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Cytotoxic and cytogenetic effects of α-copaene on rat neuron and N2a neuroblastoma cell lines

Hasan Turkez[1], Basak Togar[2]*, Abdulgani Tatar[3], Fatime Geyikoglu[2] & Ahmet Hacimuftuoglu[4]

[1]*Department of Molecular Biology and Genetics, Faculty of Sciences, Erzurum Technical University, Erzurum, Turkey*
[2]*Department of Biology, Faculty of Science, Atatürk University, 25240 Erzurum, Turkey; e-mail: basaktogar@gmail.com*
[3]*Department of Medical Genetics, Medical Faculty, Atatürk University, Erzurum, Turkey*
[4]*Department of Medical Pharmacology, Medical Faculty, Atatürk University, Erzurum, Turkey*

Abstract: Alpha-copaene (α-COP), a tricyclic sesquiterpene, is present in several essential oils of medicinal and aromatic plants and has antioxidant and antigenotoxic features. Its cytotoxic, cytogenetic and oxidative effects have not been investigated in neuron and N2a neuroblastoma (NB) cell cultures. Therefore, we aimed to describe *in vitro*: (i) cytotoxic properties by 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenlytetrazolium bromide test; (ii) antioxidant/oxidant activity by total antioxidant capacity (TAC) and total oxidative status (TOS) analysis; and (iii) genotoxic damage potential by single cell gel electrophoresis – of α-COP in healthy neuron and N2a-NB cell cultures for the first time. Significant ($P < 0.05$) decrease in cell proliferation were observed in cultured primary rat neurons starting with the concentration of 150 mg/L and in N2a-NB cells starting with 100 mg/L. In addition, 25 mg/L of α-COP treatment caused increase of TAC levels and α-COP treatments at higher doses led to increase of TOS levels in neuron N2a-NB cell cultures. Moreover, none of the tested concentrations of α-COP have shown a genotoxic effect on both cell lines. Our findings clearly demonstrate that α-COP exhibited mild cytotoxic effects on N2a-NB cell line. In conclusion, α-COP may have potential as an anticancer agent, which needs to be further studied.

Key words: α-copaene; antiproliferative; neuroblastoma; single cell gel electrophoresis; total antioxidant capacity; total oxidative status.

Abbreviations: α-COP, α-copaene; EDTA, ethylenediaminetetraacetic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NB, neuroblastoma; SCGE, single cell gel electrophoresis; TAC, total antioxidant capacity; TOS, total oxidative status.

Introduction

Neuroblastoma (NB) is an embryonal tumour that originates from primitive cells (Cornero et al. 2012; Aydin et al. 2013; Dauphin et al. 2013). It is the most common solid tumour responsible for 15% of all cancer-related deaths in childhood. This tumour accounts for more than 7% of malignancies in patients fewer than 15 years of age (Stiller et al. 1992; Brodeur 2003; Carlson et al. 2013). NBs grow rapidly and often give rise to metastasis (Wassberg et al. 1999). Therapy resistance to anticancer drugs represents the major limitation to the effectiveness of clinical treatment. This is a major reason for the high frequency of foetal outcome of the disease (Svensson & Larsson 2003). The incidence of cancer is a global health problem and cancer treatments do not have effective medicine as the currently available drugs are causing side effects in some instances. Therefore, the natural products derived from medicinal plants have gained significance in the treatment of cancer (Sithranga et al. 2010). Recently, some biological compounds from plants, such as farnesyl pyrophosphate (Hooff et al. 2010), bilobalide (Shi et al. 2010), phenylbutyric acid (Wiley et al. 2010), artesunate (Michaelis et al. 2010), catechol (Lima et al. 2008), huperzine A (Hemendinger et al. 2008) and zoledronic acid (Bäckman et al. 2008) were found to be effective against NB cells.

Alpha-copaene (α-COP) is a tricyclic sesquiterpene derived from different plants, e.g. *Ceratitis capitata* (Nishida et al. 2000), *Annona reticulate* (Chavan et al. 2011), *Cedrelopsis grevei* (Afoulous et al. 2013), *Xylopia laevigata* (Quintans et al. 2013). Previous studies on α-COP-containing essential oils indicated anticarcinogenic, antioxidant, hepatoprotective and anti-inflammatory activities (Veiga Junior et al. 2007; Vinholes et al. 2013). In addition, the limited numbers of recent investigations have revealed that α-COP possesses important biological activities, including antigenotoxic and antioxidant activity (Turkez et al. 2013, 2014c).

