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ORIGINAL ARTICLE / ÖZGÜN MAKALE

APOPTOTIC AND CYTOTOXIC EFFECT OF *ORIGANUM MINUTIFLORUM* ON NB2A NEUROBLASTOMA CELLS

ORIGANUM MINUTIFLORUM'UN NB2A NÖROBLASTOMA HÜCRELERİNE APOPTOTİK VE SİTOTOKSİK ETKİSİ

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ABSTRACT

Objective: *The aim of the present study is to investigate the cytotoxic and apoptotic effects of oregano oil from Origanum minutiflorum, which is an endemic medicinal plant in Turkey, on NB2a neuroblastoma cells.*

Material and Method: *Cell proliferation, apoptosis, and expression of inducible and endothelial nitric oxide synthase have been determined by MTT, TUNEL, and immunohistochemistry, respectively.*

Result and Discussion: *The cell viability was significantly decreased gradually in NB2a cells when the concentrations of both oregano oil and doxorubicin were increased. IC_{50} doses were found to be10.75 µl/ml for oregano oil and 5µM for doxorubicin. Furthermore, expression of inducible and endothelial nitric oxide*

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synthase, also the number of apoptotic cells was significantly enhanced in NB2a cells after treatment with oregano oil and doxorubicin. There was a remarkable increase in inducible and endothelial nitric oxide synthase expression levels and in the number of apoptotic cells by the dual application of these agents. In addition, oregano oil enhanced the apoptotic effect of doxorubicin. In conclusion, our results indicated that oregano oil of O. minutiflorum has a cytotoxic and apoptotic effect on NB2a cells and these effects may be related to its enhancing effects on inducible and endothelial nitric oxide synthase levels.

Keywords: *apoptosis, cytotoxicity, neuroblastoma, nitric oxide synthase, Origanum minutiflorum*

ÖZ

Amaç: *Bu çalışmanın amacı, Türkiye'de endemik bir tıbbi bitki olan Origanum minutiflorum'dan elde edien Oregano yağının NB2a nöroblastoma hücrelerine sitotoksik ve apoptotik etkilerini incelemektir.*

Gereç ve Yöntem: *Hücre proliferasyonu, apoptoz, İndüklenebilir ve endotelyal nitrik oksit sentazın ekspresyonu sırasıyla MTT, TUNEL ve immünohistokimya ile belirlendi.*

Sonuç ve Tartışma: *Hem Oregano yağı hem de doksorubisin derişimleri artırıldığında NB2a hücrelerinin canlılığı kademeli olarak azaldı. IC50 dozu Oregano yağı için 10.75 ul / ml doksorubisin için 5µM olarak belirlendi. Ayrıca, Oregano yağı ve doksorubisin ile muamele edildikten sonra NB2a hücrelerinde indüklenebilir ve endotelyal nitrik oksit sentazın ekspresyonu ve apoptotik hücre sayısı anlamlı olarak yükseldi. Bu ajanların ikili uygulaması ile indüklenebilir ve endotelyal nitrik oksit sentaz ekspresyon seviyelerinde ve apoptotik hücre sayısında dikkate değer bir artış olmuştur. Ek olarak, Oregano yağı, doksorubisinin apoptotik etkisini arttırmıştır. Sonuç olarak, bulgularımız O. minutiflorum'a ait Oregano yağının NB2a hücreleri üzerinde sitotoksik ve apoptotik bir etkiye sahip olduğuna ve bu etkilerin Oregano yağının indüklenebilir ve endotelyal nitrik oksit sentaz düzeylerini arttırıcı etkileri ile ilişkili olabileceğine işaret etmektedir.*

Anahtar Kelimeler: *apoptoz, sitotoksisite, nöroblastom, nitrik oksit sentaz, Origanum minutiflorum.*

INTRODUCTION

Origanum minutiflorum (O. Schwarz and P.H. Davis), is an endemic species growing in the eastern Mediterranean and southwestern Anatolia region in Turkey, especially in Isparta [1,2]. This species is mostly used as a spice, herbal tea, and also traditional medicinal herb for cold and stomach aches [3]. The herbal parts of *Origanum* species are rich in essential oils that have antioxidant, antibacterial, antiviral and antifungal activities. In this context, this genus has been gained a great deal of interest in the pharmaceutical and medicinal areas [4] and these plants are largely exported to Europa [5]. In recent years, a lot of studies have been done about the chemical composition and biological activity of the *O. minutiflorum* [6-8]. Previous studies have revealed that extracts of *O. minutiflorum* have many biological and pharmacological properties, such as analgesic [9], antifungal [10], antimicrobial [8], antioxidant [11] anticancer [12] and antidiabetes [13] properties. However, few studies have focused on the cytotoxic properties of *Origanum* species [14]. To our knowledge, there are no studies that evaluate the cytotoxic effects of the extracts from *O. minutiflorum* on neuroblastoma cells. Furthermore, there are no available reports on its effects on apoptosis, inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) levels.

