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ANTIPROLIFERATIVE, GENOTOXIC AND OXIDANT ACTIVITIES OF CYCLOSATIVENE IN RAT NEURON AND NEUROBLASTOMA CELL LINES

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Abstract – Cyclosativene (CSV) is a tetracyclic sesquiterpene found in the essential oils of *Centaurea cineraria* (Asteraceae) and *Abies magnifica* A. Murray (Pinaceae) plants. To the best of our knowledge, its cytotoxic, genotoxic and oxidant effects have never been studied on any cell lines. Therefore, we aimed to investigate the *in vitro* antiproliferative and/or cytotoxic properties, antioxidant/oxidant activity and genotoxic damage potential of CSV in healthy neurons and N2a neuroblastoma (N2a-NB) cell cultures. After treatment with 10-400 μg/ml of CSV for 24 h, cell proliferation was measured by the MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The antioxidant activity was assessed by the total antioxidant capacity (TAC) and total oxidative stress (TOS) assays. To evaluate the level of DNA damage, single cell gel alkaline electrophoresis (SCGE) was used. The MTT assay showed that the application of CSV significantly reduced cell viability in both cell types. CSV treatments at higher doses led to decreases of TAC levels and increases of TOS levels in neuron and N2a-NB cells. The mean values of the total scores of cells showing DNA damage were not found to be significantly different from the control values in both cells. In conclusion, this study suggests that CSV has weak anticancer potential.

Key words: Cyclosativene; neuroblastoma; MTT assay; total antioxidant capacity; total oxidative stress; single cell gel electrophoresis.

INTRODUCTION

Neuroblastoma (NB), a peripheral nervous system cancer that can be highly invasive and metastatic. NB is the most common solid tumor and is responsible for 15% of all cancer-related deaths in childhood (Mueller et al., 2012; Wojtalla et al., 2012; Waheed Roomi et al., 2013). NB tumors from patients are often characterized by deregulation of many key signaling pathways regulating growth, survival, apoptosis and proliferation with concomitant resistance to chemotherapy (Li and Thiele, 2007; Peirce and Findley, 2009). The acquisition of multidrug resistance upon treatment with anticancer drugs is a common phenomenon for NBs (Svensson and Larsson, 2003). Therapy resistance to anticancer drugs is the major limitation to the effectiveness of clinical treatment. This is a main reason for the high frequency of fetal outcome of the disease (Svensson and Larsson, 2003). To improve patients' prognoses with conventional therapies where currently available drugs cause side effects in some instances, the development of safer

treatments is needed and the use of natural products is one strategy currently explored for the treatment of cancer (Park et al., 2012; Liu et al. 2013).

Naturally occurring plant components from traditional herbs are a significant source of potential therapeutic compounds for cancer treatment (Tan et al., 2012). Sesquiterpenes, which are one of the most common terpenes, are a class of natural products with a diverse range of attractive industrial properties (Scalcinati et al., 2012). They are compounds containing three isoprene units, with fifteen carbons and twenty-four hydrogens per molecule ($C_{15}H_{24}$). There are more than 10 000 types of sesquiterpenes (Davis and Croteau, 2000), and some of the biological activities of some of these compounds have been investigated: such as their anticarcinogenic (Afoulous et al., 2013), antimicrobial (Wang et al., 2013), antifungal (Kundu et al., 2013), anti-inflammatory (Wang et al., 2013), and more recently antioxidant activities (Abolaji et al., 2013).

Cyclosativene (CSV) is a tetracyclic sesquiterpene. The volatile organic compounds unique to *Helminthosporium sativum*, *Helminthosporium victoriae* and *Centaurea cineraria* include CSV (Crutwell-McFadyen, 1998; Lodewyk et al., 2008; Beck et al., 2008). To our best knowledge, the antiproliferative, genotoxic and oxidant effects of CSV on any cell lines have never been explored. Therefore, we aimed to assess the antiproliferative (by the MTT assay), oxidative (by measuring TAC and TOS levels) and genotoxic (by the SCGE assay) effects of CSV in cultured rat neuron and N2a-NB cells.