* Corresponding author

To our best knowledge, α-COP sesquiterpene have not been studied for cytotoxic and cytogenetic effects in neuron and NB cells yet. There is absence of knowledge about cellular activities of α-COP. Therefore, the aim of the present study was to investigate the cytotoxic, antioxidant/oxidant, and genotoxic effects by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, total antioxidant capacity (TAC) and total oxidative status (TOS) levels, and single cell gel electrophoresis (SCGE) assay, respectively, by using the neuron and N2a-NB cell lines.

Material and methods

Test compound and chemicals

α-COP (CAS: 3856-25-5, $C_{15}H_{24}$, M_W 204.34), Dulbecco modified eagles medium, Hank's balanced salt solution, neurobasal medium, NaH_2PO_4, KH_2PO_4, ethylenediaminetetraacetic acid (EDTA), phosphate buffer solution, dimethylsulfoxide, Triton-X-100, DNase type 1, Tris, low melting point agarose, normal melting point agarose, dimethyl sulphoxide and ethidium bromide were purchased from Sigma–Aldrich® Germany. Foetal calf serum and trypsin-EDTA were purchased from Biol Ind®Israel.

Cell cultures

Primary rat cerebral cortex neuron cultures were prepared using rat foetuses as described previously (Ban et al. 2006). Briefly, a total of nine new-born Sprague-Dawley rats were used in the study. The rats were decapitated by making a cervical fracture in the cervical midline and the cerebral cortex was dissected and removed. The cerebral cortex was placed into 5 mL of Hank's balanced salt solution, which had already been placed in a sterile petri dish and macromerotomy was performed with two lancets. This composition was pulled into a syringe and treated at 37°C for 25-30 min as 5 mL Hank's balanced salt solution plus 2 mL trypsin-EDTA (0.25% trypsin, 0.02% EDTA) and chemical decomposition was achieved. Eight μL of DNase type 1 (120 U/mL) was added to this solution and treated for 1–2 min, and centrifuged at 800 rpm for 3 min. After having thrown away the supernatant, 31.5 mL of neurobasal medium and 3.5 mL foetal calf serum were added to the residue. The single cell which was obtained after physical and chemical decomposition was divided into 3.5 mL samples in each of 10 flasks coated with poly-D-lysine formerly dissolved in phosphate buffer solution. The flasks were left in the incubator including 5% CO_2 at 37°C. The flasks were then changed with a fresh medium of half of their volumes every three days until the cells were branched and had reached a certain maturity and *in vitro* experiments were performed eight days later. This study was conducted at the Medical Experimental Research Centres in Ataturk University (Erzurum, Turkey). The Ethical Committee of Ataturk University approved the study protocol (B.30.2.ATA.0.23.85-73).

We employed a cell line N2a-NB used widely as a model for brain cancer. The rat brain NB cell line N2a was obtained from Turkey FMD Institute, Ankara, Turkey. Prior to the experiments, the cells were thawed and grown in tissue culture flasks as a monolayer in Dulbecco modified eagles medium supplemented with 1% glutamine, 0.5% penicillin/streptomycin (PAN Biotech®, Germany) and 10% foetal bovine serum at 37°C in a humidified (95%) incubator with CO_2 (5%). The cultured cells were trypsinised with trypsin/EDTA for a maximum of 5 min and seeded with a subcultivation ratio of 1:3–1:8.

Treatments

α-COP was dissolved in ethanol and ethanol was evaporated to dryness at ambient temperature. α-COP was applied into cultures at concentrations of 10, 25, 50, 75, 100, 150, 200 and 400 mg/L for 24 h. The concentrations were selected according to the work of Wang et al. (2009).

MTT assay

MTT substrate solution was used according to the manufacturer's instructions (Cayman Chemical Company®, USA). Briefly, MTT (final amount 10 μL) was added to the cell cultures for 3 h. Formed formazan crystals were dissolved in dimethyl sulphoxide and the plates were analyzed using Microquant reader at 570 nm wavelength (Lewerenz et al. 2003).

