (Koocheki and Rezvani Moghaddam, 2012). Previous chemical investigation of *N. binaloudensis* has shown the presence of nepetalactone and 1,8 cineole (Rustaiyan and Nadji, 1999). Some

studies were done about antibacterial activities of *N. binaloudensis* but its cytotoxicity and anticancer effects have not been studied yet. Therefore, the purpose of the study was to determine the anticancer effects of *N. binaloudensis* extract on two breast cancer cell lines (MCF-7, MDA-MB-231) and normal breast cell line (MCF-10A). Also, the present study is focused on evaluating the level of ODC1 and ADA gene expression in the normal and cancerous breast cells.

## Material and methods

### Plant collection and extraction

Shoots of *Nepeta binaloudensis* Jamzad, Lamiaceae, were collected from Binalud Mountains in Neyshabur, Khorasan Razavi province, northeast of Iran. The plant was identified by Mr. M.R. Joharchi, from Ferdowsi University of Mashhad Herbarium (FUMH). Voucher specimen (No. 1025) was deposited with herbarium of Islamic Azad University, Neyshabur Branch. The extraction process was performed from dried shoots. The dried shoots (100 g) were percolated with 100 ml of each solvent (EtOH 70%, hexane and $H_2O$) for 4 h separately. The extracts were filtered and the solvents were evaporated under vacuum at 45 °C to afford crude extracts. Dry extracts were dissolved in dimethyl sulfoxide (DMSO) and then subjected to cytotoxic and gene expression assays.

### Cell lines and culture medium

Two human breast cancer cell lines (MCF-7, MDA-MB-231) and one normal breast cell line (MCF-10A) were obtained from cell culture laboratory of the Faculty of Sciences, Ferdowsi University (Mashhad, Iran) and Pasteur Institute (Tehran, Iran). The cells were cultured in 25 cm$^2$ flasks with RPMI-1640 (RPMI Sigma) and supplemented with 10% fetal bovine serum (FBS, Gibco) and 100 U/ml penicillin and 100 μg/ml streptomycin solutions, at 37 °C with 5% $CO_2$ and 95% humidity. The medium was exchanged every day and the cells were passaged every 3–4 days.

### Cytotoxicity assay

The cytotoxic activity of the extracts was determined using MTT assay (2, 3, 5 diphenil tetrazolium bromide). Cells were plated with density of $3 \times 10^4$ cells/dish in 96 well plates and were incubated for 24 h at 37 °C. Then, the cells were treated with different concentrations of extracts (0, 10, 20, 40, 80, 160 and 320 μg/ml) and were incubated for 24, 48 and 72 h. A 2 mM MTT solution was added to each well and plate was incubated for 4 h. The medium was then discarded and 100 ml DMSO (dimethyl sulfoxide) was added and the plate was shaken for 10 min. Finally, optical density was determined in 540 nm using ELISA microplate reader (Awareness, Palm City, FL, USA). Each experiment was performed in triplicate. Results are expressed as the percentage growth inhibition with respect to the untreated cells.

### Colony formation assay

The effect of hexane extract on MCF7 and MDA-MB-231 cells was investigated by colony formation assay (Franken et al., 2006). Briefly, the cells (500 cells/ml) were allowed to grow in 60 mm Petri dishes for 12 h. Subsequently, the cells were treated for 48 h with hexane extract (10, 30 and 60 μg/ml), or 0.1% DMSO. The colonies were fixed and stained with 0.2% crystal violet and counted under stereomicroscope.

### Real-time PCR

The expression levels of ODC1 and ADA genes, key genes in breast cancer cells, were analyzed using Real time PCR assay. The cells were cultured in three groups and treated with increasing concentrations (0–60 μg/ml) of the hexane extract dissolved in DMSO for 48 h. After 48 h total cellular RNA was extracted from the treated and untreated (control) cells using Easy BLUE total RNA extraction kit (iNtRON). The treatments were repeated for three times. The quality of RNA was determined by absorption at 260 nm using T80 UV-Visible Spectrophotometer (PG Instruments). For each specimen 2 μg of RNA was reverse transcribed into cDNA with reverse transcription reaction mixture of Power cDNA Synthesis Kit (iNtRON). Primers of ODC1, ADA and βACT were designed using Gene Runner software (version 4).