MATERIAL AND METHODS

This study was conducted at the Medical Experimental Research Centers in Ataturk University (Erzurum, Turkey). The Ethical Committee of Ataturk University approved the study protocol (B.30.2.ATA.0.23.85-73).

Test compound and chemicals

Cyclosativene (Cas: 22469-52-9, $C_{15}H_{24}$), Dulbecco modified eagles medium, sodium phosphate (NaH_2PO_4), potassium phosphate monobasic (KH_2PO_4), ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), Triton-X-100, Tris, low melting point agarose, Normal melting point agarose, and ethidium bromide were purchased from Sigma-Aldrich® (Steinheim, Germany).

Neuron and N2a neuroblastoma cell cultures

Primary rat cerebral cortex neuron cultures were prepared using rat fetuses as described previously (Ban et al., 2006). Briefly, nine newborn 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 HBSS in a sterile Petri dish, and macromerotomy was performed with two lancets. The material was pulled into a syringe and treated at 37°C for 25-30 min with 5 ml HBSS, 2 ml Trypsin-EDTA (0.25% trypsin- 0.02% EDTA). Eight µl of DNase type 1 (120 U/ml) was added to the solution and treated for 1-2 min, and centrifuged at low speed for 3 min. After discarding the supernatant, 31.5 ml of NBM and 3.5 ml FCS were added to the residue. The single cell 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 PBS. The flasks were maintained at 5% CO2 at 37°C. The flasks were then changed with half of the volume of a fresh medium every three days until the cells were branched and reached maturity; *in vitro* experiments were performed eight days later. 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 the *FMD Institute*, Ankara, Turkey. The concentrations were selected according to Wang et al. (2009). CSV was dissolved in sterile ethanol (final concentration in culture, 0.1%) and then applied to the cultures at of the following concentrations: 10, 25, 50, 75, 100, 150, 200 and 400 µg/ml for 24 h.

MTT assay

The cells were seeded in 96-well plastic plates at a

density of 1×10^4 cells/well and incubated in a humidified atmosphere of 5% CO2 at 37°C for 24 h. The cell viability assay was performed using the MTT cell proliferation kit Cayman Chemical Company®, USA. At the end of the experiment, the neuron and N2a-NB cell lines were incubated with 0.7 mg/ml MTT for 30 min at 37^0C. After washing, blue formazan was extracted from cells with isopropanol/formic acid (95:5) and absorbance was measured at 570 nm (Lewerenz et al., 2003)

TAC and TOS analysis

The automated total antioxidant capacity (TAC) and total oxidant status (TOS) assays were carried out using commercially available kits (Rel Assay Diagnostics®, Turkey) on GYZ-treated cell cultures for 24 h. Antioxidants in the sample reduced the dark blue-green colored ABTS radicals to a colorless reduced ABTS form. The change of absorbance at 660 nm was related to the total antioxidant level of the sample. The assay was calibrated with a stable antioxidant standard solution, traditionally named Trolox Equivalent, that is a vitamin E analog. Oxidants present in the sample oxidize the ferrous ion-chelator complex to ferric ion. The oxidation reaction is prolonged by enhancer molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with chromogen in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed as μmol H_2O_2 Equiv./l (Erel, 2004; Erel, 2005; Jozanov-Stankov et al., 2009).

SCGE assay

After the application of cover slips, the slides were allowed to gel at 4^0C for 30-60 min. The slides were immersed in freshly prepared cold lysis solution and refrigerated overnight, followed by alkali treatment, electrophoresis and neutralization. The dried slides were then stained using ethidium bromide after appropriate fixing. The whole procedure was carried out in dim light to minimize artifacts. DNA damage analysis was performed at a magnification of 100x using a fluorescence microscope (Nicon Eclips E6600, Japan) after one observer (Toğar B) coded the slides. 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 all grades (giving a maximum possible score of 400, corresponding to 100 cells at grade 4).