Therefore, the main objectives of the present study are (i) to evaluate the cytotoxic activity of the extracts from *O. minutiflorum*in *in vitro* conditions (ii) to investigate the effect of its extracts on apoptosis, e-NOS and iNOS levels in neuroblastoma cells.

MATERIAL AND METHOD

Plant materials

O. minutiflorum plants used in this study were obtained from the Agriculture Faculty, Süleyman Demirel University, Isparta, Turkey. Dried oregano leaves (50 g) and tap water (250 ml were placed in the flask (500 ml) connected to the condenser of a Clevenger hydrodistillation apparatus according to the standard procedure described in the European Pharmacopoeia [15]. The extracted oil was dried over anhydrous sodium sulfate. The extraction yield was 2.50%± 0.09%. All kinds of essential oil were kept at refrigerator temperature (4°C) and stored in the dark when they were not in use. Before cell culture experiments, the essential oil was dissolved in 0.5% (v/v) dimethylsulfoxide (DMSO) (Sigma,USA), then filtered by sterile acrodisc filter (pore size: 0.2 µm), and further dilutions were made in 0.5% DMSO. The appropriate amount of DMSO (0.5 %) was added to DMEM in control and doxorubicin flasks.

The NB2a neuroblastoma cell line was provided from the European Collection of Cell Cultures (ECACC; cell line: 89121404). Cells were cultured in 5 % (v/v) horse serum, 5 % (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml gentamicin containing high glucose Dulbecco's modified Eagle medium (DMEM) at 37° and 5 % CO2 [16] . The culture media was changed once every two days.

Determination of IC_{50} dose

The IC50 doses of oregano oil and doxorubicin (KOCAK Farma) were defined by 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT, M5655, Sigma, Steinheim, Germany) assay. NB2a cells were seeded into 96-well (2.5×105 cells/well) plate for 24 h. Then, oregano oil (0, 0.1, 1, 10, 30, 100 µl/ml) and doxorubicin (0, 01, 1, 10, 30, 100 µM) were directly applied to the cells for 24 h. The media was removed and 100 µl of fresh media and 10 µl MTT (5 mg/ml in distilled water) was pipetted into all well and incubated for 4 h at 37°C. After incubation media with MTT was thrown away and 100 µl dimethyl sulphoxide (DMSO, A3672, AppliChem, Darmstadt, Germany) was pipetted into all well. The IC50 doses of agents were calculated by measuring the absorbance at 570 nm using a UV/Vis spectrophotometer multi-plate reader (ELx800UV, BioTek) [17]. Each experiment was performed at least three times.

Agents' treatments and immunocytochemistry

NB2a cells were passaged into 8-well plates at a density of 2.5×105 cells/per well and allowed to seed for 24 h. Cells were randomly allocated to four groups to treat with the oregano oil (IC50 dose, 10,75 µl/ml), doxorubicin (IC50 dose, 5 µM), a dual combination of these agents, and 0.5% DMSO

(Control). After 24 h of application with these agents, cells were fixed with 4 % paraformaldehyde in PBS at +4°C for 30 min. Following washing in PBS three times for 5 min, the permeabilization of cells was performed with 0.1 % Triton X-100 (A4975, AppliChem, Darmstadt, Germany) in PBS at +4° for 15 min. The 3 % hydrogen peroxide (1 08600, Merck, Darmstadt, Germany) was used to inhibit endogenous peroxidase activity. After washing in PBS, cells were incubated with primary antibodies: anti-endothelial nitric oxide synthase (eNOS, sc-654, Santa Cruz Biotechnology) and anti-inducible nitric oxide synthase (iNOS, GTX15322, Gene Tex) at +4°C overnight. Then, primary antibodies were then removed from the cells and washed with PBS.. The secondary antibodies, biotinylated secondary antibodies and peroxidase conjugated with streptavidin (Histostain kit, , Zymed, Carlsbad, USA), were applied to the cells. To make the immunoreactivities visible, the cells were incubated with diaminobenzidine/hydrogen peroxide (DAB, 00-2014, İInvitrogen, CA, USA). Cells were stained with Mayer's hematoxylin (800-729-8350, ScyTek, UT, USA) for counterstaining and were covered by mounting medium (DBS, Pleasanton, USA). The images of dyed cells were taken using a camera attached (SC50, Olympus, Germany) light microscope (IX71 inverted-florescence-phase microscope) (Olympus, Japan). Antibodies were not applied to the cells for staining control. Each experiment was performed at least three times [18].