Primer sequences for ODC1 were: ODC1-FW: 5′-GTGGGTGATTGGATGCTCTTTG-3′ ODC1-RV: 5′-ACCAGGCTAACTACTCGCTCAA-3′.

Primer sequences for ADA were: ADA-FW: 5′-ACCAGGCTAACTACTCGCTCAA-3′ ADA-RV: 5′-TCAGTAAAGCCCATGTCCCGTT-3′.

Primer sequences for βACT were: ACT-FW: 5′-TCCATCATGAAGTGTGACGT-3′ ACT-RV: 5′-GAGCAATGATCTTGATCTTCAT-3′.

Real-time PCR was carried out using SYBR Green method on an ABI Step-One instrument (Applied Biosystems, USA). The cDNA was taken as a template for PCR amplification of ADA and ODC1 genes and βACT gene (control gene). Each reaction was repeated for three times. A PCR reaction mixture of 20 μl contained 10 μl SYBR Green master mix, 0.4 μl Reverse primer and 0.4 μl Forward primer, 6.2 μl $dH_2O$ (RNase free) and 3 μl cDNA (4 ng). Three pairs of primers were used separately. The thermal cycling conditions were as follow: 1 denaturation cycle of 95 °C for 10 min and 40 cycles of 95 °C for 15 s, 60 °C for 60 s (annealing and extension temperature). A negative control was used in each run to assess specificity of primers and possible contamination. The realtime PCR data were analyzed using the relative gene expression ($\Delta\Delta$Ct) method, as described in Applied Biosystems User Bulletin No. 2 (Livak and Schmittgen, 2001). Briefly, the data are presented as the fold change in gene expression normalized to the reference gene (β-actin) and were determined using the equation fold change = $2^{-\Delta\Delta Ct}$.

### Statistical analysis

One-way analysis of variance and Fisher's LSD were used for data analysis. All results were expressed as mean ±SEM, and *p* values lower than 0.05 were judged as significant.