RESULTS

Cell viability measured by MTT test after 24 h significantly decreased in all neuron cells tested at therapeutically relevant CSV concentrations up to 150 μg/ml. In addition, when N2a-NB cells were treated for 24 h with 100, 150, 200 and 400 μg/ml of CSV, relative cell proliferation progressively decreased in a dose-dependent manner, as shown in Fig. 1. The results indicated that CSV exerted an antiproliferative activity on N2a-NB cells at concentrations greater than 100 μg/ml.

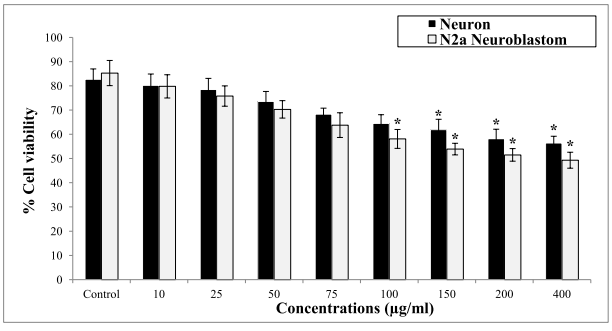

Fig. 1. Viability of rat primary cortical neurons and N2a-NB cells after 24 h exposure to (0-400 μg/ml) CSV. *$p<0.05$ was considered significant.

TAC and TOS automated colorimetric assays were used to determine the oxidative status. Table 1 shows the effects of CSV on the TAC parameter in cultured rat neurons and N2a-NB cells. Cells treated with 25 μg CSV/ml showed antioxidant capacity in neuron and N2a-NB cells compared with the controls. TOS levels increased at higher concentrations of CSV (100, 150, 200, 400 μg/ml) in N2a-NB cells

Table 1. *In vitro* levels of TAC (as mmol Trolox Equiv./L) and TOS (as mmol H_2O_2 Equiv./L) in cultured rat cortical neurons and N2a-NB cells maintained in the presence of CSV for 24 h.

	Healthy neuron cells		N2a neuroblastoma cells	
Concentration (μg/ml)	TAC (mmol Trolox Equiv./L)	TOS (mmol H_2O_2 Equiv./L)	TAC (mmol Trolox Equiv./L)	TOS (mmol H_2O_2 Equiv./L)
Control	28.6 ± 3.0	1.7 ± 0.1	6.1 ± 0.5	2.3 ± 0.2
10	27.1 ± 2.9	1.6 ± 0.2	7.2 ± 0.6	2.7 ± 0.3
25	33.8 ± 3.6*	1.8 ± 0.2	7.3 ± 0.5*	2.4 ± 0.2
50	30.4 ± 2.6	1.8 ± 0.1	6.2 ± 0.5	2.4 ± 0.2
75	30.1 ± 4.0	1.9 ± 0.1	6.7 ± 0.6	2.6 ± 0.1
100	29.8 ± 2.6	1.9 ± 0.2	5.9 ± 0.4	2.7 ± 0.1*
150	27.7 ± 3.0	2.0 ± 0.3*	5.0 ± 0.5*	2.9 ± 0.2*
200	21.9 ± 3.1*	2.2 ± 0.2*	4.8 ± 0.3*	2.8 ± 0.2*
400	20.2 ± 2.7*	2.4 ± 0.3*	5.2 ± 0.4*	2.9 ± 0.2*

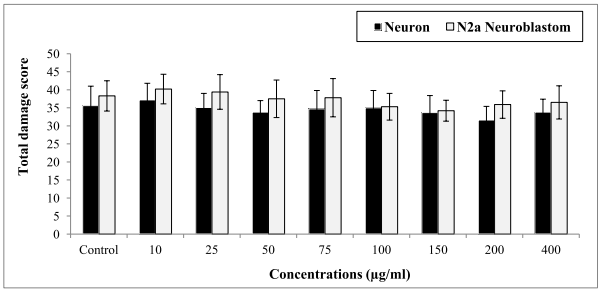

Fig. 2. Effect of increasing concentrations of CSV on DNA damage *in vitro* for 24h.

