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Scientific paper

ANTICANCER AND ANTIOXIDANT PROPERTIES OF TERPINOLENE IN RAT BRAIN CELLS

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Terpinolene (TPO) is a natural monoterpene present in essential oils of many aromatic plant species. Although various biological activities of TPO have been demonstrated, its neurotoxicity has never been explored. In this *in vitro* study we investigated TPO's antiproliferative and/or cytotoxic properties using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) test, genotoxic damage potential using the single-cell gel electrophoresis (SCGE), and oxidative effects through total antioxidant capacity (TAC) and total oxidative stress (TOS) in cultured primary rat neurons and N2a neuroblastoma cells. Dose-dependent effects of TPO (at 10 mg L^{-1}, 25 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1}, 200 mg L^{-1}, and 400 mg L^{-1}) were tested in both cell types. Significant (P<0.05) decrease in cell proliferation were observed in cultured primary rat neurons starting with the dose of 100 mg L^{-1} and in N2a neuroblastoma cells starting with 50 mg L^{-1}. TPO was not genotoxic in either cell type. In addition, TPO treatment at 10 mg L^{-1}, 25 mg L^{-1}, and 50 mg L^{-1} increased TAC in primary rat neurons, but not in N2a cells. However, at concentrations above 50 mg L^{-1} it increased TOS in both cell types. Our findings clearly demonstrate that TPO is a potent antiproliferative agent for brain tumour cells and may have potential as an anticancer agent, which needs to be further studied.

KEY WORDS: *antiproliferative agent, comet assay, MTT assay, neurotoxicity, N2a neuroblastoma cell line, oxidative status*

Brain cancers (such as astrocytoma, ependymoma, glioblastoma, oligodendroglioma, and neuroblastoma) are among the most devastating tumours in humans and often progress rapidly to the fatal outcome, despite aggressive treatment (1, 2). Neuroblastoma (NB) is a paediatric cancer originating in the primitive cells of the sympathetic nervous system and is the most common and deadly solid tumour in childhood (3, 4). Being an embryonic tumour, it accounts for 7 % to 10 % of childhood cancers, with an annual incidence of eight per million children under the age of 15 (5, 6). At an advanced stage, the tumour remains aggressive and frequently resistant to chemotherapy in spite of recent advances in multi-modality treatment protocols, with an overall 5-year survival rate of only 30 % to 40 % (7). In adults, the incidence of NB is rather low (8). Random reports in adults with this disease suggest that, at least in some cases, long-term survival may be worse than in younger patients, even with the localised disease, although the course may be longer (9-16).

Oxidative stress has long been confirmed to play a key role in neurodegenerative disorders (17-19). Recent investigations have focused on therapeutic substances capable of reducing the genotoxicity or carcinogenicity of various natural and man-made mutagens. These substances include antibodies, fatty acids, amino acids, minerals, plant extracts, phenolic

compounds, and boron compounds such as boric acid and borax (20-26).

Essential oils are a diverse group of natural products that are largely composed of terpenes (27). Monoterpenes extracted from vegetables, herbs, spices, and fruits (28) have long been used to flavour food and beverages and to enhance perfumes (29). Terpinolene (TPO, 1-isopropenyl-4-methylcyclohex-3-ene) is a monoterpenic constituent of essential oils of plants such as *Melaleuca alternifolia, Melaleuca trichostachya, Manilla elemi*, *Nectranda elaiophora*, and *Dacrydium colensoi* (30-32). TPO has a broad spectrum of biological activities such as anticancer (33), antioxidant (34), antifungal (35), and larvacidal (36). These important biological effects have raised interest in research of the antiproliferative effects induced by several monoterpenes such as carvacrol and α-pinene on human ovarian, hepatocellular, and metastatic breast cancer cells (37, 38). To the best of our knowledge, however, neurotoxic and/or anticancer potentials of TPO in brain cells have never been investigated. Our study was intended to address this gap by evaluating different *in vitro* biological activities of TPO, including cytotoxic, genotoxic and oxidative effects on primary rat neurons and N2a neuroblastoma cells.

