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 fatal 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 dimethylthiazol -2-yl]-2,5 diphenyltetrazolium bromide [MTT] assay), cytogenetic (single cell gel electrophoresis [SCGE] assay)
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, C15H18), Dulbecco modified eagles medium, sodium phosphate (NaH2PO4), potassium phosphate monobasic (KH2PO4), 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% CO2 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% CO2 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 (95:5) 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 Na2-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 Na2-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 Eclipse E600, 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.

Figure 1: Chemical structure of guaiazulene
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.

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 $\mu$g/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 $\mu$g/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 $\mu$g/mL in comparison with control values on rat neuron cell line. Also, 75, 100, 150, 200, and 400 $\mu$g/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,

### 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 ($\mu$g/mL) | Healthy neuron |  |  |  |  | N2a-NB |  |  |  |  |
|-------------------|----------------|---|---|---|---|----------------|---|---|---|---|
|                   | TAC (mmol      | TOS (mmol | TAC (mmol | TOS (mmol | TAC (mmol | TOS (mmol | | | | |
|                   |  Trolox Equiv./L) | $H_2O_2$ Equiv./L) |  Trolox Equiv./L) | $H_2O_2$ Equiv./L) |  Trolox Equiv./L) | $H_2O_2$ 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 | 6.2±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, *$P<0.05$
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 derive 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 artemesunate (a sesquiterpene lactone) induced significant DNA damage in liver cells and high doses of artemesunate 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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REFERENCES

1. Wojtal I, Salm F, Christiansen DG, Cremo I, Cwik P, Shalaby T, et al. Novel agents targeting the IGF-1R/P13K pathway impair cell proliferation and survival in subsets of medulloblastoma and neuroblastoma. PLoS One 2012;7:e47109.

2. Mueller WP, Coppenrath E, Pfugger T. Nuclear medicine and multimodality imaging of pediatric neuroblastoma. Pediatr Radiol 2013;43:418-27.

3. Peri A, Cellai I, Benvenuti S, Luciani P, Baglioni S, Serio M. PPARgamma and genotoxicity of zingiberene on different neuron cell lines amplified neuroblastoma is induced by the combination treatment nutilin-3 and doxorubicin and enhances chemosensitivity. Oncol Rep 2009;22:1443-9.

4. Li Z, Thiele CJ. Targeting Akt to increase the sensitivity of neuroblastoma to chemotherapy: lessons learned from the brain-derived neurotrophic factor/TrkB signal transduction pathway. Expert Opin Ther Targets 2007;11:1611-21.

5. Svensson K, Larson C. A protein kinase Cbeta inhibitor attenuates multidrug resistance of neuroblastoma cells. BMC Cancer 2003;3:10.

6. Togar, B, Tatar, A, Geyikoglu F, Hacimuftuoglu A. Cytotoxic activity of guaiazulene against human oral cancer cell lines. Cytotechnology 2014.

7. Togar B, Tatar A, Geyikoglu F, Hacimuftuoglu A. Cytotoxic activity of azulenes against human oral tumor cell lines. Anticancer Res 2003;23:4747-55.

8. Erel O. A new automated colorimetric method for measuring total antioxidant status. Clin Biochem 2005;38:1103-11.

9. Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem 2004;37:277-85.

10. Perovic A, Perovic S, Erdinger L, Hollert H. Assessment of the genotoxic potential of Lake Skadar sediments using Ames test and comet assay on the fish cell line rlt-1w. Arch Biol Sci Belgrad 2012;64:249-56.

11. Wakabayashi H, Hashiba K, Yokoyama K, Hashimoto K, Kikuchi H, Nishikawa H, et al. Cytotoxic activity of azulenes against human oral tumor cell lines. Anticancer Res 2003;23:4747-55.

12. Kourounakis AP, Rekka EA, Kourounakis PN. Cytotoxic activity of guaiazulene and protection against paracetamol hepatotoxicity in rats. J Pharm Pharmacol 1997;49:938-42.

13. Dovolou E, Clemente M, Amiridis GS, Messinis IE, Kalitsaris A, Gutierrez-Adan A, et al. Effects of guaiazulene on in vitro bovine embryo production and on mRNA transcripts related to embryo quality. Reprod Domest Anim 2011;46:862-9.

14. Kourounakis AP, Rekka EA, Kourounakis PN. Effect of guaiazulene on some cytochrome P450 activities. Implication in the metabolic activation and hepatotoxicity of paracetamol. Arch Pharm (Weinheim) 1997;330:7-11.

15. Vinholes J, Goncalves P, Martel F, Coimbra MA, Rocha SM. Assessment of the antioxidant and antiproliferative effects of sesquiterpenic compounds in in vitro Caco-2 cell models. Food Chem 2014;156:204-11.

16. Aquino I, Perazzo FF, Maistro EL. Genotoxicity assessment of the antimarial compound artesunate in somatic cells of mice. Food Chem Toxicol 2011;49:1335-42.

17. Orhan G, Ekmecki A, Menevse S. The effect of the male contraceptive agent gossypol acetic acid on mouse bone marrow cells in vivo: micronuclei and mitotic index. Contraception 1993;47:377-85.