(Table 1). In addition, the TOS level was significantly increased in ≤150 μg/ml CSV-treated neuron cultures in comparison with the control group.

The potential genotoxic effect of CSV was assayed on cultured primary rat neurons and N2a-NB cell lines using the SCGE assay. Results obtained after treatment by CSV are shown in Fig. 2. *In vitro* exposure of both cell types at different doses of CSV did not result in comet formation in the SCGE assay, indicating the non-genotoxic nature of CSV.

DISCUSSION

The results demonstrate that CSV induced a significant cytotoxic effect on N2a-NB cancer cell lines. To our knowledge, there is no information regarding the cytotoxic effect of CSV in cell culture. Several sesquiterpenes such as irofulven (human colon and ovarian carcinoma cells), parthenolide (human lung carcinoma (A549), human medulloblastoma (TE671), human colon adenocarcinoma (HT-29), human umbilical vein endothelial cells (HUVEC)) and artesunate (human hepatocarcinoma SMMC-7721 cell line) β–elemene (human RCC 786-0 cells, human breast carcinoma MCF-7 and doxorubicin resistant MCF-7 cells), zerumbone (human cervix (HeLa) cells), and liver (DEN/AAF-induced) cancer cell lines) exhibit cytotoxic effects, as observed in the MTT assay (Poindessous et al., 2003; Parada-Turska et al., 2007; Abdel Wahab et al., 2009; Taha et al., 2010; Zhan et al., 2012; Xu et al., 2012).

This study indicates that increasing concentrations of CSV cause oxidative stress in both cell types. Previous studies reported that different mechanisms underlie the cytotoxic effects of plant products, including (i) proteasome inhibition, (ii) topoisomerase inhibition, (iii) inhibition of fatty acid synthesis, (iv) accumulation of p53, (v) induction of cell cycle arrest, (vi) inhibition of phosphatidylinositol 3-kinase, (vii) enhanced expression of c-fos and c-myc, and oxidative stress (Constantinou et al., 1995; Lepley et al., 1996; Plaumann et al., 1996; Agullo et al., 1997; Chen et al., 1998; Kazi et al., 2004; Chen et al., 2005).

Studies have shown that sesquiterpenes have antioxidant/prooxidant properties at different concentrations. Our results revealed that CSV at a low concentration of 25 µg/ml caused an increase in the TAC level in neuron and N2a-NB cells (Table 1). It was found that 3-vinylguaiazulene has the most potent, possessing antioxidant activity (Pratsınıs and Haroutounian, 2002). Al-maskri et al. (2011) reported that the essential oil of *Ocimum basilicum* (containing farnesene sesquiterpene) showed higher antioxidant activity. In addition, it was demonstrated that the essential oil of *Centaurea Centaurium* L. (containing alpha-zingiberene, beta-farnesene, beta-santalene, beta-bisabolene, beta-himachalene and azulene) exhibited higher DPPH radical inhibition (Conforti et al., 2008).

In this study, the sesquiterpene CSV did not induce DNA damage in neuron and N2a-NBs cell lines, as judged by the SCGE assay. Zerumbone was not found to be mutagenic in cultured human peripheral blood lymphocytes (Al-Zubairi et al., 2010). Beta-caryophyllene was reported to be non-genotoxic according to the results of micronuclei (MN) assay (Di Sotto et al., 2010). In contrast, gossypol exhibited a genotoxic effect in cultured mouse bone marrow cells (evaluated by the MN assay), human lymphocyte cells (evaluated by sister chromatid exchange) (Orhan et al., 1993). It was reported that nivalenol displayed genotoxicity in cultured Chinese hamster ovary (CHO) cells and in several mouse organs and tissues such as the liver, kidney, thymus, bone marrow and mucosa of stomach, jejunum, and colon, as evaluated by the SCGE assay (Tsuda et al., 1998).

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