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Original Article

Effect of *Melaleuca alternifolia* oil on cytotoxicity and neuropeptide y gene expression

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ABSTRACT

Background and Aims: This study aims to investigate the effect of tea tree oil (TTO), which is an oil produced by steam distillation of the *Melaleuca alternifolia* (Maiden & Betche) Cheel plant terminal branches and leaves, on changes in neuropeptide Y (NPY) gene expression in SH-SY5Y neuroblastoma cells.

Methods: The first stage of this study investigates the cytotoxic/proliferative effects of TTO solutions prepared at different concentrations on the SH-SY5Y cell line using the tetrazolium reduction (MTT) assay. The next stage analyzes the effects of the determined TTO concentrations on NPY gene expression using the real-time polymerase chain reaction (qPCR) method.

Results: TTO concentrations prepared at a ratio of 1:32, 1:64, and 1:28 (v/v) showed statistically significant effects on cells in the cytotoxicity test and were used in the gene expression analysis. The highest significant gene expression change was seen in the cells in which the TTO solution had been applied at a ratio of 1:64 for 24 hours. NPY gene expression in these cells was determined to have increased 2.24 times compared to control cells.

Conclusion: Upon evaluating the results from the MTT assay and gene expression analysis, the solutions of the cells prepared at different TTO ratios were determined to have caused changes in gene expression. Future studies will be able to reveal all affected molecular pathways by increasing research involving TTO.

Keywords: Gene expression, Neuroblastoma, NPY, SH-SY5Y, Tea tree oil

INTRODUCTION

Tea tree oil (TTO) is an essential oil obtained mainly from the Australian endemic plant *Melaleuca alternifolia* (Maiden & Betche) Cheel. TTO is produced through steam distillation of the terminal branches and leaves of *M. alternifolia*. Used largely for its anti-microbial properties, TTO has also been included as a major ingredient in many formulations used to treat skin infections. It is widely available in the Australian, European, and North American markets and is marketed as a medicine for a variety of ailments (Carson, Hammer, & Riley, 2006). TTO consists of terpene hydrocarbons to which monoterpenes, sesquiterpenes, and alcohols are attached. TTO has a relative density of 0.885-0.906 g/mL at 25ºC (Kumari, 2013), is only slightly soluble in water, and is miscible with non-polar solvents (Carson, et al., 2006). Studies conducted between 1940-1980 in the literature have revealed TTO's anti-bacterial (Atkinson & Brice, 1955; Low, Rawal & Griffin, 1974) and later its anti-fungal (Inouye et al., 1998; Inouye et al., 2000; Inouye, Uchida, & Yamaguchi, 2001) effects. Although studies are found on the anti-microbial and anti-inflammatory properties of TTO, few studies have been done regarding the oil's toxicity and safety. Some cases of toxic effects have been reported as a result of the dermal application of TTO on cats and dogs. Typical observed symptoms are muscle tremors, incoordination, weakness, and depression (Villar, Knight, Hansen, & Buck,1994). Another

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study that dermally applied TTO on dogs and cats showed 443 cases to cause depression, weakness, coordination disorder, and muscle tremors (Khan, McLean, & Slater, 2013). One case study showed a 4-year-old child who'd swallowed a small amount of TTO to have come down with ataxia and loss of consciousness (Morris, Donoghue, Markowitz, & Osterhoudt, 2003).

Neuropeptide Y (NPY) is a peptide found in the autonomic nervous system and brain and is associated with depression, anxiety, obesity, learning and memory, epilepsy, sleep, and circadian rhythms. NPY has recently received much attention as an endogenous anti-epileptic and anti-depressant agent, because drugs with anti-epileptic or mood-stabilizing properties can reduce or increase the seizure threshold by changing NPY concentrations. NPY is also a tumor biomarker for neuroblastomas (Farrelly, Savage, O'Callaghan, Toulouse, & Yilmazer-Hanke,2013). This study has chosen to use the SH-SY5Y (ATCC® CRL-2266™) cell line that originates from malignant neuroblastoma and expresses epithelial morphology because it contains many neuron cells responsible for NPY synthesis (Kitlinska, 2007; Silva, Cavadas, & Grouzmann, 2002).

As seen, a few studies have examined TTO in the literature, and it has become an increasingly popular topical antiseptic found in many products in recent years. Due to its anti-fungal and anti-microbial properties, TTO is applied in the cosmetics industry without a warning label on market products used for various purposes, especially acne treatment and fungal infections, and people use it without being aware of it. However, upon examining the limited number of studies in the literature, TTO is seen to have a potential toxicity. In addition to its toxicity, case studies are also available in the literature that have shown this component to cause acute ataxia in humans, as well as scientific articles that have shown it to act as a depression-triggering agent when used long-term.