## Results

### Cytotoxicity of various extracts of Nepeta binaloudensis

The cytotoxic activity of *N. binaloudensis* extracts were studied against cultured MCF-10A (Normal), MBA-MD-231 and MCF-7 (cancer) breast cell lines using MTT assay. The cell lines were subjected to increasing doses of EtOH 70%, hexane and $H_2O$ extracts ranging from 10 to 320 μg/ml for 24, 48 and 72 h. The results showed EtOH 70% and hexane extracts decreased viability of malignant cells in a concentration and time-dependent manner. Whereas $H_2O$ extract showed no marked cytotoxicity on the cell lines tested. EtOH and hexane extracts in the same concentrations caused less toxicity to MCF-10A than tumor cells indicating a degree of specificity for malignant cell lines (Fig. 1). Concentrations inducing 50% cell growth inhibition ($IC_{50}$) against MBA-MD-231 and MCF-7 cells are presented in Table 1. MCF-7 cells were found to be more
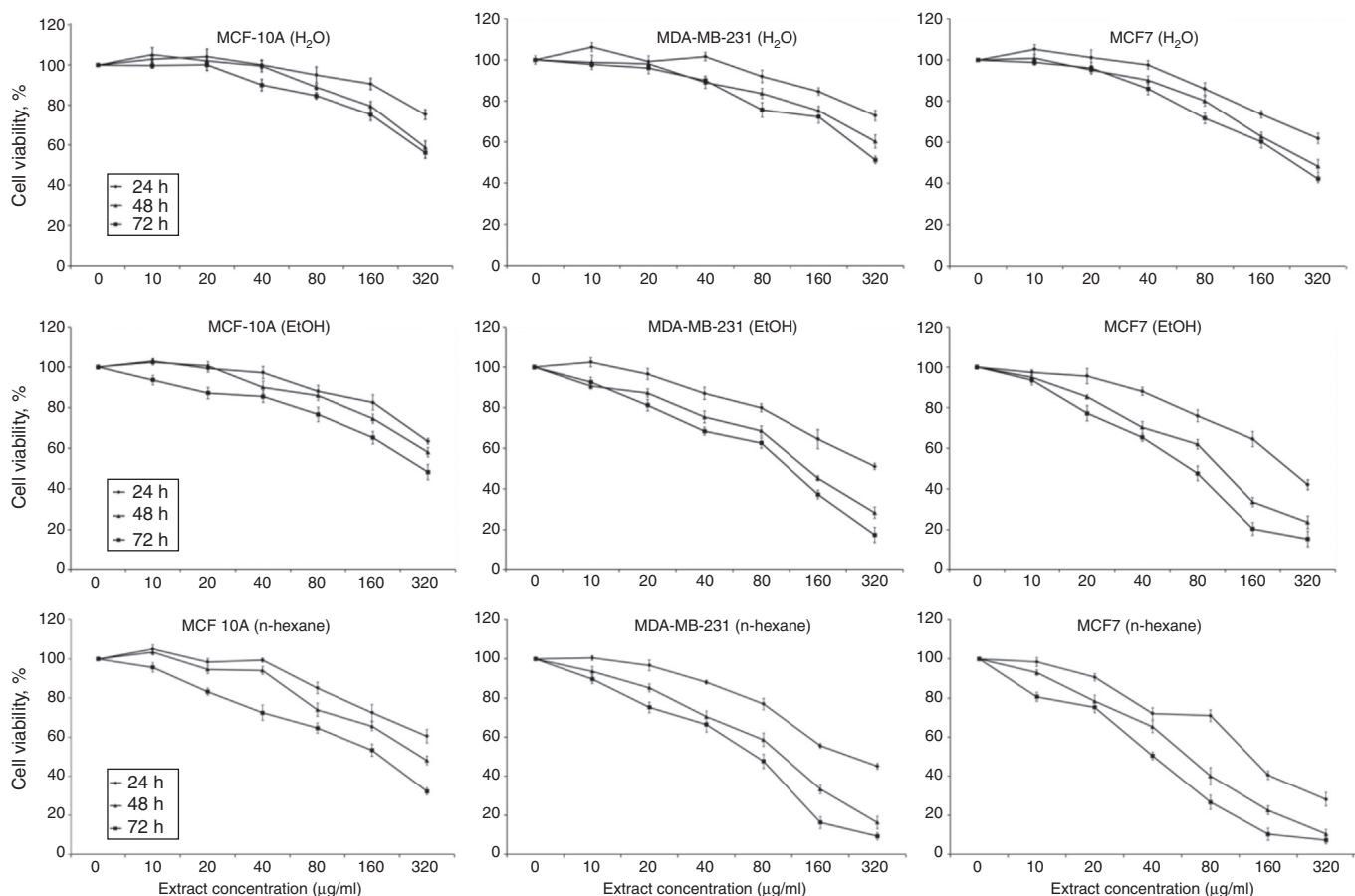
**Fig. 1.** Cytotoxic effects of *Nepeta binaloudensis* various extracts on MCF-10A (Normal), MBA-MD-231 and MCF-7 (cancer) breast cell lines. Cells were treated for 24, 48 and 72 h with different concentrations of extracts. Cytotoxicity was determined by MTT assay. Results are the mean ± SEM of three independent experiments.

**Table 1**
IC$_{50}$ values (µg/ml) for different extracts of *Nepeta binaloudensis* in cell lines.

| Plant extract | Cell line | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | MBA-MD-231 | | | MCF-7 | | | MCF-10A | | |
| | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| EtOH 70% | >300 | 138.4 | 97.42 | 249.5 | 101.9 | 65.96 | >300 | >300 | >300 |
| Hexane | 234.17 | 92.18 | 61.29 | 132 | 60.89 | 39.24 | >300 | >300 | 147.8 |
| H$_2$O | >300 | >300 | >300 | >300 | 281.7 | 229.5 | >300 | >300 | >300 |

sensitive to the cytotoxic effects of the extracts compared to MBA-MD-231cells.

Fig. 2 shows the photomicrographic images of MCF-7 and MBA-MD-231cell lines treated by hexane extract of *N. binaloudensis*. Cytotoxicity effects were coupled with morphological changes including decrease in cell volume and shrinking of the cells.