DNA damage assay

In this study, the DNA damage evaluation was performed by SCGE (Comet) assay (Singh et al. 1988). After the application of coverslips, the slides were allowed to gel at 4°C for 30–60 min. The slides were immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris; pH = 10 in which 10% dimethyl sulphoxide and 1% Triton X-100 were added) and refrigerated overnight followed by alkali treatment (300 mM NaOH, 1 mM EDTA; pH > 13), electrophoresis (25 V, 300 mA) and neutralization (0.4 M Tris; pH = 7.5). The dried slides were then stained using ethidium bromide (20 μg/mL) after appropriate fixing. The whole procedure was carried out in dim light to minimize artefact. DNA damage analysis was performed at 100× magnification using a fluorescence microscope (Nikon Eclips E6600, Japan) after coding the slides by one observer (author: Basak Togar). A total of 100 cells were screened per slide. A total damage score for each slide was derived by multiplying the number of cells assigned to each grade of damage by the numeric value of the grade and summing over all grades (giving a maximum possible score of 400, corresponding to 100 cells at grade 4).

TAC and TOS analyses

The automated TAC and TOS analyses were carried out by commercially available kits (Rel Assay Diagnostics®, Turkey) on α-COP-treated cell cultures for 24 h (Erel 2004, 2005).

Statistics

Statistical analysis was performed using the SPSS software (version 18.0, SPSS, Chicago, IL, USA). For statistical analysis of obtained data, Duncan's test was used. Statistical decisions were made with a significance level of 0.05.

Results

MTT assay was used to assess the cytotoxic activity of α-COP. The effect of α-COP on cultured primary rat neuron and N2a-NB cells are shown in Figure 1. The results of MTT analysis showed that α-COP significantly suppressed the proliferation of N2a-NB cells, at concentrations higher than 100 mg/L (150, 200 and 400 mg/L) compared to control value. However, α-COP treatments showed cytotoxic activity on healthy neuron cells at concentrations over 150 mg/L (200 and 400 mg/L).

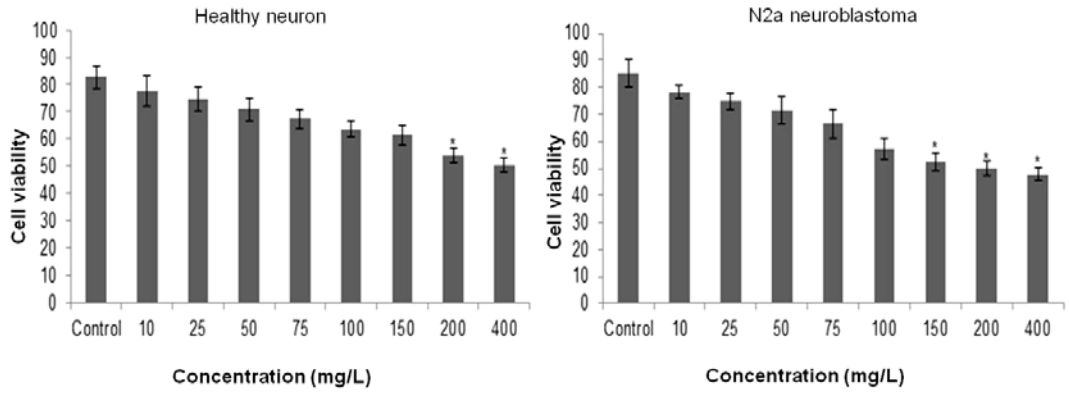

Fig. 1. Cytotoxic effect of α-COP on cultured rat neuron and N2a-NB cells. The results are given as the means ± SD from six independent experiments. Compared with control, $^*P < 0.05$.

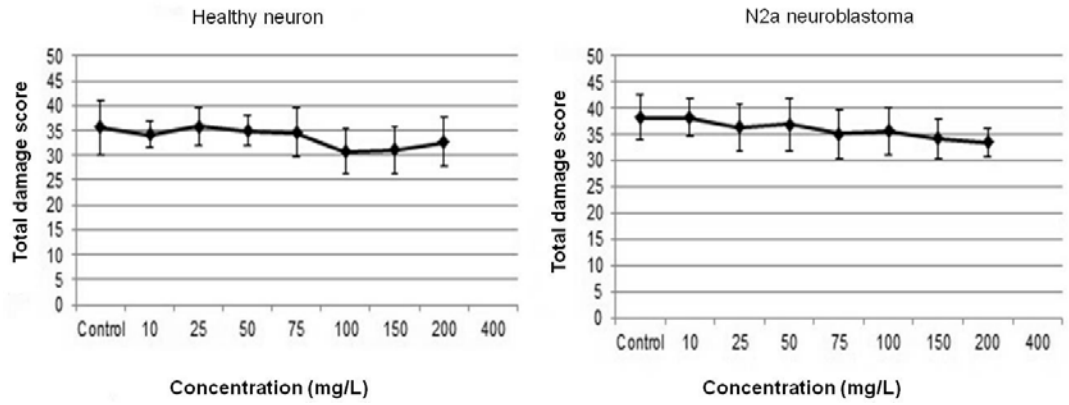

Fig. 2. Effect of varying concentrations of α-COP on inducing DNA damage *in vitro* for 24 h. The results are given as the means ± SD from six independent experiments (all the values are not significantly different from the control at the 5% level).