Investigating the potential toxicity of TTO, which has a wide range of uses in the cosmetics industry from skin creams to perfumes and soaps, and showing whether it affects the expression of genes involved in various cellular processes are important for public health.

This study investigates the effects of TTO, which is used in various cosmetic and ointment formulations due to its properties, with regard to neuroblastoma cells using cytotoxicity studies and NPY gene expression analyses. The study aims to contribute to the literature in this way by revealing the cytotoxicity of TTO and its effect on NPY gene expression, about which insufficient information is found.

MATERIALS AND METHODS

Mammalian cell culture and cytotoxicity assay

The cytotoxic activity of different concentrations of TTO (purchased from Sigma-Aldrich [Cat. No. W390208]) was assessed on the human neuroblastoma cell line (SH-SY5Y, ATCC CRL-2266). The SH-SY5Y cell line was obtained from Associate Professor Belkıs Atasever Arslan (Üsküdar University, Faculty of Engineering and Natural Sciences) as a gift.

The study uses the mammalian cell culture method alongside the tetrazolium reduction (MTT; Sigma, M-5655) assay, as previously described by Kaya, Atasever-Arslan, Kalkan, Gür, & Ülküseven,. (2016). The cell culture was incubated 24 h before each treatment. 10 µL of sterile 0.5% v/v DMSO-DMEM (Gibco, 41966) was used in place of TTO as a negative control, and the cell viability for this sample was regarded as 100%. Cells that were treated with different concentrations of TTO were incubated for 24 h and 48 h. The MTT assay results are given in the Figure 1.

The cell viability was calculated as the percentage of viable cells in the experimental group (exp.) versus the untreated (negative) control group (cont.) using the following formula, where A = absorbance of related groups:

$$\text{Cell viability (\%)} = (A_{exp.} / A_{cont.}) \times 100 \quad (1)$$

Statistical analysis of cytotoxicity assay

Two independent experiments with at least three repeats were carried out, and the results were evaluated using the program GraphPad Prism® 8. One-way analysis of variance (ANOVA) and Dunnett's test were used in order to determine the differences between the groups. The limit of significance was accepted as $p < 0.5$.

Total RNA isolation and gene expression analysis with Real-Time PCR

A commercial Biobasic E210 RNA Mini Preps Kit (Markham ON, Canada) was used for total RNA isolation, with the kit's recommended procedures being followed. The obtained RNAs were stored at -80°C. Purity and concentration measurements for all the RNAs obtained from the SH-SY5Y cells were performed with the Implen NanoPhotometer (CA, USA) instrument.

The OneScript cDNA Synthesis Kit (BC, Canada) was used for cDNA synthesis based on the isolated total RNA. Blastaq 2x qPCR MasterMix (BC, Canada) was used for the real-time polymerase chain reaction (qPCR) method. This kit's recommended procedures were followed.

The GAPDH housekeeping gene was used as a control gene. The primer sequences of the target NPY gene and GAPDH control gene are given in Table 1.

Table 1. Primer sequences of NPY and GAPDH genes.

Gene	Forward Primer	Reverse Primer	Product Size
NPY	3′- GAGTTTGGGCAAGAAGGGAGA -5′	3′- GCTCCACCTGAAAACTTCGC -5′	156 bp
GAPDH	3′- AGGGCTGCTTTTAACTCTGGT -5′	3′- CCCCACTTGATTTTGGAGGGA -5′	425 bp

RESULTS

Cell viability effects of *M. alternifolia* oil and its serial dilutions on SH-SY5Y Cells

This study has investigated the effects of TTO and its serial dilutions (different concentrations) on the cell viability of human neuroblastoma cell lines (SH-SY5Y).

The results obtained as a result of the 24 h exposure of SH-SY5Y cells with TTO prepared in pure and other various dilution ratios are given in Figure 1A. The highest test concentration (Absolute TTO) of TTO was applied for 24 h, and its inhibition percentage was 61.23 ± 12.53%. The cytotoxic effect of the 1:1 dilution was calculated as 64.95 ± 12.52%. The proliferative effects of the six different dilutions [1:2, 1:4, 1:16, 1:32, 1:64, and 1:128(v/v)] were determined respectively as 121 ± 12.53%, 129 ± 12.52%, 150.96 ± 12.44%, 337.3 ± 14%, 342± 12.5%, and 288 ± 14.02%. Neither cytotoxic nor proliferative activity was observed in the other dilution (1:8; $p > 0.05$).