Apoptosis assay

After the application of the agents, the terminal Transferase dUTP Nick End Labeling (Promega G7130) kit was used to detect the apoptotic cells. The cells were fixed in 4 % paraformaldehyde for 30 min and washed in three times in PBS for 5 min. Then they were incubated with 0.1 % Triton X-100 (A4975, AppliChem, Darmstadt, Germany) for 15 min and washed three times in PBS. Cells were treated with 3 % hydrogen peroxide to eradicate endogenous activity and rinsed in PBS. Following the equilibration buffer for 5 min, cells were incubated with Tdt-enzyme for 1 hours at 37°C and treated with 2×SCC solution for 15 min. Secondary antibodies were performed for 45 min. DAB and Mayer's hematoxylin stainings were done, and the cells were mounted using mounting medium. An experiment was performed in triplicate and staining was examined independently by 2 histologists [19]. TUNEL positive cells were counted under an Olympus BX40 light microscope with 100 cells from randomly chosen fields. The percentage of apoptotic cells was calculated by a blinded observer as follows: 0: no apoptosis; 1: 1%–10% apoptosis; 2: 11%–25% apoptosis; 3: 26%–50% apoptosis; 4: 51%–75% apoptosis; and 5: more than 75% apoptosis [20].

Statistical analysis

The immunocytochemical staining of the cells was evaluated by two observers. In grading the staining intensity, three categories were used: weak (+), moderate (++) and, strong (+++). For iNOS

and eNOS expression, immunohistochemical reactions were assessed in three different microscopic areas of the cells. To evaluation of the expression of iNOS, eNOS in the investigated cells, the H-Score obtained by multiplying the staining intensity by the percentage of immunoreactive cells (0-100 %). The data was statistically analyzed by repeated-measures ANOVA, the Tukey-Kramer multiple comparisons test, and was given mean±SD. Significance level was set at $p < 0.05$.

RESULT AND DISCUSSION

To detect the IC50 doses of oregano oil and doxorubicin MTT assay was performed with oregano oil (0, 0.1, 1, 10, 30, 100 µl/ml) and doxorubicin (0, 01, 1, 10, 30, 100 µM) for 24 h. Cell proliferation was decreased with increasing concentrations of agents when compared with control (non-treated group; medium with 0 µL/mL oregano oil, 0 M doxorubicin and an appropriate amount of DMSO which is used to dissolve the essential oil). IC50 doses of agents were calculated as 10.75 µl/ml, and 5 µMfor oregano oil and doxorubicin, respectively (fig. 1).

