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RESEARCH ARTICLE

Is UR-144 Synthetic Cannabinoid Toxic to The Neuronal Cells: An *In Vitro* Evaluation

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SUMMARY

The use of synthetic cannabinoids (SCs) increases dramatically among youth from different cultures. UR- 144 is one of these SCs that was reported in 2012 for its first abuse. Case reports indicate symptoms of neuronal toxicity following UR-144 treatment, while several laboratory studies have demonstrated that UR-144 induces apoptosis and oxidative stress in various cell lines. However, none of those studies evaluate the effects of UR-144 in neuronal cells. This study aims to explore the molecular impacts of UR-144 on human neuroblastoma (SH-SY5Y) cells. MTT and neutral red uptake (NRU) assays were performed to evaluate the cytotoxicity. Oxidative stress was examined using the total antioxidant capacity test (TAC) and the changes in reactive oxygen species (ROS) and malondialdehyde (MDA) levels. The Comet assay was used to assess DNA damage. Results indicate that UR-144 did not cause cell death after 24 hours of treatment by MTT test. However, the cell death ratio at 50 µM was 19.34%. UR-144 significantly decreased the levels of ROS and MDA in the cells, but no change in TAC levels was noticed. The comet assay results indicate no genotoxicity of UR-144 in SH-SY5Y cells. While these results indicate no significant toxicity of UR-144 SCs, further studies should be planned to illuminate the mechanism underlying the neuronal symptoms in UR-144 cases and approve or disapprove these results.

Key Words: *Synthetic cannabinoids, UR-144, neurotoxicity, oxidative stress.*

Sentetik Kannabinoid UR-144 Nöronal Hücreler İçin Toksik Midir? Bir In Vitro Değerlendirme.

ÖZ

Sentetik kannabinoidlerin (SK) kullanımı, farklı kültürlerdeki gençler arasında önemli ölçüde artmaktadır. UR-144, bu SK'lerden biridir ve ilk kez 2012'de kötüye kullanımı rapor edilmiştir. Vaka raporları, UR-144 maruziyetinden sonra nöronal toksisite belirtilerine işaret ederken, çeşitli laboratuvar çalışmaları UR-144'ün farklı hücre hatlarında apoptoz ve oksidatif stresi indüklediğini göstermektedir. Ancak bu çalışmalardan hiçbiri UR-144'ün nöronal hücreler üzerindeki etkileri değerlendirmemektedir. Bu çalışmada, UR-144'ün insan nöroblastoma (SH-SY5Y) hücrelerinde moleküler etkilerinin araştırılması amaçlanmıştır. Sitotoksisiteyi değerlendirmek için MTT ve nötral kırmızı tutulum (NRU) testleri yapılmıştır. Oksidatif stres, toplam antioksidan kapasite testi (TAC) ile değerlendirilmiş ve reaktif oksijen türleri (ROS) ile malondialdehit (MDA) seviyelerindeki değişimler ölçülmüştür. DNA hasarını değerlendirmek için Comet testi kullanılmıştır. Sonuçlar, MTT testine göre UR-144'ün 24 saat maruz kalımından sonra hücre ölümüne neden olmadığını göstermektedir. Ancak 50 µM'de hücre ölüm oranı %19.34'tür. UR-144 hücrelerdeki ROS ve MDA seviyelerini önemli ölçüde azaltırken, TAC seviyelerinde bir değişiklik fark edilmemiştir. Comet testi sonuçları, UR-144'ün SH-SY5Y hücrelerinde genotoksisiteye neden olmadığını göstermektedir. Bu sonuçlar UR-144'ün nöronal hücreler üzerinde önemli bir toksisitesi olmadığını gösterse de, UR-144 vakalarındaki nöronal semptomların altında yatan mekanizmayı aydınlatmak ve bu sonuçları doğrulamak veya reddetmek için daha fazla çalışma planlanmalıdır.

Anahtar Kelimeler: *Sentetik kannabinoidler, UR-144, nörotoksisite, oksidatif stres.*