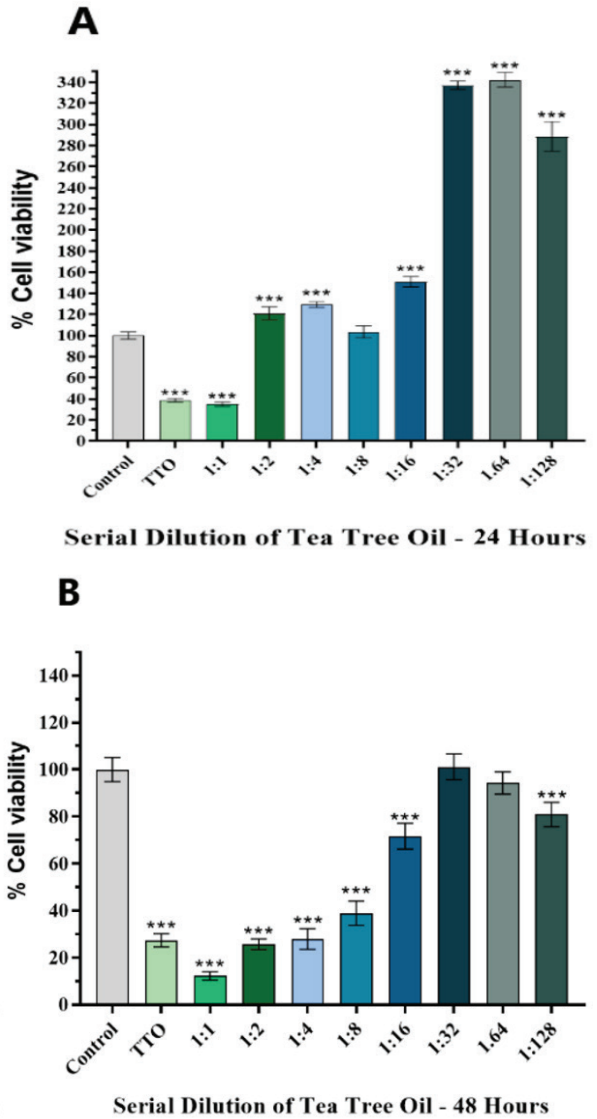

Figure 1. Comparing the effects of TTO applied on SH-SY5Y cells at different rates by volume for 24 h (A) and 48 h (B) regarding % viability (***p < 0.001, vertical bars show standard deviation values).

The results obtained after 48 h of exposure to SH-SY5Y cells with pure and prepared TTO at different dilution ratios are given in Figure 1B. The highest test concentration was TTO for 48 h, and its inhibition percentage was 72.64 ± 10.54 %. The cytotoxic effect of the six dilutions [1:1, 1:2, 1:4, 1:8, 1:16, and 1:128 (v/v)] were determined respectively as 87.75 ± 9.83%, 74.30 ± 9.84, 72.05 ± 9.83, 61.18 ± 9.84, 28.31 ± 9.83, and 19.07 ± 9.87%. Neither cytotoxic nor proliferative activity was observed in the other dilutions [1:32 and 1:64 (v/v); $p > 0.05$].

The TTO concentrations of 1:32, 1:64, 1:128 (v/v) as determined according to the cytotoxicity assay were given to the cells for 24 h and 48 h. After incubation with TTO, the total RNA isolation from the cells was performed.

Gene expression analysis

NPY gene expression levels were determined in cells exposed to different TTO concentrations for different durations. The rates of gene expression change (fold change values) determined after the 2^-ΔΔCt analysis are given in Table 2.

Table 2. Fold change values of target gene according to different TTO concentrations and durations.

Sample	Fold Change
TTO 1:32 24 h	1.14
TTO 1:32 48 h	0.45
TTO 1:64 24 h	2.24
TTO 1:64 48 h	0.40
TTO 1:128 24 h	0.13
TTO 1:128 48 h	0.01

The fold change values of NPY gene expression in cells exposed to TTO at a ratio (v/v) of 1:32 for 24 h and 48 h, were 1.14 and 0.45, respectively. The fold change values of NPY gene expression in cells exposed to TTO at a ratio of 1:64 for 24 h and 48 h, were 2.24 and 0.40, respectively. The fold change values of NPY gene expression in cells exposed to TTO at a ratio of 1:128 for 24 h and 48 h, were 0.13 and 0.01, respectively.

According to the real-time PCR results, the 24 h 1:32 and 1:64 TTO applications were observed to have a greater effect on NPY gene expression compared to the other concentrations.

DISCUSSION

NPY regulates the behavioral consequences of stress through its activities in the brain (Heilig, 2004). One of the reasons why the behavioral anti-stress effects of NPY are important is that similar effects have been observed in a wide variety of animal models (Fendt & Fanselow, 1999; Sajdyk, Vandergriff, & Gehlert,1999). This indicates the potential effect of NPY on behavioral stress responses to be a common mechanism in many organisms.