MATERIALS AND METHODS

Cell cultures

This study was conducted at the Atatürk University Medical Experimental Research Center (Erzurum, Turkey). The Ethics Committee of Atatürk University approved the study protocol. All procedures were performed in accordance with the National Institute of Health Principles of Laboratory Animal Care (39). Primary rat neuron cultures were prepared from brains of five newborn male Sprague-Dawley rats. The cerebral cortices were dissociated with Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich®, Steinheim, Germany) + Trypsin-EDTA (0.25% trypsin + 0.02 % EDTA; Sigma-Aldrich®), treated with DNAse type 1 (120 units mL^{-1}, Sigma, St Louis®, MO, USA) and centrifuged. After having thrown away the supernatant, neurobasal medium and foetal calf serum (PAN Biotech®, Germany) were added to the residue. Single cell suspension, which was obtained after physical and chemical decomposition, was divided into 3.5 mL samples pipetted into 10 flasks coated with poly-D-lysine dissolved in phosphate buffer solution. The flasks were left in the incubator with 5 % CO_2 at 37 °C. Fresh medium was added every three days until the cells branched out (40).

The rat brain neuroblastoma cell line N2a is widely used as a model for brain cancer. It was obtained from the FMD Institute, Ankara, Turkey. Prior to the experiments, the cells were thawed and grown in tissue culture flasks as a monolayer in Dulbecco's Modified Eagle's medium, (DMEM; Sigma-Aldrich®) supplemented with 1 % glutamine, 0.5 % penicillin/streptomycin (PAN Biotech, Germany) and 10 % foetal bovine serum at 37 °C in an incubator with 95 % air humidity and 5 % CO_2. The cultured cells were trypsinised with Trypsin-EDTA for a maximum of 5 min and seeded with a subcultivation volume ratio of 1:3 to 1:8.

TPO treatment in vitro

Cells were incubated at 37 °C in a humidified 5 % CO_2/95 % air mixture and treated for 24 h with the following TPO ($C_{10}H_{16}$, CAS No. 586-62-9; Fluka, Sigma-Aldrich®) concentrations: 10 mg L^{-1}, 25 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1}, 200 mg L^{-1}, and 400 mg L^{-1}. The selection of the doses was based on earlier studies (41-44).

MTT bioassay

The cells were seeded in 48-well plates incubated at 37 °C in a humidified 5 % CO_2/95 % air mixture and treated with TPO as described above. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) substrate solution was used according to the manufacturer's instructions (Cayman Chemical Company, USA). Briefly, MTT was added to the cell cultures for 3 h. The obtained formazan crystals were dissolved in dimethyl sulphoxide (Sigma-Aldrich, Steinheim, Germany), and the plates analysed using a Microquant reader (Bio-Tek Instruments, USA) at the 570 nm wavelength.

Comet assay

The comet assay was performed and scored according to slightly modified protocols reported in literature (45-51) using the OxiSelect 96-Well Comet Assay kits (Cell Biolabs®, San Diego, CA, USA). Approximately $5x10^3$ neuron cells were suspended in 100 μL^{-1} of agarose and layered on slides. Gel was covered with cover slips and allowed to solidify at

4 °C for 30 min to 60 min. The slides were immersed in freshly prepared cold lysing solution and refrigerated overnight. Followed alkali treatment, electrophoresis, and neutralisation. The slides were then fixed and stained using the Vista Green dye. The whole procedure was carried out in dim light. After coding, the slides were analysed using a Nikon Eclipse E600 fluorescence microscope (Japan) at a magnification of 100x. One well-trained observer (Elanur Aydın) performed all scorings. A total of 100 cells per slide were screened. Total damage score for each slide was calculated by multiplying the number of cells assigned to each grade of damage by the numeric value of the grade and by summing up all grades (giving a maximum possible score of 400, corresponding to 100 cells at grade 4) (52, 53).

TAC and TOS analysis

Total antioxidant capacity (TAC) and total oxidant status (TOS) of cell cultures were analysed using automated commercial kits (Rel Assay Diagnostics®, Gaziantep, Turkey). The major advantage of the TAC assay is that it measures the antioxidant capacity of all antioxidants in a biological sample and not just of a single compound (54). In this test, antioxidants in the sample reduce dark blue-green coloured 2,2'-azinobis-(3- ethylbenzothiazoline-6-sulphonate radical to its colourless form. The change in absorbance at 660 nm corresponds to the total antioxidant level in a sample. The assay was calibrated with a stable antioxidant standard solution of vitamin E analogue, (Trolox-equivalent) (55). The TOS assay used here is based on the oxidation of the ferrous ion-chelator complex to ferric ion (Fe^{3+}), which is mediated by oxidants contained in the tested sample. The reaction is further enhanced by other molecules from the reaction medium. The reaction of Fe^{3+} with chromogen in an acidic medium produces a coloured complex. Its intensity corresponds to the total amount of oxidants in the sample and can be measured spectrophotometrically. The TOS assay was calibrated with hydrogen peroxide (56), and the results are expressed in terms of micromolar hydrogen peroxide equivalent per litre.

