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Guaiazulene biochemical activity and cytotoxic and genotoxic effects on rat neuron and N2a neuroblastom cells

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ABSTRACT

Aim: Neuroblastoma (NB)cells are often used in cancer researches such as glioblastoma cells since they have the potential of high mitotic activity, nuclear pleomorphism, and tumor necrosis. Guaiazulene (GYZ 1,4-dimethyl-7-isopropylazulene)is present in several essential oils of medicinal and aromatic plants. Many studies have reported the cytotoxic effect of GYZ; however, there are no studies that compare such effects between cancer cell lines and normal human cells after treatment with GYZ. **Materials and Methods:** In this study, we aimed to describe *in vitro* antiproliferative and/or cytotoxic properties (by 3-[4,5 dimetylthiazol -2-yl]-2,5 diphenlytetrazolium bromide [MTT] test), oxidative effects (by total antioxidant capacity [TAC] and total oxidative stress [TOS] analysis)and genotoxic damage potentials (by single cell gel electrophoresis)of GYZ. **Result:** The results indicated that GYZ have anti-proliferative activity suppressing the proliferation of neuron and N2a-NB cells at high doses. In addition, GYZ treatments at higher doses led to decreases of TAC levels and increases of TOS levels in neuron and N2a-NB cells. On the other hand, the mean values of the total scores of cells showing DNA damage were not found different from the control values. **Conclusion:** From this study, it is observed that GYZ has *in vitro* cytotoxic activity against neuron and N2a-NB cells.

KEY WORDS: Cytotoxicity, genotoxicity, guaiazulene, neuroblastoma, single cell gel electrophoresis assay, 3-(4,5 dimetylthiazol -2-yl)-2,5 diphenlytetrazolium bromide assay

INTRODUCTION

Neuroblastoma (NB) is an embryonal tumor that originates from primitive cells. It's the most common solid tumor and is responsible for 15% of all cancer-related deaths in childhood [1,2]. This tumor accounts for more than 7% of malignancies in patients fewer than 15 years of age [3,4]. NB tumors from these patients are often characterized by deregulation of many key signaling pathways regulating growth, proliferation, survival, and apoptosis, with concomitant resistance to chemotherapy [4,5]. The acquisition of multidrug resistance upon treatment with anti-cancer drugs is a common phenomenon for NBs. This is a major reason for the high frequency of fetal outcome of the disease [6]. Recently, there are strong epidemiological evidence and laboratory studies that are naturally occurring terpenes may exert cytotoxic effects against NB cells [7,8].

Terpenes are the largest group of natural substances biosynthetically derived from isoprene units [9]. Guaiazulene (1,4-dimethyl-7-isopropylazulene GYZ, Figure 1)is a bicyclic sesquiterpene derived from different plants, guaiac wood oil, *Callis intratropica* blue and *Matricaria chamomilla* L and has attracted much attention due to its beneficial biological activities [10,11]. Moreover, previous reports indicated that GYZ has antioxidant, antifungal, antimicrobial, anti-inflammatory, anti-spasmodic, anti-ulcer, antitumoral activities and relaxant properties [12-18]. Although it has been demonstrated to have interesting biological effects, GYZ has been not proven to be cytotoxic, genotoxic and antioxidant/oxidant effects on neuron and NB cell lines. Therefore, the aim of the present study was to firstly evaluate the cytotoxic/antiproliferative (3-[4,5 dimetylthiazol -2-yl]-2,5 diphenlytetrazolium bromide [MTT] assay), cytogenetic (single cell gel electrophoresis [SCGE] assay)

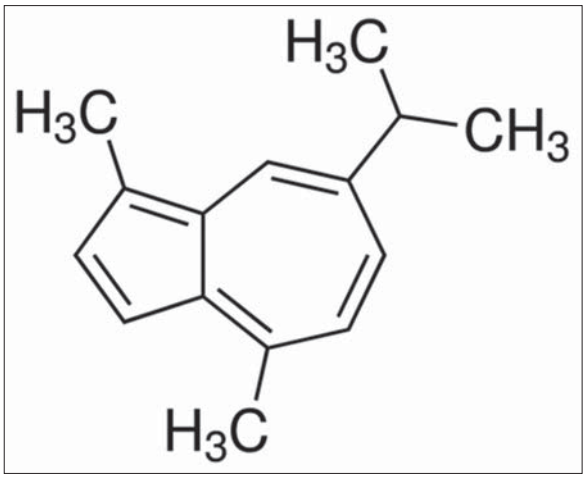

Figure 1: Chemical structure of guaiazulene

and oxidative effects (total antioxidant capacity [TAC] and total oxidative stress [TOS] analysis)of GYZ on neuron and NB cell cultures for its possible use in the complementary and alternative medicine practices.