Paclitaxel (700 nM) was used as a positive control. Paclitaxel at this concentration decreased the viability of MCF-7 and MBA-MD-231 cells to 6.3% ± 0.6 and 12.2 ± 0.3 in comparison with untreated control received an equal volume of the solvent respectively (data not shown).

In vitro *clonogenic survival assay*

As given in Fig. 3 there is a significant dose dependent inhibition in colony formation in MCF-7 and MBA-MD-231 after hexane extract treatments. The results were showed a decrease in colony formation by 30–50% in MBA-MD-231 while a 45–70% decrease

was observed in MCF-7 when treated with 30 µg/ml and 60 µg/ml *n*-hexane extract respectively.

*Changes in the expression of ODC1 and ADA*

Our results showed that exposure of cells to increasing concentrations of *N. binaloudensis* hexane extract for 48 h caused significant decrease in the mRNA levels of ODC1 and ADA in cancer cell lines but not non-cancer cells. The effect of *N. binaloudensis* on selected genes expression was shown to be concentration-dependent. *N. binaloudensis* hexane extract at concentrations of 10, 30 and 60 µg/ml significantly decreased the ODC1 expression by 1.4, 2.3 and 4.97 fold, respectively in MCF-7 cell line. Moreover, this extract significantly reduced mRNA levels of ODC1 in MDA-MB-231 cell line by 1.2, 1.7 and 3.6 fold with 10, 30 and 60 µg/ml, respectively (Fig. 4).

Similar to ODC1, *N. binaloudensis* extract significantly decreased the expression of ADA at all the concentrations. In MCF-7 cell line *N. binaloudensis* extract decreased the expression of ADA in a
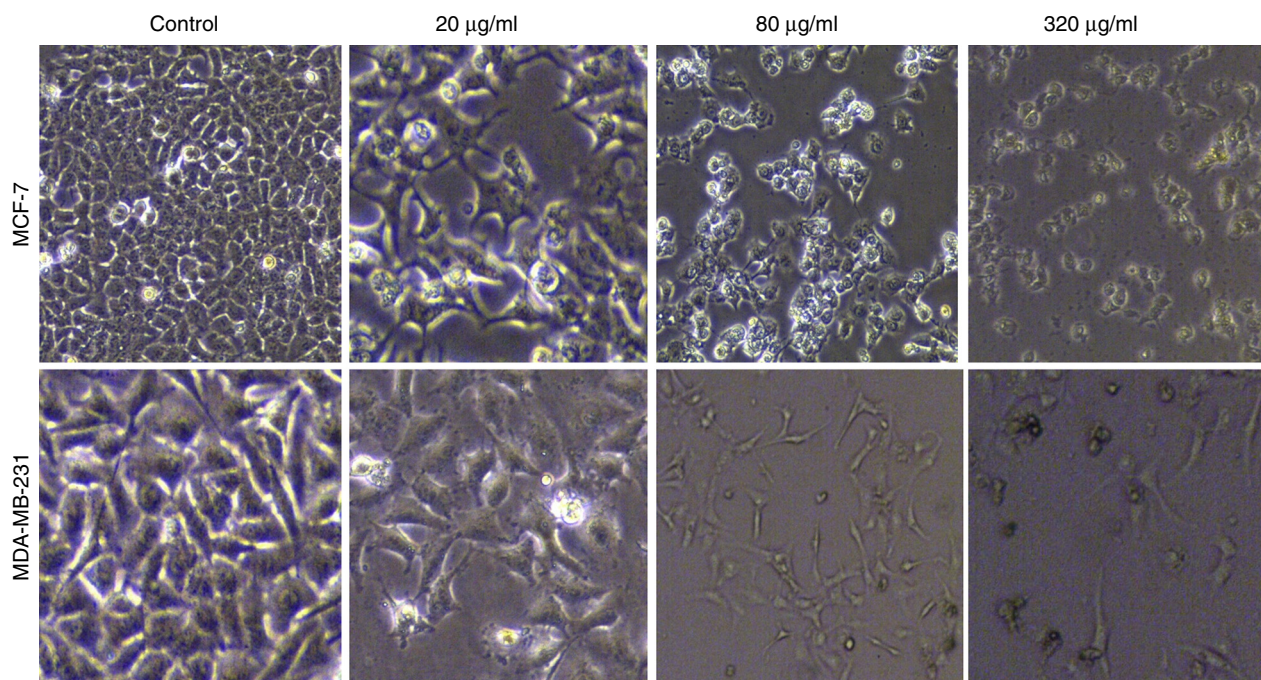
**Fig. 2.** Morphological changes of MCF-7 and MDA-MB-231 cells treated with different concentrations of the hexane extract of *Nepeta binaloudensis* for 48 h viewed under inverted phase-contrast microscope (200× magnification). Reduce in cell population was noted with the increase in the concentration of the treatment as compared to the control (untreated cells).
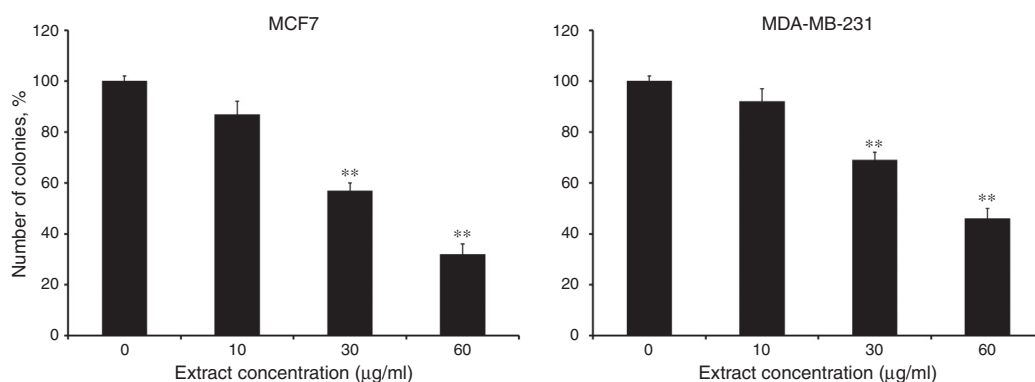