SCGE assay was performed in healthy neuron and N2a-NB cell lines to measure the genotoxicity of α-COP. α-COP at all tested concentrations did not alter the total damage scores as compared to the control group (Fig. 2.). But the neuron and N2a-NB cultures were found to be sterile at higher concentration (400 mg/L) of α-COP. The scoring criteria which determine DNA damage levels using comet formations in cultured neuron and N2a-NB cells treated with the α-COP are shown in Figure 3.

Table 1 and Table 2 reflect the comparison of oxidant-antioxidant profile of α-COP on cultured primary rat neuron and N2a-NB cell cultures. It is seen that one concentration of α-COP (25 mg/L) caused a significant increase of TAC level, while concentrations higher than 200 mg/L (400 mg/L) significantly decreased TAC levels on neuron cells compared to the control value. Also, high concentrations of α-COP for 24 h led to a significant elevation in the level of TOS as compared to that of the control group. Similar to these findings, 25 mg/L concentration of α-COP caused increase of TAC level, while its concentrations above 150 mg/L (200 and 400 mg/L) significantly decreased TAC levels on N2a-NB cells when compared with the control group. On the other hand, the TOS levels increased at 100, 150, 200, and 400 mg/L concentrations of α-COP in cultured N2a-NB cells, respectively.

Table 1. *In vitro* TAC level in cultured neuron and N2a-NB cells maintained 24 h the presence of α-COP.

α-COP	Neuron cells	N2a-NB cells
Treatments (mg/L)	TAC (mmol trolox equiv./L)	
Control	28.6 ± 3.0	6.1 ± 0.5
10	29.8 ± 4.1	7.1 ± 0.7
25	30.4 ± 2.7[a]	7.2 ± 0.7[a]
50	26.7 ± 3.0	6.0 ± 0.5
75	28.3 ± 2.9	6.2 ± 0.5
100	27.8 ± 2.1	5.9 ± 0.4
150	26.5 ± 2.2	5.0 ± 0.5[a]
200	22.7 ± 2.9[a]	4.8 ± 0.4[a]
400	21.1 ± 3.1[a]	4.7 ± 0.3[a]

[a] Significant differences from control groups at the $P < 0.05$ level. Values are expressed as means + SD of three experiments.

Discussion

In this study the *in vitro* cytotoxic, genotoxic and antioxidant/oxidant activities of α-COP were examined for the first time. MTT assay was used to measure