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INTRODUCTION

According to the World Health Organization (WHO), "substance abuse refers to the harmful or hazardous use of psychoactive substances, including alcohol as well as illicit drugs". "Psychotropic substance" is defined as a chemical substance that acts primarily upon the central nervous system, where it alters the person's brain function, resulting in temporary changes in the person's level of awareness, mood or attitude, consciousness, and demeanor. WHO further explains that psychoactive substance use can lead to dependence or an addictive syndrome, characterized by a cluster of behavioral, cognitive, and physiological phenomena that develop after repeated substance use (Atumeyi, Ligom & Tivkaa 2021; Bulska et al., 2020; Modrzyński, Pisarska, & Mańkowska 2022). New psychoactive substances or "legal highs" are synthetic compounds engineered to mimic the effects of illegal drugs like cocaine, ecstasy, amphetamines, and cannabis. Synthetic cannabinoids are a class of legal highs, designed to mimic the psychoactive effects of delta-9-tetrahydrocannabinol (THC), the primary active compound in **Cannabis sativa** (marijuana) (O'Hagan & Smith 2017). The use of *Cannabis sativa* (marijuana) as a hallucinogenic agent dates back to ancient human history. Although the identification of cannabinoid (CB) receptors was at the end of the 1980s, the research to develop chemicals that work similarly to THC was started in the 1960s, and the aim was to develop analogs with high analgesic and anti-inflammatory properties and low toxicity and side effects (Cacciola et al., 2010; Musselman & Hampton 2014).

Opposite to the expectations, synthetic cannabinoids show higher hallucination and psychological effects compared to the *Cannabis sativa*. In addition to their high addiction potential, synthetic cannabinoids such as JWHs and others were reported to cause toxic effects on the cardiovascular, kidney, lung, and neuronal systems (Riederer et al., 2016). While acute toxicity is the primary concern, some studies have highlighted the chronic toxicity effects of synthetic cannabinoids, including psych-symptoms, change in the emotional state, damage in the nervous tissue, and changes in the release of the neurotransmitter, carcinogenic potential (Evren & Bozkurt 2013) and reproductive toxicity as depresses spermatogenesis are examples of this chronic effects (Cacciola et al., 2010)

UR-144 (indole-3-yl cycloalkyl ketone; Figure 1.) is one of the synthetic cannabinoids that developed in 2006. It was reported due to abusive use for the first time in 2012 (Al-Matrouk, Alqallaf, AlShemmeri, & BoJbarah 2019; Krotulski, Cannaert, Stove, & Loganet 2021). UR-144 has been used alone or as a mixture with other herbs, tobacco, or other psychoactive drugs (Labay et al., 2016; Adamowicz et al., 2017). In general, UR-144 is primarily used in the form of cigarettes; however, some reports also suggest its use through vapor inhalation and oral consumption (Adamowicz & Lechowicz 2015; Adamowicz et al., 2017). Due to its distinct chemical structure compared to other synthetic cannabinoids, it became a ''legal" alternative for marijuana in some countries at that time. The binding affinity of UR-144 to CB2 receptors is higher than that of CB1 receptors; however, According to WHO reports in 2014, the affinity of UR-144 to CBS receptors is lower than THC (WHO, 2014). WHO in 2017 reports mentioned a higher affinity of UR-144 to CBS compared to THC (WHO, 2017). The acute intoxication symptoms of UR-144, as reported in various clinical studies, include depression, convulsions, tachycardia, euphoria, hallucinations, and other symptoms (Adamowicz & Lechowicz 2015; Adamowicz et al., 2017). However, the toxicological and pharmacological effects of UR-144 and the mechanisms underlying those effects are still unclear and based generally on case reports and the abuser's experiences (WHO, 2014). Studies indicate the carcinogenic effects of some synthetic cannabinoids and show the oxidative stress, genotoxicity, and cytotoxicity effects as mechanisms underlying their toxicity. In this study, it was hypothesized that UR-

144 could induce oxidative stress leading to cell death and DNA damage effects in the SH-SY5Y cells, which show neuroblast-like morphology, express some characteristics of catecholaminergic neurons' enzymes (Kovalevich & Langford, 2013), and important *in vitro* model for investigating neurotoxicology (Lopez-Suarez, Al Awabdh, Coumoul, & Chauvet 2022). For that, human neuroblastoma cells were exposed to UR-144 for 24 hours, and MDA level, ROS production, and TAC depletion were evaluated as endpoints for oxidative stress. Cytotoxicity was assessed using the MTT and NRU tests, while genotoxicity was evaluated using the comet assay.