**Fig. 3.** Colony formation assay shows a significant reduction in colony formation in MCF-7 and MDA-MB-231 cell lines treated with different concentration of hexane extract of *Nepeta binaloudensis*. The results were presented as mean ± SEM, $n = 3$ (**$p < 0.01$).

dose-dependent manner by 1.32, 1.83 and 3.49 fold with 10, 30 and 60 μg/ml, respectively. As shown in Fig. 5, at 10, 30 and 60 μg/ml concentration of *N. binaloudensis* extract ADA mRNA expression was reduced 1.21, 1.56 and 2.56 fold in MDA-MB-231 cell line, respectively (Fig. 5). In cancer cell lines, mRNA expression of MCF-7 was reduced more than that of MDA-MB-231.

## Discussion

Several studies suggest an increased polyamine concentration in breast cancer tissue compared to normal breast tissue and polyamines biosynthesis inhibitors decrease the growth of cancerous tumors (Brown et al., 2009; Arisan et al., 2012; Zhu et al., 2012). Ornithine decarboxylase (ODC1), is a key enzyme in polyamine biosynthesis which decarboxylates Ornithine to putrescine (Wang and Jiang, 2014). Furthermore, adenosine deaminase (ADA) enzyme, which catalyzes the hydrolytic deamination of adenosine to inosine, plays an important role in purine metabolism and Adenosine homeostasis (Garcia-Gil et al., 2015). It has been proved to be a direct correlation between breast cancer and

increased ADA enzyme activity in cancer tissue of patients (Gocmen et al., 2009; Ri et al., 2010; Ogata et al., 2011). Therefore, ODC1 and ADA genes are the appropriate target in anticancer research.

According to previous studies and the importance of recognizing anti-cancer mechanisms of plant extracts, in present study the effects of various extracts of *N. binaloudensis* on the cell proliferation and the expression of ADA and ODC genes in breast cancer cell lines (MCF-7, MDA-MB-231) with respect to non-cancer lines (MCF-10A) were evaluated.

The results showed that after treatment of cells (at 24, 48 and 72 h) with extracts of hexane and EtOH 70%, excluding $H_2O$ extract, a linear relationship between the doses with the percentage of cell viability was observed. According to the results of this study hexane and EtOH extract of this plant has anti-cancer effects.