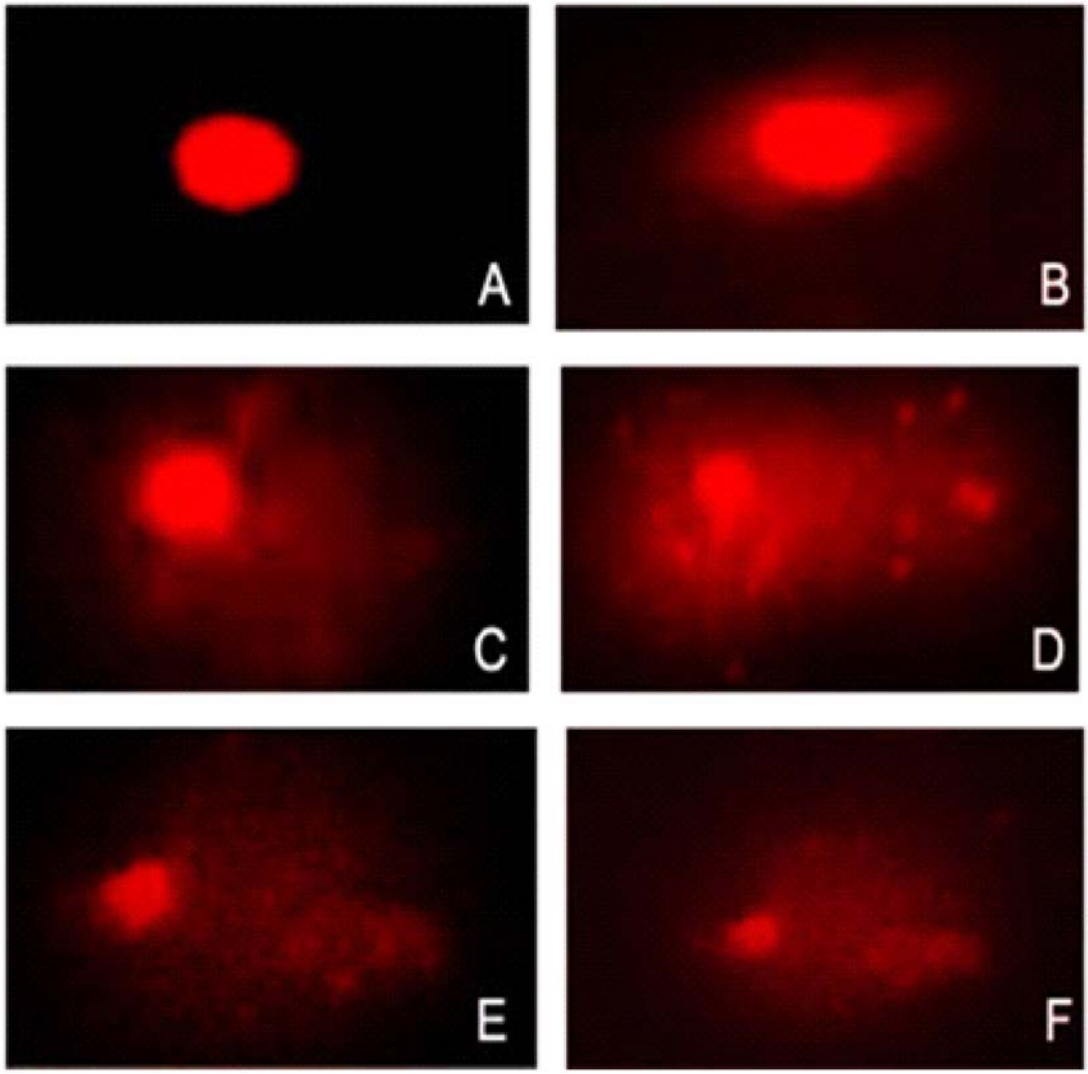

Fig. 3. The scoring criteria for determining damage levels in cultured neurons and N2a-NB cells. A – class 0 (undamaged); B – class 1 (slightly damaged); C – class 3 (damaged); D – class 4 (highly damaged); E – class 5 (very highly damaged); F – class 6 (extremely damaged).

Table 2. *In vitro* TOS level in cultured neuron and N2a-NB cells maintained 24 h the presence of α-COP.

α-COP	Neuron cells	N2a-NB cells
Treatments (mg/L)	TOS (mmol H_2O_2 equiv./L)	
Control	1.7 ± 0.1	2.3 ± 0.2
10	1.7 ± 0.2	2.3 ± 0.1
25	1.7 ± 0.3	2.6 ± 0.3
50	1.5 ± 0.2	2.4 ± 0.2
75	1.7 ± 0.3	2.4 ± 0.1
100	1.6 ± 0.1	2.5 ± 0.2[a]
150	1.8 ± 0.3	2.6 ± 0.3[a]
200	2.1 ± 0.2[a]	2.7 ± 0.2[a]
400	2.2 ± 0.1[a]	2.8 ± 0.2[a]

[a] Significant differences from control groups at the $P < 0.05$ level. Values are expressed as means + SD of three experiments.

the cytotoxic activity of α-COP on neuron and N2a-NB cells. MTT assay results indicated significant ($P < 0.05$) decreases of the cell proliferation rates in healthy neurons treated with α-COP at 150, 200 and 400 mg/L, while significant decreases were observed in N2a cells at 100, 150, 200 and 400 mg/L (Fig. 1). This means that cytotoxicity analysis of samples suggested a direct dose-response relationship, in which cell proliferation decreased in higher concentrations. In another study (Turkez et al. 2013), we studied the cytotoxic effect of α-COP in human blood cell cultures and found that α-COP (at high concentrations) has a distinct cytotoxic effect on human blood cells. Based on these results, we concluded that α-COP exhibit the same effects in different cell lines. Our results are in agreement with previous studies, which have reported that several sesquiterpenes, such as foveolide A (in SW620, HepG2, BT474 and KATO-III cancer cell lines), irofulven (human colon and ovarian carcinoma cells), artesunate (human hepatocarcinoma SMMC-7721 cell line), β-elemene (human renal-cell carcinoma 786-0 cells), parthenolide (human lung carcinoma) demonstrated cytotoxicity in the MTT *in vitro* assay (Poindessous et al. 2003; Parada-Turska et al. 2007; Zhan et al. 2012). Likewise, Fiori et al.