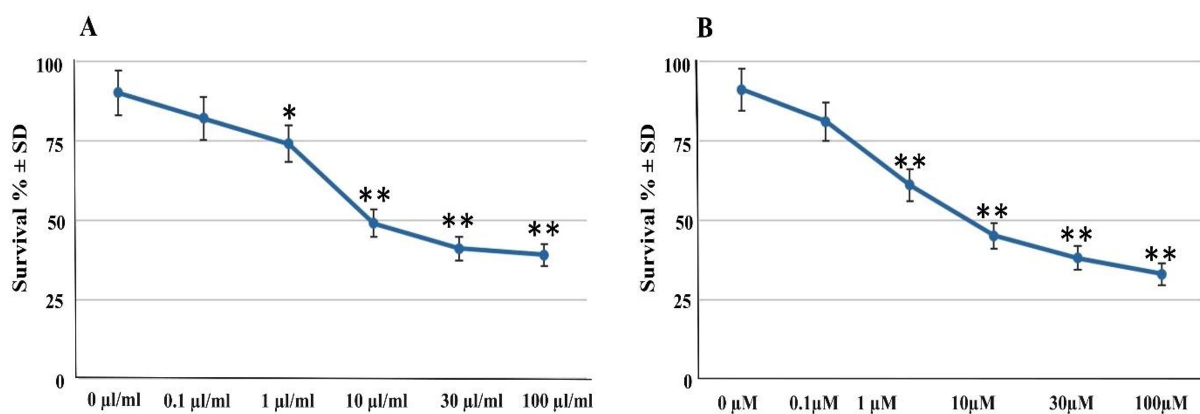

Figure 1. The percentage of cell survival of NB2a neuroblastoma cells after the application of oregano oil (A) and doxorubicin (B). Each point represents a mean ± SD of three experiments.*$p < 0.05$; **$p < 0.01$ compared to control.

Immunocytochemistry and apoptosis

The immunocytochemical stainings of eNOS and iNOS were evaluated by H-score (fig. 2). The immunoreactivity of eNOS was increased in the presence of oregano oil and doxorubicin alone, a dual combination of these agents enhanced the eNOS staining significantly ($p<0.001$) when compared with the control group (non-treated group). iNOS was notably raised by the dual application of these agents. The staining level of eNOS was lower than iNOS staining in the dual combination group (figs. 2 and 3).

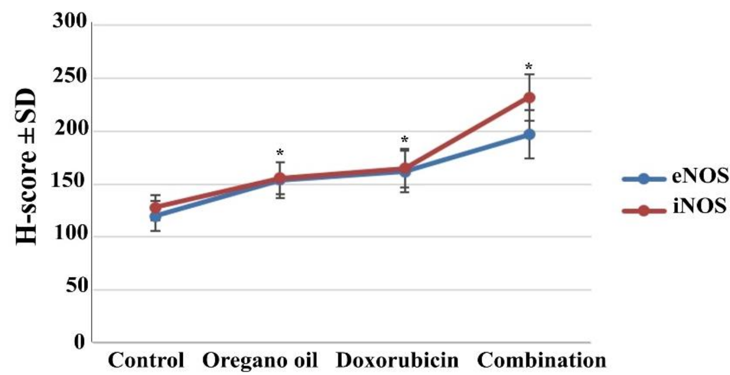

Figure 2. Immunoreactivities of eNOS and iNOS were evaluated by H-score method. —●— eNOS; —●— iNOS. H-score results were analyzed using one-way ANOVA, and asterisks indicate significant difference (*$p < 0.05$) for both iNOS and eNOS compared with control.

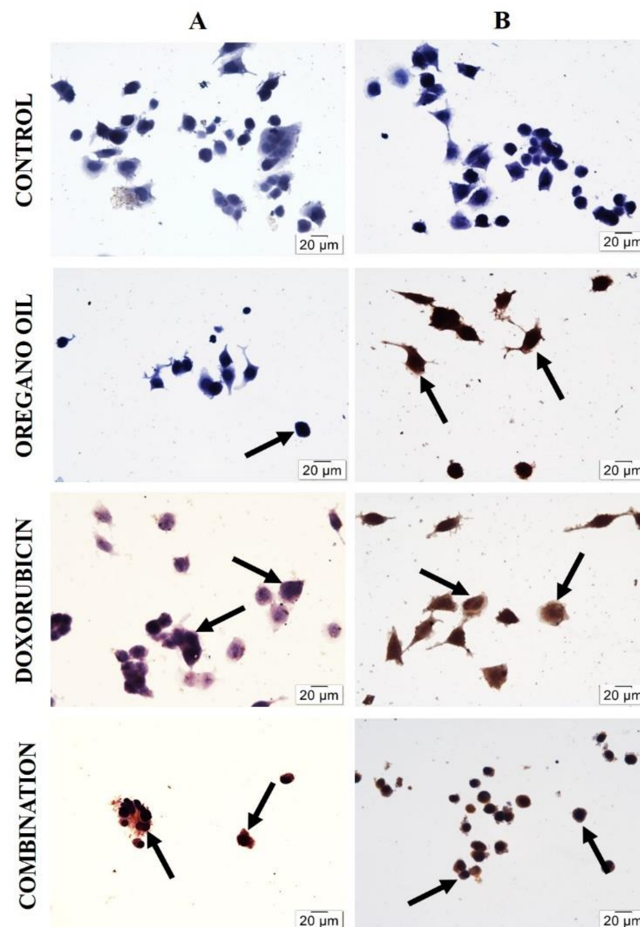

Figure 3. The distributions of eNOS (A) and iNOS (B) immunoreactivities in the NB2a neuroblastoma cells after the application of oregano oil, doxorubicin and dual combination of these agents. Arrows: immunopositive cells. Scale bars: 20 µm.