Different studies have shown the antiproliferative activity of *Nepeta* species including *N. ucrainica* (Khakdan and Rassam, 2014), *N. cataria* (Formisano et al., 2011) and *N. glomerata* (Rigano et al., 2011). Chemical composition analyses of *N. binaloudensis* oil revealed that 1,8-cineol (68.31%), α-terpineol (5.24%), β-pinene (4.74%), δ-terpineol (2.57%), α-pinene (1.54%) were main
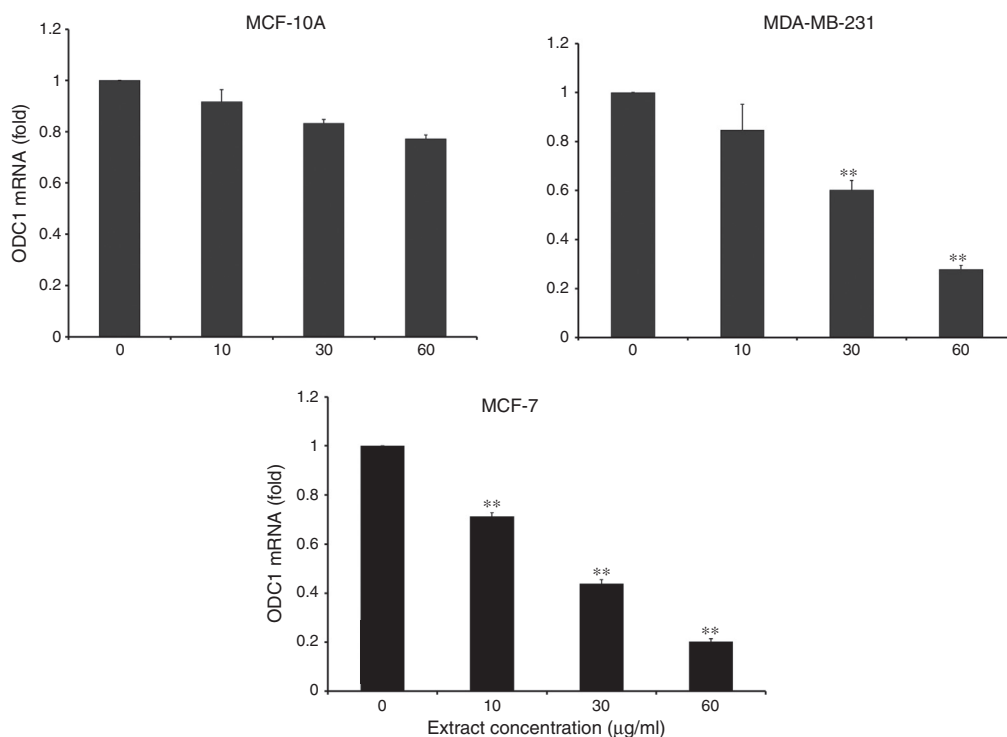
**Fig. 4.** Quantitative real-time PCR analysis of ODC1 mRNA expression in one normal breast cell line (MCF-10A) and two cancerous breast cell lines (MDA-MB-231 and MCF-7). The cells were incubated with the indicated concentrations of the hexane extract of *Nepeta binaloudensis* for 48 h. ODC1 mRNA levels decrease in cancer cell lines in dose-dependent manner. Relative abundance of mRNA is obtained by normalization to β-actin expression. Results are the mean ± SEM of three independent experiments. **$p < 0.01$ compared to control.
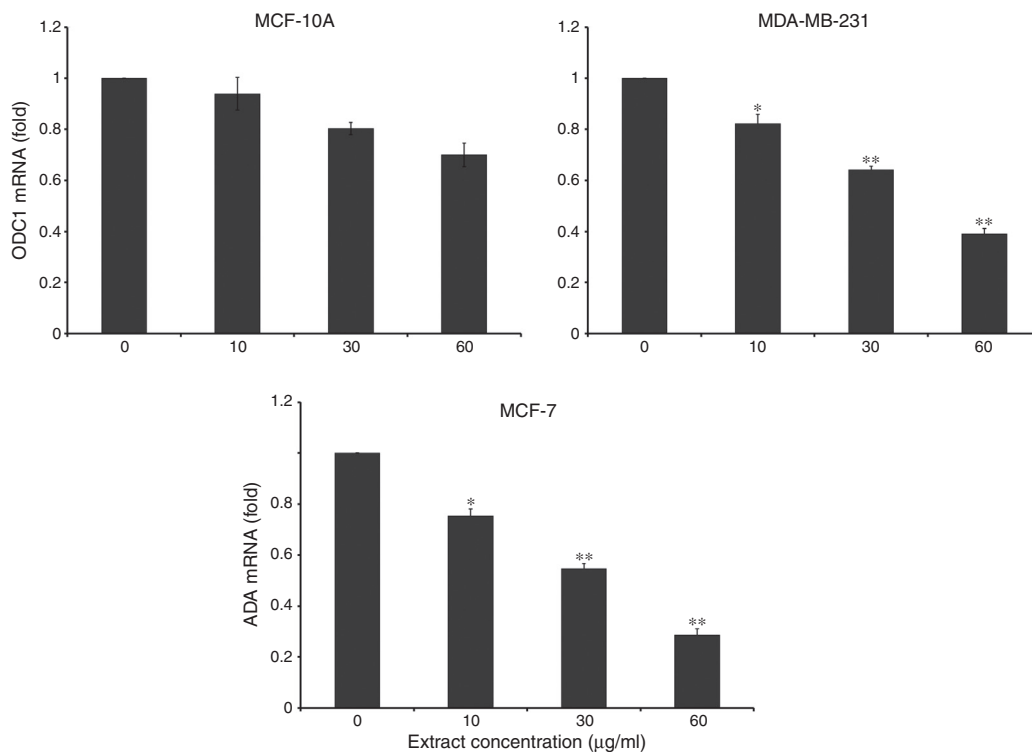