Behavioral studies in genetically modified animals also support this hypothesis, with studies observing increased emotionality upon inactivation of NPY transmission, whereas the opposite

was observed when NPY signaling was over-activated (Heilig, 2004). Studies on rats (Stogner & Holmes, 2000) and mice (Redrobe, Dumont, Fournier, & Quirion, 2002) have shown the central NPY signals to have an anti-depressant-like effect.

With regard to the suggested organization of the corticotropin releasing factor (CRF) and NPY signaling process within the amygdala concerning fear and stress responses, various stress factors are seen to initiate a rapid release of CRF in the amygdala and facilitate the emergence of the stress response. NPY release, which begins at a later stage, is thought to mediate the termination of the acute response or act as a coping mechanism during repeated/prolonged exposure to stress (Heilig, 2004).

In the brain, some regions in the septum, especially the amygdala, hippocampus, and locus coeruleus are involved in regulating the anti-stress effects of NPY (Heilig & Murison, 1987; Naveilhan et al., 2001).

Studies are also found to have shown NPY to be involved in the neuroinflammation process (Álvaro et al., 2007; Barnea, Roberts, Keller, & Word, 2001). NPY and neuropeptide Y (Y1) receptor levels are elevated following microglia activation, as demonstrated in an endotoxin-mediated model of inflammation in a microglial cell line (Ferreira et al., 2010). This increase in NPY gene expression is thought to be a feedback mechanism for preventing the inflammatory response (Ferreira et al., 2010). *In vitro* studies have shown NPY to inhibit microglial motility through Y1 receptor activation, to inhibit phagocytosis, and to limit the effect of the inflammatory response by reducing proinflammatory cytokines and reactive oxygen species production (Duarte-Neves, Pereira de Almeida, & Cavadas, 2016).

These data in the literature reveal the potential the NPY system has to be a target for pharmacological treatments of stress-related disorders, including anxiety and depression.

Although studies on TTO in this area are limited in the literature, an examination of current studies shows TTO application to reduce tumor sizes by 80% in mice with glioblastoma (Arcella & Sanchez, 2021). In line with the results obtained from cell viability tests, the 24 h and 48 h exposures were observed to cause serious differences in cell viability when compared to similar rates, with the 24 h application of the TTO mixture prepared by diluting at a ratio of 1:2 showing a 21% proliferative effect, while the 48 h application showed a 74.30% cytotoxic effect. A similar situation was observed in the 24 h (29% proliferative) and 48 h (72.05% cytotoxic) applications prepared with the 1:4 dilutions. While the 24 h application of the TTO mixture prepared at a ratio of 1:128 (v/v) had a proliferative effect of 88% on the SH-SY5Y cells, the 48 h application showed a low toxic effect (19.07%). These results emphasize the importance of exposure time regarding the cytotoxic or proliferative effect of the relevant chemical.

In addition, when examining the results obtained from the 24 h exposure period, the inductive or inhibitory effect of the solutions obtained by diluting the same substance at different percentages with regard to cell proliferation did not vary linearly based on the dilution rate. This situation is explained by the phenomenon referred to in the literature as the hormetic dose response, which is expressed as different effects in living things as a result of different chemicals being taken into the cell/living body at varying amounts. Within the scope of this study, different proliferative or cytotoxic effects were determined for different application rates of the same substance. The literature has also shown regarding different cell culture studies that different application concentrations of the same molecule can cause different responses in a cell (Bao, Wang, Zhou, & Sun, 2014; Hayes, 2007; Wang, Calabrese, Lian, Lin, & Calabrese, 2018).

When evaluating the real-time PCR results, the 24 h 1:32 and 1:64 TTO applications were observed to have a greater effect on NPY gene expression compared to the other concentrations. While TTO at a ratio of 1:32 and applied for 24 h increased NPY gene expression 114%, TTO at a ratio of 1:64 increased the NPY gene expression 224% in the experiment group, both compared to the control group.

The data obtained within the scope of this study is thought to contribute to the literature on TTO, which has wide daily use through various drugs and cosmetic products. The study has determined the inducing and inhibitory effects of TTO and solutions prepared by diluting it with various ratios of SH-SY5Y cells. The study also investigated the effect of selected concentrations on NPY gene expression. When considering the cellular mechanisms in which the NPY gene takes an active role, investigating the effect of TTO on NPY gene expression is considered important. According to the results of the cytotoxicity test, TTO was additionally determined to inhibit cell proliferation at various concentrations.

CONCLUSION

The motivation underlying this study was to determine the effects of TTO on cytotoxicity and NPY gene expression. Excessive use of this substance in humans, consciously or unconsciously, is thought to be able to result in toxicity. As the present study shows, the use of TTO at certain ratios changes gene expression and affects molecular processes. Increasing the number of studies on TTO, which has a limited number of publications in the literature, determining the cytotoxicity of TTO with regard to different cells, and investigating its effect on gene expression will shed light on future studies.

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