Statistical analysis

The obtained data were analysed using SPSS for Windows version 18.0 statistical software (SPSS Inc., Chicago, IL, USA). Differences between the groups were compared using the one-way analysis of variance (ANOVA) and Duncan's post-hoc test. The significance level was set at $P<0.05$.

RESULTS

Antiproliferative activity

The effects of TPO on cultured primary rat neurons and N2a neuroblastoma cells are shown in Figure 1. The addition of TPO at concentrations below 100 mg L^{-1} for 24 h did not cause any change in cell viability in primary rat neurons. However, at higher doses, (100, 200, and 400) mg L^{-1}, it became cytotoxic. In N2a neuroblastoma cells, TPO treatments started to show antiproliferative effects at the concentrations of 50 mg L^{-1} and above.

Genotoxicity

In vitro exposure to TPO of either cell type did not result in comet formation, regardless of the dose, indicating the non-genotoxic nature of TPO (Figure 2).

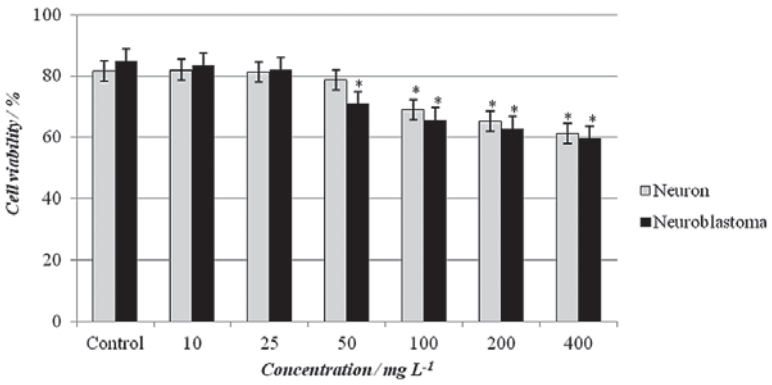

Figure 1 *Cytotoxic effects of TPO on cultured primary rat neurons and N2a neuroblastoma cells.*
* P<*0.05* vs. *control*

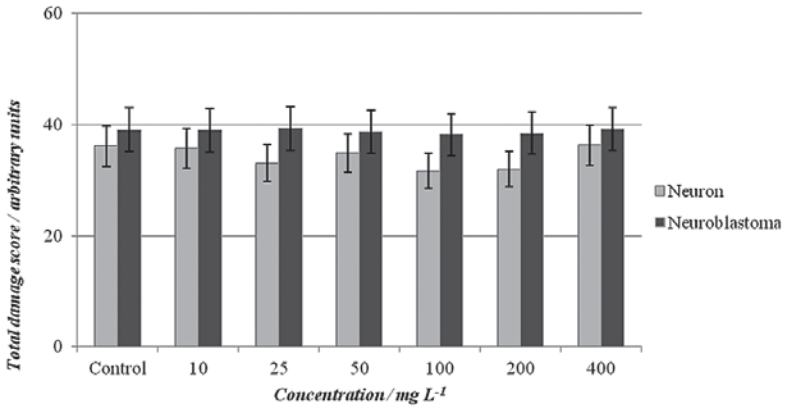

Figure 2 *DNA damage induced by varying concentrations of TPO over 72 h*

Antioxidant activity

TPO at the concentrations of 100 mg L^{-1} and 200 mg L^{-1} did not affect TAC in primary rat neuron cells, increased it significantly at the concentrations of (10, 25, and 50) mg L^{-1}, and decreased it significantly at the highest concentration (400 mg L^{-1}) compared to control (Table 1). Similarly, in N2a neuroblastoma cells TPO (at 10 mg L^{-1} and 25 mg L^{-1}) did not change TAC levels, but decreased them significantly at (50, 100, 200, and 400) mg L^{-1}, compared to control.

On the other hand, TOS levels increased at (100, 200 and 400) mg L^{-1} of TPO in primary rat neurons, but had no effect at doses lower than 100 mg L^{-1}. In N2a neuroblastoma cells, TOS significantly rose with TPO concentrations of 50 mg L^{-1} and above.