**Fig. 5.** Quantitative real-time PCR analysis of ADA mRNA expression in one normal breast cell line (MCF-10A) and two cancerous breast cell lines (MDA-MB-231and MCF-7). The cells were incubated with the indicated concentrations of the hexane extract of *Nepeta binaloudensis* for 48 h. ADA mRNA levels decrease in cancer cell lines in dose-dependent manner. Relative abundance of mRNA is obtained by normalization to β-actin expression. Results are the mean ± SEM of three independent experiments. *$p < 0.05$ and **$p < 0.01$ compared to control.

components (Mohammadpour et al., 2013). The cytotoxic effects of these monoterpenes have been previously shown against different cell lines. 1,8-cineole, the main compound present in the *N. binaloudensis* oil, has been reported to inhibit the growth of liver-derived (HepG2) and extrahepatic (A549) cell lines (Rodenak Kladniew et al., 2014). The results of another study indicated 1,8-cineole suppressed human colorectal cancer proliferation by inducing apoptosis (Murata et al., 2013). Alpha terpineol another key component of the oil, has also been reported to inhibits the growth of tumor cells through a mechanism that involves inhibition of the NF-κB pathway (Hassan et al., 2010). δ-Terpineol inhibits cell growth and induces apoptosis in human liver cancer BEL-7402 cells (Wu et al., 2014). Anti-tumor effect of α-pinene on human hepatoma cell lines through inducing G2/M cell cycle arrest has been reported (Chen et al., 2015). It was demonstrated that α-pinene inhibited BEL-7402 cells by arresting cell growth in the G2/M phase of the cell cycle, down regulating Cdc25C mRNA and protein expression, and reducing cycle dependence on kinase 1(CDK1) activity (Chen et al., 2014).

Among the extracts, hexane extract exhibited the largest cytotoxic effects on cancer cells. This can be described by the low polarity of hexane, which extract low-polar compounds that are either favorably absorbed through the cell or have cytotoxic activity. Therefore, it is assumed that most of the cytotoxic compounds were concentrated in the hexane extract (Tayarani-Najaran et al., 2013).