After the TUNEL assay, the apoptotic index was calculated to determine the number of dead cells. Both the oregano oil and doxorubicin caused cell death in NB2a neuroblastoma cells. The numbers of apoptotic cells were increased significantly in the group of oregano oil and doxorubicin in comparison with the control group (non-treated group) ($p<0.05$). In parallel with the findings of immunocytochemistry, the numbers of the apoptotic cells were the highest in the group of dual combinations of these agents (fig. 4 and Table 1).

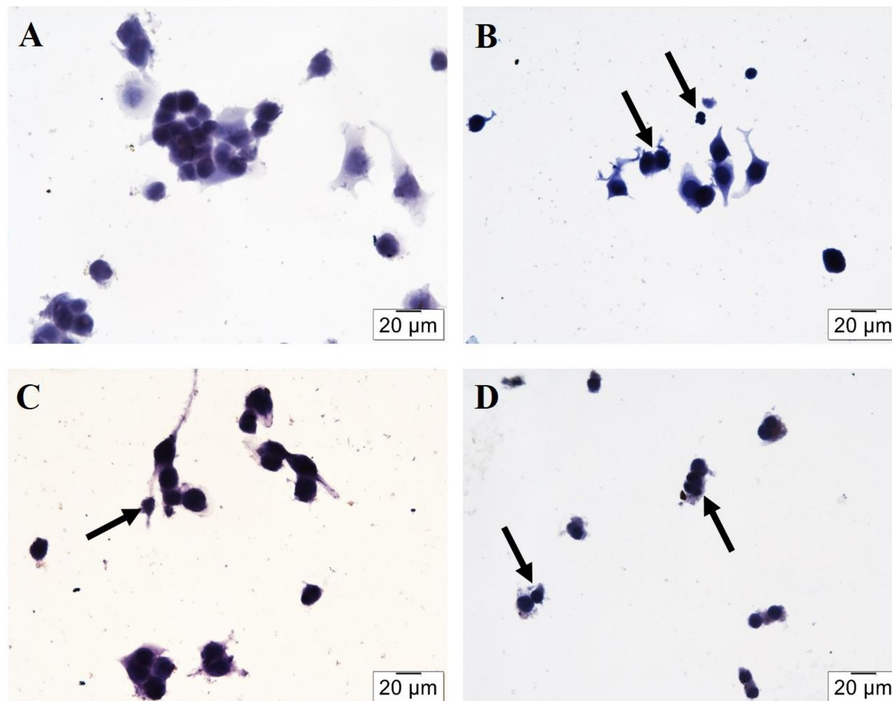

Figure 4. Apoptotic cells in different groups TUNEL staining image in the control (A), of oregano oil (B), doxorubicin (C) and combination (D) groups. Arrows: apoptotic cells. Scale bars: 20µm.

Table 1. Apoptotic Index After The Application Of Oregano Oil, Doxorubicin And Dual Combination Of These Agent

Group	Apoptotic index
Control	7.2±0.8
Oregano Oil	10.4±1.2*
Doxorubicin	11.1±1.4*
Combination	14.6±1.5*

**p*<0.05

Cancer is a leading cause of mortality worldwide and its incidence is expected to increase continuously due to the aging population [21]. Therefore, the number of studies related to the treatment, diagnosis, and prevention of cancer is increasing. In this field, researches attempting to demonstrate the anticancer, antiproliferative and apoptotic activities of different plant species' extracts are also growing progressively due to their potential applications in biological systems [22,23].

Origanum species used in folk medicine have also been widely investigated and it is found that their extracts and/or their essential oils have an antioxidant [5], anticarcinogenic [24], antigenotoxic [22], antibacterial [3] and antifungal [10] properties. *Origanum minutiflorum* is an endemic plant in Turkey among these species. Although we have knowledge about antibacterial, antifungal, antioxidant properties of *O. minutiflorum* from the data obtained from many different studies, cytotoxic effects of the extracts from *O. minutiflorum* on neuroblastoma cells has not been investigated previously. Therefore, this study was aimed to evaluate the cytotoxic effect of *O.minutiflorum* extracts on neuroblastoma cells.