DISCUSSION

Terpinolene and other monoterpenes are lipophilic and highly soluble in blood (57). Inhaled monoterpenes are absorbed almost entirely by the human and animal lungs and then delivered to the liver, where they are completely metabolised by detoxification enzymes (58-61). In addition, Igimi et al. (58) have shown that orally administered limonene, a monoterpene similar to terpinolene, is highly absorbed in the intestine. Although there is no information about TPO metabolites in literature, limonene has been shown to metabolise to oxygenated metabolites in rats and in humans (62). Metabolites of limonene in serum are perillic acid and dihydroperillic acid in rats (63) plus limonene-1,2-diol additionally produced in humans (64). In urine, glycine and glucuronide conjugates of perillic acid and uroterpenol (p-mentha-8,9-diol) have been detected in many limonene-fed mammals (65, 66). Yeruva et al. (67) reported that perillic acid elicited dose-dependent cytotoxicity, induced cell cycle arrest and apoptosis, and increased the expression of bax, p21, and caspase-3 activity in non-small cell lung cancer lines (NSCLC, A549, and H520). In bone

Table 1 *Total antioxidant capacity (TAC) and total oxidative stress (TOS) levels in cultured primary rat neurons and N2a neuroblastoma cells exposed to TPO for 24 h*

Treatments with TPO / mg L^{-1}	Primary rat neurons		N2a neuroblastoma cells	
	TAC (Trolox Equiv. / mmol L^{-1})	TOS (H_2O_2 Equiv. / mmol L^{-1})	TAC (Trolox Equiv. / mmol L^{-1})	TOS (H_2O_2 Equiv. / mmol L^{-1})
Control	34.5±5.0	2.1±0.3	5.9±0.6	2.8±0.4
10	38.2±5.2*	1.9±0.3	5.5±0.7	2.6±0.3
25	38.9±5.1*	2.0±0.1	6.1±0.7	2.9±0.3
50	39.5±4.9*	2.1±0.2	4.9±0.6*	3.4±0.5*
100	34.8±5.0	2.4±0.2*	4.5±0.5*	3.7±0.6*
200	34.1±4.8	2.7±0.1*	3.9±0.5*	3.9±0.4*
400	27.2±4.7*	2.9±0.3*	3.5±0.6*	4.0±0.5*

* P<*0.05* vs. *control*

marrow of mice exposed to gamma radiation it reduced DNA damage (68).

Because of the widespread use of TPO as a synthetic food flavouring additive or fragrance enhancer in perfumes, some terpene-based air fresheners, and cleaners, the potential for human exposure is high (69, 70). Our findings that TPO significantly reduces cell viability in primary rat neurons and N2a neuroblastoma cells are in agreement with earlier reports of the cytotoxic effects of several monoterpenes such as d-limonene (on human normal epithelial prostate PZ-HPV-7 cells), α-pinene, myrcene, linalool (on Vero monkey kidney cells), and stylosin (on human foetal fibroblast cells) (71-73). Likewise, Chen et al. (74) reported a strong anticancer effect of α-terpinolene against breast and cervical cancer cell lines. In a recent study by Okumura et al. (70), TPO reduced protein expression of AKT1 in K562 human leukemic cells and inhibited cell proliferation. In turn, Matsuo et al. (75) showed that α-pinene was able to induce apoptosis, as evidenced by early disruption of the mitochondrial potential, production of reactive oxygen species, and increased caspase-3 activity in B16F10 murine melanoma cells. While the exact mechanism of the cytotoxic action of TPO is not known, it is mainly attributed to oxidative stress. In addition to oxidative stress, a number of studies (76-83) have associated the cytotoxicity of plant products with the following mechanisms: proteasome inhibition, topoisomerase inhibition, inhibition of fatty acid synthesis, accumulation of p53, induction of cell cycle arrest, inhibition of phosphatidyl-inositol 3-kinase, and enhanced expression of c-fos and c-myc.

Our findings also indicate that TPO is not genotoxic to primary rat neurons and N2a neuroblastoma cells. Similarly, Turner et al. (84) reported that limonene was not genotoxic and mutagenic in male Big Blue rats. Slamenova et al. (85) reported that chromosomal aberration assay (CA) in rat primary hepatocytes did not confirm any genotoxic activity of carvacrol and thymol. Gomes-Carneiro et al. (86) also found no mutagen activity of β-myrcene, α-terpinene, and (+) and (−)-α-pinene in *Salmonella typhimurium* TA100, TA98, TA97a, and TA1535 strains. Horvath et al. (87) reported that carvacrol and thymol significantly reduced DNA damage induced by hydrogen peroxide (H_2O_2) in K562 cells. Mademtzoglou et al. (88) found no genotoxic effects with *S*-(+)-carvone, while *R*-(−)-carvone turned out to be a potent mutagen even at low concentrations in *Drosophila melanogaster*. In contrast to our findings, National Toxicology Program reported d-carvone-induced changes in sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells (89).