(2011) revealed that guaiazulene inhibited cell growth in human gingival fibroblasts (under UV irradiation) in a dose-dependent manner and decreased cell viability. According to the literature, the mechanisms of the cytotoxic action of α-COP are not known, but oxidative stress is thought to be the main responsible mechanism in its cellular toxicity. In addition to oxidative stress, previous studies reported that different mechanisms have been linked to cytotoxicity of plant products, including proteasome inhibition, topoisomerase inhibition, inhibition of fatty acid synthesis, accumulation of p53, induction of cell cycle arrest, inhibition of phosphatidyl-inositol 3-kinase or enhanced expression of c-fos and c-myc (Constantinou et al. 1995; Lepley et al. 1996; Plaumann et al. 1996; Agullo et al. 1997; Chen et al. 1998; Kazi et al. 2004; Brusselmans et al. 2005; Chen et al. 2005).

In our study, the tricylic sesquiterpene α-COP showed to lack mutagenic activity in cultured neuron and N2a-NBs when assessed for induction of DNA damage in the SCGE assay (Fig. 2). To the best of our knowledge, present data represent the first complete evaluation of the genotoxicity for this compound. α-COP was found to be not genotoxic on human lymphocyte cells (Turkez et al. 2013). In parallel to this finding it was reported that zerumbon (a sesquiterpene phytochemical from a type of edible ginger) did not induce genotoxicity in cultured human peripheral blood lymphocytes (Al-Zubairi et al. 2010). Similarly, Di Sotto et al. (2010) found that β-caryophyllene concentrations up to 100 mg/L did not produce any cytotoxicity and genotoxicity, as shown by the value of nuclear division index and the frequency of micronuclei in cultured human lymphocytes. In the same way, Molina-Jasso et al. (2009) found that β-caryophyllene did not induce genotoxic damage in mouse bone marrow cells. In contrast to our findings, nivalenol (a sesquiterpene mycotoxin) showed organ specific genotoxicity in cultured Chinese hamster ovarium cells and in several mouse organs and tissues like liver, kidney, thymus, bone marrow and mucosa of stomach, jejunum, and colon using SCGE assay (Tsuda et al. 1998). In addition, Best & Mckenzie (1988) and de Peyster & Wang (1993) investigated the genotoxic effects of gossypol by structural and numerical sister chromatid exchange in human lymphocytes culture. They reported that gossypol increased the number of numerical sister chromatid exchange. These divergent results suggest the relevance of the chemical structure in the biological effect of sesquiterpenes as well as indicate the importance of using various test models to reach a valid conclusion.

For assessing the antioxidant/oxidant effects of α-COP, TAC and TOS assays were performed. Our results reported that one concentration of α-COP (25 mg/L) caused increase of TAC level in neuron and N2a-NB cell cultures. On the other hand, our results showed that α-COP (concentration higher than 150 mg/L) caused increases in TOS levels in healthy neurons. And high concentrations of α-COP (higher than 100 mg/L) caused significant increases in TOS levels in N2a-NB cells (Tables 1 and 2). In parallel with our findings, Turkez et al. (2014a,b) demonstrated that α-farnesene, β-farnesene and cyclosativene exhibited antioxidant effect on primary neuron cell cultures. They have also found that these natural sesquiterpene compounds were capable of protecting against H_2O_2-induced cytotoxicity and oxidative DNA damage in cultured rat primary cortical neuron cells. Likewise, it was demonstrated that low concentrations of α-COP showed antioxidant activity on human blood cell cultures (Turkez et al. 2013). Our results document that α-COP, as other sesquiterpenes, exhibits antioxidant effect at low concentrations.

In conclusion, it can be stated that α-COP possesses a non-genotoxic/mutagenic feature, weak antioxidant and cytotoxic activity *in vitro* for normal and cancer cell lines. α-COP might be a new agent which can inhibit proliferation in N2a-NB cells in a dose-dependent manner. α-COP may therefore have a clinical application in anticancer therapy, however, the dose should be carefully used as an ingredient of functional foods as well as for pharmaceutical purposes.

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