MATERIALS AND METHODS

Test Compounds and Chemicals

GYZ (CAS 489-84-9, $C_{15}H_{18}$), Dulbecco modified eagles medium, sodium phosphate (NaH_2PO_4), potassium phosphate monobasic (KH_2PO_4), ethylenediaminetetraacetic acid (EDTA), dimethylsulfoxide (DMSO), triton-X-100, tris, low melting point agarose, normal melting point agarose, ethidium bromide were purchased from Sigma-Aldrich® (Steinheim, Germany).

Experimental Design

Primary rat cerebral cortex neuronal cultures were prepared using rat fetuses as described previously [19]. 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 (HBSS, Sigma-Aldrich®, Steinheim, Germany), 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 HBSS plus 2 mL trypsin-EDTA (0.25% trypsin- 0.02% EDTA)and chemical decomposition was achieved. 8 μL of DNase Type 1 (120 U/mL), was added to this solution, 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 (Life Technologies, Inc.)and 3.5 mL fetal calf serum (Sigma-Aldrich®, Steinheim, Germany)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 3 days until the cells were branched and had reached a certain maturity. Further *in vitro* experiments were performed 8 days later. This study was conducted at the Medical Experimental Research Centre 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.

Treatments

GYZ was dissolved in ethanol and ethanol was evaporated to dryness at ambient temperature. GYZ 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 Si *et al*. [20].

MTT assay

The cells were seeded in 96-well plastic plates at a density of 1×104 cells/well and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 24 h. Cell viability assay was performed using the MTT cell proliferation kit (Cayman Chemical Company®, USA). Pure water was added in the control group. At the end of the experiment, the neurons, and N2a-NB cells were incubated with 20 μL of MTT for 30 min at 37°C. After washing the blue formazan was extracted from cells with isopropanol/formic acid (955)and was photometrically determined at 570 nm [8].

TAC and TOS analysis

The automated TAC and TOS assays were carried out by commercially available kits (Rel Assay Diagnostics, Turkey)on cell cultures of GYZ-treated cultures for 24 h [21,22].

SCGE assay

DNA damage evaluation was performed by SCGE also known as comet test assay. After the application of coverslips, the slides were allowed to gel at 4°C for 30-60 min. The coverslip was removed, and the slide was immersed in cell lysis solution (2.5 M NaCl, 0.1 M Na_2-EDTA, 1% Na-sarcosinate, 10 mM Tris-HCl (pH 10.0), 10% DMSO, and 1% triton X-100)for 1 h. and refrigerated overnight followed by alkali treatment (1 mM Na_2-EDTA and 0.3 M NaOH, pH > 12.0), electrophoresis (at 1.6 V cm−1 for 20 min, 300 mA)and neutralization (0.4 M tris, pH 7.5). The dried slides were then stained using ethidium bromide (20 lg/mL)after appropriate fixing for 10 min. The whole procedure was carried out in dim light to minimize artifact [23]. DNA damage analysis was performed at a magnification of ×100 using a fluorescence microscope (Nikon Eclips E6600, Japan)after coding the slides by one observer (Togar B.). 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).

Statistical Analysis

Statistical analysis was performed using 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.

Table 1: *In vitro* **TAC and TOS levels in cultured healthy neuron and N2a-NB cells maintained 24 h in the presence of GYZ**

Doses (μg/mL)	Healthy neuron		N2a-NB	
	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	27.8±2.7	1.6±0.2	5.8±0.8	2.8±0.3
25	28.0±3.5	1.5±0.2	6.1±0.7	2.4±0.2
50	28.3±3.8	1.7±0.1	6.1±0.5	2.4±0.3
75	26.4±3.6	1.8±0.2	5.0±0.7*	2.6±0.2
100	26.7±2.9	1.9±0.2	4.8±0.5*	2.7±0.2*
150	21.6±2.3*	2.2±0.1*	4.1±0.6*	2.8±0.2*
200	20.7±3.0 *	2.3±0.2*	3.9±0.5*	2.9±0.2*
400	20.5±3.7*	2.5±0.1*	3.8±0.6*	2.9±0.1*

TAC: Total antioxidant capacity, TOS: Total oxidative stress, GYZ: Guaiazulene, NB: Neuroblastoma, *$P<0.05$

RESULTS

MTT assay, a colorimetric method for determining the number of viable cells in proliferation, was used to examine the inhibitory activities of GYZ on cell proliferation. Cell viability measured by MTT test after 24 h was significantly decreased in neuron, and N2a-NB cells tested at therapeutically relevant GYZ concentrations up to 150 μg/mL [Figure 2].