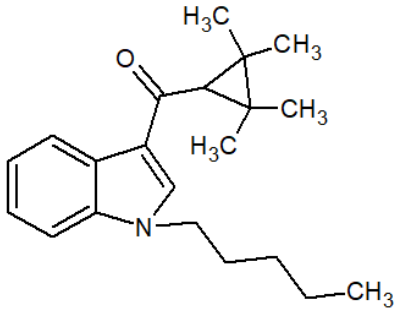


Figure 1. Chemical structure of UR-144

MATERIAL AND METHODS

Chemicals

UR-144 was purchased from Lipomed (Weil am Rhein, Germany). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), neutral red dye (NR), dimethylsulfoxide (DMSO), Triton-x, acetic acid, ethyl alcohol, Sodium chloride (NaCl), Ethylene diamine tetra acetic acid (EDTA), and ethidium bromide from Sigma Aldrich (Missouri, USA). Low melting agarose (LMA) and high melting agarose (HMA) from Himedia (Mumbai, India). Cell culture medium, fetal bovine serum (FBS), phosphate buffer saline (PBS), antibiotic solution, and trypsin solution were taken from Multicell Wisent Bioproducts (Quebec, Canada). Cell culture materials were taken from Nest Biotechnology (Wuxi, China).

Cell culture and treatment

The SH-SY5Y cell line was purchased from ATCC (CRL-2266™). Cells were continued in EMEM cell culture medium with 10% FBS and 1% antibiotic-antimycotic mixture and incubated in 5% CO_2, 37 °C, and 85% humidity. Cells were passaged by trypsinization when they reached about 70% confluence. About 10^4 cells /100 µL/ well were used for cytotoxicity assays, 10^5 cells /2 mL/ well for comet assay, and 5 x10^6 cells /12 mL/ flask for oxidative stress assays. Cells were treated with UR-144 for 24 hours. The treatment concentrations were chosen according to the preliminary study results and according to the previous articles. The unexposed cells were evaluated as the growth control group, and DMSO was used as the negative control.

Cell viability determination

MTT assay: This test is based on the reduction of MTT dye to formazan crystals by a mitochondrial enzyme in viable cells (Fotakis & Timbrell 2006). After UR-144 treatment, 20 µL of 5 mg/ mL MTT dye solution was added to each well and incubated for 3 hours at 37 °C. The upper phase was thrown, and the formazan crystals were dissolved in 100 µL of DMSO. The absorbance (OD) was measured at 590 nm by a microplate reader (Biotek, Germany), and the cell viability was calculated as the relative percentage of the negative control.

Neutral red uptake (NRU) assay: To evaluate whether UR-144 causes cell death by affecting the lysosomal activity, the NRU assay was used (Borenfreund & Puerner 1985). While NR dye could be held in the viable cells, the disrupted cell membrane disallows it in the dead cells. At the end of the treatment period, the medium was replaced with a fresh medium containing 50 µg /mL of neutral red dye and incubated for three hours. After that, wells were washed with PBS, and the accumulated NR dye was dissolved with 100 µL/ well of glacial acetic acid: ethanol: water [1:49:50]. After 10 minutes of gentle shaking, the OD was determined at 540 nm by a microplate reader (Biotek Germany).

Oxidative stress determination

Reactive oxygen species (ROS) level: ROS levels in the cells were investigated with molecular probe H_2DCF-DA, which is converted to the fluorescent DCF in the presence of ROS. After the exposure to UR-144 treatment for 24 hours, the supernatant was removed, cells were trypsinized, washed with PBS (1X), and resuspended in PBS. 1 mL of 20 µM H_2DCF-DA was added to the cells and incubated for 30 minutes. Then the cells were washed with PBS and suspended in 150 µL bovine serum albumin (BSA, 1%). The fluorescence intensity was measured in the FITC channel (488 nm/530 nm) on an ACEA NovoCyte flow cytometer (ACEA, San Diego, CA, USA). The results were represented as median fluorescence intensity ± standard deviation.

Malondialdehyde (MDA) level: Lipid peroxidation produces MDA as a final product, and an increase in MDA levels is commonly used as an indicator of oxidative stress in cells (Del Rio, Stewart, & Pellegrini 2005). For that, MDA levels in the cells were quantified manually by the thiobarbituric acid reactive substances assay (TBRAS) method, as previously described by Draper and Hadley (1990). The ODs were measured at 532 nm using a microplate reader (Epoch, Biotek). The protein amount of each sample was assessed with a Bicinchoninic acid (BCA) protein assay (ThermoScientific), whose principle is the reduction of Cu^{2+} to Cu^{1+} by amino acids in alkaline conditions using a commercial kit, and the results were calculated as the mean (µg/mg protein) ± standard deviation.

Total antioxidant capacity (TAC) level: TAC is a marker of antioxidant defense capacity in the cells. In this study, TAC levels were determined using a commercially available kit (Sigma, Missouri, USA) according to the manufacturer's instructions. 100 µL of Cu^{2+} working solution was added to the standards and samples wells and incubated for 90 minutes in the dark at room temperature. The OD values were measured at 570 nm in a microplate reader (Epoch, Biotek). Trolox was used as a standard for the calibration curve. The results are presented as the mean (nmol/ µL) ± standard deviation.