Our data from the MTT assay showed that proliferation of NB2a neuroblastoma cells decreased gradually and significantly when the concentrations of both oregano oil and doxorubicin (a drug that is used in cancer therapy since long) were increased and IC_{50} doses were 10.75 and 5 µl/ml for oregano oil and doxorubicin, respectively. These results suggested that Oregano oil of *O. minutiflorum* has a cytotoxic effect on NB2a neuroblastoma cells.

Erenler *et al*. demonstrated that *Origanum majorana,* another species of *Origanum* genus, has an anticancer activity on C6 and HeLa cell lines [25]. Furthermore, Demir *et al* reported that essential oil from *Origanum onites*, *Origanum minutiflorum* has an antigenotoxic effect and this observed effect was associated with antioxidant properties of the essential oils [22]. In addition, in many researches, it was found that the major component of the essential oil from *O. minutiflorum* was carvacrol (68.23 - 92.3 %) and the ratio of carvacrol differs according to its habitat or the subspecies of *O. minutiflorum* [6, 26]. It is suggested that the different biological effects of essential oils such as antioxidant and tumor-suppressive activities may result from the main components of *Origanum* species, especially carvacrol, thymol, p-cymene, and γ-terpinene [27, 28].

In summary, our results are consistent with previous studies [22, 25] and the cytotoxic effect of Oregano oil on NB2a neuroblastoma cells may be attributed to the primary compounds in the Oregano oil, mainly carvacrol.

In addition, we have also investigated the effect of the Oregano oil on apoptosis, eNOS and iNOS levels in NB2a neuroblastoma cells, because there is a lack of information about its effect on these parameters in NB2a neuroblastoma cells. Our results have indicated that expression of iNOS and eNOS, also the numbers of apoptotic cells have been significantly increased in NB2a neuroblastoma cells after treatment with oregano oil and doxorubicin, which is an anticancer drug that induces apoptosis. iNOS,

eNOS expression levels and the number of apoptotic cells have notably been increased by the dual application of these agents, as well. Although there is no study about the apoptotic effect of oregano oil obtained from *Origanum minutiflorum* on Nb2A cells, in some studies, it has been shown that oregano oil from different plant species induces apoptosis in different cancer cell lines such as 5RP7 and MCF-7 cells [29-31]. The results of these studies are consistent with our study results.

Nitric oxide synthases are a family of proteins catalyzing the generation of nitric oxide (NO) from l-arginine, and especially iNOS and eNOS are known to play a central role in the production of NO. NO is involved in several biological processes such as modulation of vascular tonus, regulation of immune response and nervous systems [32,33]. On the other hand, NO is a gaseous free radical, and it becomes hazardous if it is synthesized in excess. Thus, increased production of NO can cause oxidative stress and finally cellular damage [34]. From this point of view, we consider that the Oregano oil induces apoptosis via elevated levels of iNOS and eNOS, which may promote the production of NO, is a reactive nitrogen species.

In several studies, it has been reported that increased NO levels cause the induction of apoptosis and cytotoxicity. Furthermore, it has been shown that above physiological limits NO, give rise to loss of mitochondrial membrane potential and for this reason induces cytochrome c release to the cytosol [35-37]. These results support our hypothesis of a link between the cytotoxic, apoptotic effect of Oregano oil on NB2a neuroblastoma cells and elevated levels of eNOS, iNOS.

In conclusion, the current study firstly has demonstrated that Oregano oil of *O. minutiflorum* has a cytotoxic and apoptotic effect on NB2a neuroblastoma cells. Furthermore, oregano oil has enhanced the apoptotic effect of doxorubicin. In addition, oregano oil-induced the expression of iNOS and eNOS, which may produce more NO, and, finally may lead to increasing the level of reactive nitrogen species in the neuroblastoma cells. Therefore, cytotoxic and apoptotic effects of oregano oil may be attributed to its enhancing effects on iNOS and eNOS levels. Collectively, it is suggested that Oregano oil of *O. minutiflorum* may be used with doxorubicin in the treatment of cancer in order to enhance its apoptotic effect. However, further *in vivo* and *in vitro* studies are needed to evaluate the effects and underlying molecular mechanisms of the Oregano oil.

DECLARATION OF INTEREST

All authors declare that they have no conflict of interest.

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