TAC and TOS analysis, rapid and reliable automated colorimetric assay, was used to determine the oxidative status by GYZ. As shown in Table 1, GYZ caused statistically important decreases in TAC levels at concentrations higher than 100 μg/mL in comparison with control values on rat neuron cell line. Moreover, GYZ caused statistically important increases in TOS levels at concentrations higher than 100 μg/mL in comparison with control values on rat neuron cell line. Also, 75, 100, 150, 200 and 400 μg/mL of GYZ applications caused significant decreases of TAC levels in comparison with control values on N2a-NB cell line. In contrast, the TOS levels increased at 100, 150, 200,

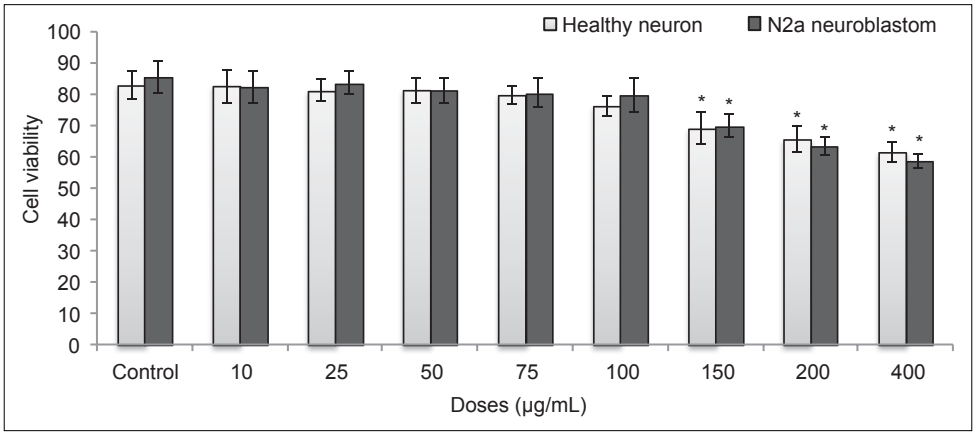

Figure 2: Cytotoxic effect of GYZ on cultured primary rat neurons and N2a NB cells. The results are given as the means ± standard deviation from six independent experiments. Compared with control, *$P < 0.05$

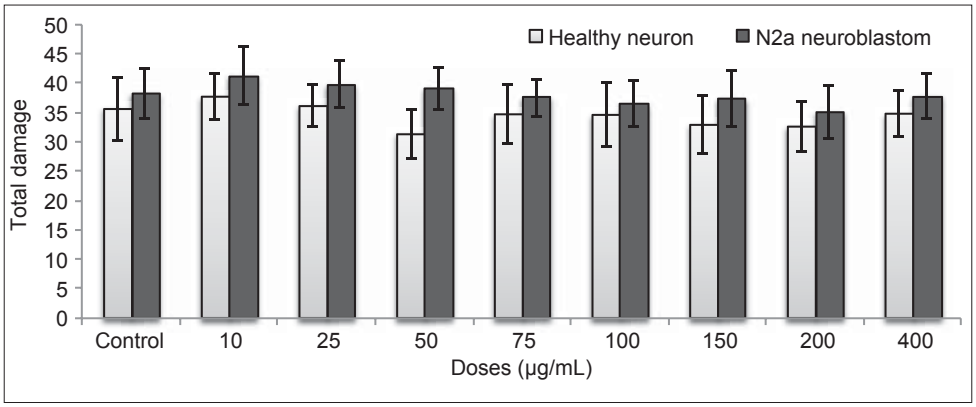

Figure 3: Effect of varying concentrations of GYZ on inducing DNA damage *in vitro* for 24 h. The results are given as the means ± standard deviation from six independent experiments. Compared with control, *$P > 0.05$

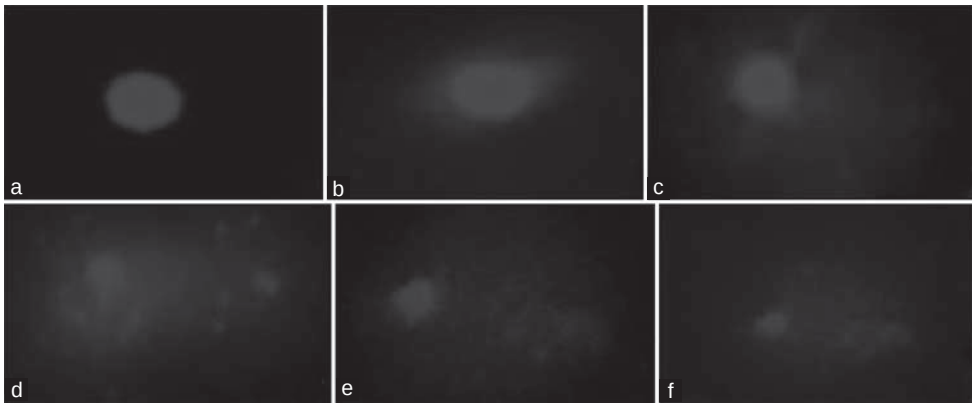

Figure 4: The scoring criteria for determining damage levels in cultured neurons (a) Class 0 (undamaged); (b) Class 1 (slightly damaged); (c) Class 3 (damaged); (d) Class 4 (highly damaged); (e) Class 5 (very highly damaged); (f) Class 6 (extremely damaged)

and 400 μg/mL concentrations of GYZ in cultured N2a-NB cells, respectively.