Genotoxicity evaluation

DNA damage was evaluated with alkaline Comet assay as described in a previous study (Alpertunga et al. 2014) using a fluorescent microscope (Olympus BX53, Japan) with Comet assay IV image analysis system (Perceptive Instruments, Suffolk, UK). Briefly, the cells were collected at the end of the treatment period, washed, and counted. Then, the cells were mixed with low-melting agarose and spread on slides coated previously with high-melting agarose. Then, the cells were lysed using a high-salt solution for 24 hours. The next day, the lysed cells were treated with an alkaline (PH about 10.5) solution for 20 minutes before electrophoresis. The percentage of DNA in the tail was used to express the degree of damage in the individual cell. More than one hundred cells/ slide were blindly assessed. The results were evaluated compared to the solvent control group and presented as the mean ± standard deviation. The cells treated with 100 µM H_2O_2 for two hours were used as the positive control.

Statistical analysis

All experiments were performed at least in three replicates and repeated three times (n= 9). Data were represented as the mean ± standard deviation (SD). The statistical differences were analyzed by one-way ANOVA and Post Hoc Tukey-test using SPSS version 20 for Windows (SPSS Inc., Chicago, IL) compared to the solvent control groups.

RESULTS and DISCUSSION

The mortal effect of high-dose intake of synthetic cannabinoids has been approved by several clinical reports (Giorgetti, Busardò, Tittarelli, Auwärter, & Giorgetti 2020; Adamowicz 2021; Al-Matrouk et al., 2019); however, their chronic toxicity and the mechanisms underlying the toxicities are still insufficient and controversial (Coronado-´Alvarez et al., 2021); While a lot of studies show the toxicity of SCs

(Krotulski et al., 2020; Maida et al., 2021; Pellegrini, Marchei, Papaseit, Farré, & Zaami 2020), especially the neural toxicity (Gugelmann et al., 2014; Tomiyama and Funada, 2014; Cha et al., 2015), cardiotoxicity (Davis and Boddington, 2015), and nephrotoxicity (Silva, Carmo, & Carvalho 2018) and mentioned the cell death, oxidative stress, and inflammation induction (Oztas, Abudayyak, Celiksoz, & Özhan 2019; Akar, Ercin, Boran, Gezginci-Oktayoglu, & Özhan 2022) as the mechanisms underlying these toxicities, other studies show the benign- therapeutic effects of these chemicals (Coronado-´Alvarez et al., 2021; Muralidhar Reddy, Maurya, & Velmurugan 2019).

For UR-144, there is very little data related to their biological effects. According to a Scopus database search on 21 Dec. 2021 (TITLE-ABS-KEY(ur 144) AND TITLE-ABS-KEY(cannab*) AND NOT TITLE-ABS-KEY(urethral)) AND (LIMIT-TO (DOCTYPE,"ar")) there are 84 articles containing UR-144 in there title, abstract, and keywords. Of these, 55 articles were related to the analytical detection methods of UR-144 and other cannabinoids. While 11 articles focused on reports of the detection of UR-144 in the various products or the biological samples of the abusers.

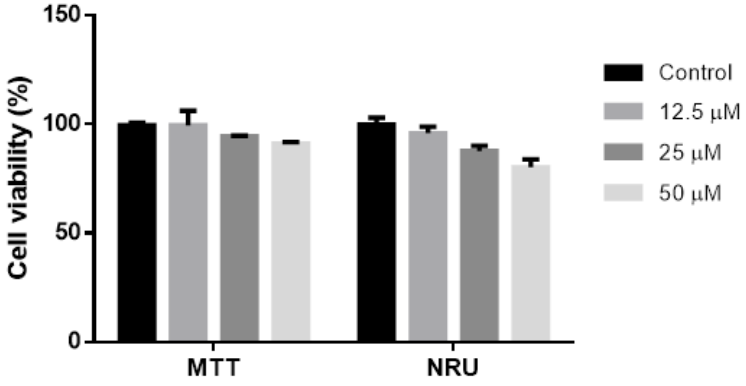
UR-144 was developed by Abbott as an analgesic in 2006; however, it was not approved by the FDA. The first reports about the abuse were in 2012. Data mentioned the uses of different street names for UR-144 as Hardcore, Sonrisa, and Tio Tieso (Fabregat-Safont et al., 2020). Additionally, reports mentioned the uses of UR-144 alone or in combination with other synthetic cannabinoids like FUB-AMB, 5F-AKB48, and AB-FUBINACA (Al-Matrouk et al., 2019; Gugelmann et al., 2014; Kronstrand, Roman, Andersson, & Eklund 2013; Krotulski et al., 2020; Turcant et al., 2017). In addition to CB2 receptor activity, UR-144 has an agonist selectivity to CB1 (Coronado-´Alvarez et al., 2021). While the high selectivity and affinity of SC to CB1 compared to THC make the side effects of SC higher than that of THC (Chung, Cha, Min, & Yun 2021), it could be concluded that UR-144 has a higher toxicity than THC.