Research into the use of antioxidants in cancer treatment has shown a rapid progress. Antioxidants are extensively studied for their ability to prevent or treat cancer in humans (90). Research has so far showed that monoterpenes such as myrtenal, vallesiachotamine, geraniol, terpinen-4-ol, and linalool exhibit anticancerogenic properties in different experimental models (liver, melanoma, prostate, and non-small cell lung and breast cancers) (91-95). TPO was also suggested to have an important *in vitro* antitumour effect against K562 human leukemic (70), breast, and cervical cancers (71) and *Ehrlich ascites* carcinoma cells (96).

Several experimental and epidemiological studies have confirmed the greater or lesser anticancer potentials of a variety of antioxidants (97-99). Furthermore, regular intake of natural antioxidants is associated with reduced risk of cancer (100-102). The antioxidant activity of TPO in our study is similar to the findings by Bourgou et al. (103) in normal human skin fibroblast (WS1) cells. Other recent studies have also demonstrated the antioxidant properties of TPO using 2,2-diphenyl-1-picrylhydrazyl (104), hexanal/hexanoic acid assay (105), thiobarbituric acid reactive species (96), and beta-carotene agar diffusion methods (34). In our study, however, TPO at 400 mg L^{-1} significantly lowered TAC, and at concentrations higher than 50 mg L^{-1} increased TOS in healthy neurons. Furthermore, at 25 mg L^{-1} it significantly lowered TAC and increased TOS in cancer (neuroblastoma) cells. These findings support earlier findings by Lima et al. (106) that high concentrations of monoterpenoid compounds could lead to neurotoxicity by reducing total glutathione levels and by lactate dehydrogenase leakage of primary rat hepatocytes.

CONCLUSION

To sum up, TPO was not genotoxic in experimental conditions applied in this study, but it showed antioxidant and antiproliferative properties. These preliminary *in vitro* findings suggest that TPO is a good and safe natural antioxidant and a potential anticancer agent. However, its dose should be carefully adjusted since it is an ingredient in food and is used

for pharmaceutical purposes. Future studies, especially on animal models, should further elucidate its possible use in the biotherapy of cancer.

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Sažetak

PROTUTUMORSKA I ANTIOKSIDATIVNA SVOJSTVA TERPINOLENA U MOŽDANIH STANICA ŠTAKORA

Terpinolen (TPO) prirodni je monoterpen prisutan u esencijalnim uljima mnogih aromatskih biljaka. Premda su otprije poznate razne biološke aktivnosti TPO-a, dosad nije ispitana njegova neurotoksičnost. Svrha je ovog istraživanja *in vitro* bila utvrditi antiproliferacijska i/ili citotoksična svojstva TPO-a pomoću testa 3-(4,5-dimetiltiazol-2-yl)-2,5 difeniltetrazolijeva bromida (MTT), njegov genotoksični potencijal pomoću komet-testa te oksidativno djelovanje kroz ukupni antioksidativni kapacitet i ukupni oksidativni stres u uzgojenim primarnim neuronima štakora i N_2a stanicama neuroblastoma. U objema staničnim linijama ispitani su učinci TPO-a u skladu sa sljedećim dozama: 10 mg L^{-1}, 25 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1}, 200 mg L^{-1} i 400 mg L^{-1}. Značajni ($p<0.05$) pad stanične proliferacije u primarnim neuronima štakora zamijećen je pri dozama od 100 mg L^{-1} naviše, a u N_2a stanicama neuroblastoma pri dozama od 50 mg L^{-1} naviše. Niti u jednoj staničnoj liniji TPO se nije pokazao genotoksičnim. Usto se primjenom TPO-a pri dozama od 10 mg L^{-1}, 25 mg L^{-1} i 50 mg L^{-1} povećao ukupni antioksidativni kapacitet primarnih štakorskih neurona, ali je takvo djelovanje izostalo u N_2a stanica. Međutim, pri koncentracijama višim od 50 mg L^{-1} TPO je povećao ukupni oksidativni stres u objema staničnim linijama. Naši rezultati nedvojbeno pokazuju da je TPO snažan antiproliferacijski agens u tumorskih stanica mozga, a njegovu potencijalnu ulogu kao protutumorskog lijeka trebalo bi dalje istraživati.

KLJUČNE RIJEČI: *antiproliferacijski agens, komet test, MTT, neurotoksičnost, N_2a stanice, neuroblastom, oksidacijski status*

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