The results of our study revealed that the cytotoxicity of the hexane extract of *N. binaloudensis* on the estrogen receptor-positive cell line (MCF7) (ER+/PR+/HER2−) was stronger than that of ER/PR/HER2 triple negative breast cancer cell line (MDA-MB231). Such findings are similar to those found in the work of Srisawat et al. (2015) who found a higher apoptosis and growth inhibition rate in MCF-7 cells than in MDA-MB-231 cells. The higher cytotoxic effect of *N. binaloudensis* extract on MCF-7 was probably due to lack of estrogen stimulation in this experiment.

The decrease of the cells viability could be because of an increase of apoptosis and/or decreased cell reproduction (Islam et al., 2013). In our study, a colony formation assay was performed to evaluate the hexane extract capability of arresting proliferation of cancerous cells by lowering reproductive ability. Our results revealed that cytotoxic effects of hexane extract could be due to a decrease of cell proliferation.

According to the cytotoxicity results, hexane extract exhibited the largest cytotoxic effects on cancer cells. Therefore, in gene expression studies hexane extract was used in safe concentrations (0–60 μg/ml). Significant reduction in expression of ODC1 gene was observed in breast cancer cell lines (MCF-7 and MDA-MB-231) after 48 h treatment with *N. binaloudensis* hexane extract. Other studies have shown the inhibitory effects of plant materials on ODC activity and expression in cancerous cell lines and tissues. Liao et al. (2008) found that enzyme activity and protein expression of ODC were reduced in curcumin treated leukemia HL-60 cells. They suggested that curcumin-induced apoptosis occurs through a down-regulating mechanism of ODC. In another study, Hakimuddin et al. (2008) reported more than 3-fold reduction in ODC1 mRNA level in tumors of wine polyphenol-treated mice.

However, the mechanism by which *N. binaloudensis* extract reduces ODC1 gene expression is unknown. In the ODC1 promoter, there are multiple binding sites for Ap-1, Ap-2 and c-Myc (Liao et al., 2008). The mechanisms of the inhibition of ODC1 expression by *N. binaloudensis* extract likely involve transcriptional inhibition of these factors.

ODC enzyme activity is related to the production of polyamines such as spermine that function as a free radical scavenger (Smirnova et al., 2012). Inhibition of ODC gene expression by *N. binaloudensis* extract decreases the concentration of polyamines.

Thus, reduction of polyamines could increase intracellular ROS and cause apoptosis in cancer cells (Wang et al., 2011).

ADA gene expression decreased in the cancerous human breast cell lines treated by *N. binaloudensis* hexane extract. Many researchers reported that plant extracts have inhibitory effect on ADA expression and activity. For example, Durak et al. (2005) showed that black grape extracts significantly inhibited the ADA enzyme activity in cancerous colon tissues. In another study, it has been found that expression of the adenosine deaminase gene was consistently decreased by all the berries in intestinal adenoma tissue of rats (Mutanen et al., 2008). Tomato juice has been reported to causes significant inhibition on ADA activity in the prostate cancer tissues (Durak et al., 2003).

Our data demonstrate that decrease of adenosine deaminase expression abolishes advantage of cancer cells in obtaining more nucleotides in DNA synthesis and proliferation (Namuslu et al., 2014). Also, inhibition of ADA expression causes increased availability of adenosine which is a protective molecule in case of tumorous condition (Mahajan et al., 2013).

In conclusion, the study described here demonstrates that *N. binaloudensis* hexane extract has inhibitory effects on ODC1 and ADA gene expression in breast cancer cell line. Therefore, a possible mechanism for observed cytotoxicity effect of this plant extract could be expression inhibition of these genes. This is the first report about the cytotoxicity of *N. binaloudensis* hexane extract by inhibition of ODC1 and ADA in mRNA level, hence further studies will be necessary to supplement our findings.

## Ethical disclosures

**Protection of human and animal subjects.** The authors declare that no experiments were performed on humans or animals for this study.

**Confidentiality of data.** The authors declare that no patient data appear in this article.

**Right to privacy and informed consent.** The authors declare that no patient data appear in this article.

## Author contributions

ASA designed and supervised the experiments, analyzed the data and drafted the paper; FSN performed experiments, analyzed data and drafted the paper; MM performed experiments and analyzed data; AK designed and analyzed the data. All the authors have read the final manuscript and approved the submission.

## Conflicts of interest

The authors declare no conflicts of interest.

## Acknowledgment

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