The induction of DNA damage in rat neuron and N2a-NB cells after exposition to GYZ was investigated using SCGE assay. The obtained results are presented as total damage score in Figure 3. None of the tested GYZ concentrations produced genotoxic effect after 24 h treatments in rat neuron and N2a-NB cells. The scoring criteria for determining DNA damage levels using comet formations in cultured neurons treated with the compounds were shown in Figure 4.

DISCUSSION

This paper describes a comprehensive *in vitro* cytotoxicity, genotoxicity and antioxidant/oxidant capacity assessments of GYZ on rat neuron and N2a-NB cells for the first time. MTT assay, based upon the ability of living cells to reduce MTT into formazan, was used to measure the cytotoxic effect of GYZ on neuron and N2a-NB cells. The results clearly demonstrate that high concentrations of GYZ induced significant cytotoxic effect on the cultured neurons and N2a-NB cell lines. These results conducted to previous studies investigated the cytotoxic effect of GYZ and azulene derivate toward gingival fibroblast, pulp cell, periodontal ligament fibroblast and human tumor cell lines including submandibular gland carcinoma, oral squamous cell carcinoma (HSC-2, HSC-3), promyelocytic leukemia (HL-60 [11,24]. Our findings are also in agreement with the results that have reported that zingiberene (a monocyclic sesquiterpene)exhibited strong antiproliferative effect against N2a-NB cell line by MTT assay [7]. In addition, Turkez *et al*. [8] demonstrated that the copaene (a tricyclic sesquiterpene), showed cytotoxic effect in N2a-NB cells. Oxidative stress is likely to be the common triggers of molecular events underlying its antiproliferative effects. The results of our study indicate that the cytotoxic activity is related to the state of pro-oxidation of GYZ.

In our study, the antioxidant/oxidant effects of GYZ were assessed in this study by the means of TAC and TOS assays. The high concentrations of GYZ caused significant increases ($P < 0.05$)of TOS levels in both cells without changing the TAC levels. In contrast to our findings, the previous studies showed that GYZ has been found to inhibit rat hepatic microsomal membrane peroxidation *in vitro* [25,26]. Similarly, the *in vitro* and *in vivo* effect of GYZ were investigated on rat hepatic cytochrome P450 (CYP)and reported that GYZ inhibited CYP1A2 activity [27]. Furthermore, Vinholes *et al*. [28] reported that GYZ showed higher scavenger capacity against DPPH *in vitro* Caco-2 cell models.

Our findings indicate GYZ is neither genotoxic nor mutagenic on healthy neurons and N2a-NB cells since the observed mean values of the total scores of cells showing DNA damage was not found significantly different from the control values on both cells. *in vitro* or *in vivo* genotoxicity of several sesquiterpenes but not GYZ in different cell types. Therefore, we discussed its genotoxic potential in comparison with other sesquiterpenes. In similar to our findings, Turkez *et al*. [19] reported that the alpha-copaene did not induce genotoxicity in cultured neuron cell lines. In addition, zingiberene did not induce genotoxic damage in cultured neuron and N2a-NB cells [7]. Furthermore, in our recently published paper, using the SCGE assay, we have demonstrated that tricyclic sesquiterpene α-copaene has neither genotoxic nor mutagenic effect neuron and N2a-NB cells [8]. In contrast to the findings, Aquino *et al*. [29] demonstrated that artesunate (a sesquiterpene lactone)induced significant DNA damage in liver cells and high doses of artesunate caused an increase in the mean number of micronucleated polychromatic erythrocytes. Likewise, Orhan *et al*. [30] reported that gossypol showed genotoxic effect in cultured mouse bone marrow cells (as shown by micronuclei index), and human lymphocyte cells (revealed by sister chromatid exchange index). These divergent results suggest the relevance of the chemical structure in the biological effect of sesquiterpenes and indicate as well the importance of using various test models to reach a valid conclusion.

CONCLUSION

In conclusion, our *in vitro* studies suggest that high concentrations of GYZ could be cytotoxic on both cells. The

efficacy of anti-cancer chemotherapy is limited by the cytotoxic effect on healthy neuron cells because of a lack of selectivity of GYZ and poor uptake of the therapeutics by N2a-NB cells.

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