Case reports mentioned different concentrations of UR-144 in the biological samples of the users; in Kronstrand et al. (2013) studies' the concentration was between 0.05 ng and 25.9 ng, and the mean was 1.26 ng per 1 mL blood sample (Kronstrand et al. 2013). In a study by Adamowicz (2021), it was reported, based on previous data analysis, that UR-144 blood concentrations in fatal cases vary between 1.4 and 12.3 ng/ mL (Adamowicz, 2021). In another study, 39 cases of UR-144 intoxication were reviewed, and 17 ng /mL was reported as the maximum concentration observed in those cases (Adamowicz et al., 2017).

Despite the possible toxicity of UR-144, there is a real lack of data related to the effect and toxicity of synthetic cannabinoids in general and UR-144 specifically (Kronstrand et al. 2013; Coronado-´Alvarez et al., 2021; Maida et al., 2021).

Behavior and cardiopathy symptoms were noticed in UR-144 intoxication cases (Adamowicz et al., 2017). Similarly, in a case reported by Al Fawaz et al. (2019), it was concluded that the UR-144 may cause prolonged epilepsy-like symptoms and stress cardiomyopathy (Al Fawaz et al., 2019). The neurotoxicity, like epileptic symptoms, was mentioned in a case that used the ''Crazy Monkey" drug, where the blood and urine samples analysis revealed the presence of UR-144 metabolites in addition to PB-22 (QUIPIC) SCs; however, the researchers suggested PB-22 as a cause of those symptoms (Gugelmann et al., 2014).

In the present study, at the tested concentrations (12.5, 25, and 50 μM; the higher dose was chosen based on solubility) UR-144 did not exhibit a significant cytotoxic effect. The median inhibition concentration (IC_{50}) could not be calculated using either the MTT assay or the NRU (Figure 2.)



Groups	Cell viability (%)	
	MTT	NRU
Control	100±2.76	100±7.92
12.5 µM	99.54±9.60	95.91±6.09
25 µM	94.52±0.57	87.78±4.77
50 µM	91.17±1.23	80.34±7.24

Figure 2. The effect of UR-144 on SH-SY5Y cell viability.

In a limited number of laboratory studies, Almada et al. (2020) evaluated UR-144 along with other SCs on BeWo human placental cytotrophoblast cells. A decrease in cell viability was observed; however, no significant changes were detected in LDH enzyme levels, a key parameter used to evaluate the damage in cells' metabolic activity. Their results also indicate the involvement of apoptotic pathways since Caspase 3, caspase 7, and caspase 9 were increased significantly. Additionally, a cell cycle arrest was noticed for all SCs including UR-144. While there was no change in the cellular ROS levels, UR-144 caused a loss of mitochondrial membrane potential. The comparison with Δ9-tetrahydrocannabinol (THC) indicates higher toxicity of SCs compared to TCH. Oppositely, Fonseca et al. (2019) hypothesized the effect of SCs on the endometrium remodeling process and tested this hypothesis using endometrial stromal (St-T1b) cells and decidual fibroblasts primary cells. A transitory increase in ROS and RNS levels and induction of the endoplasmic reticulum damage were detected following the treatment to UR-144 for 48 hours, but no cytotoxicity effects were noticed. Similarly, the current study results reveal no cytotoxicity of UR-144 on SH-SY5Y cells. The decrease in ROS and MDA levels couldn't be assessed as a toxic effect since the induction of oxidative stress concurs with the increase in both ROS and MDA levels. Since the decrease was compared to the negative control and solvent control groups and the cells in the treatment groups did not treat previously with an oxidative stress inducer agent, the reduction in ROS and MDA couldn't also be assessed as an antioxidant effect. Herewith, it could be concluded that there is no significant oxidative stress effect on SH-SY5Y cells exposed to UR-144 for 24 hours.

In our study, the tested concentrations of UR-144 significantly reduced ROS levels in the cells compared to the control group. This reduction was further supported by a significant decrease in MDA levels observed in the 12.5 and 50 µM exposure groups (Figure 3a., 3b.). However, the slight increase in TAC levels in the exposed groups was not statically significant compared to the control group (3c) ($p > 0.05$).

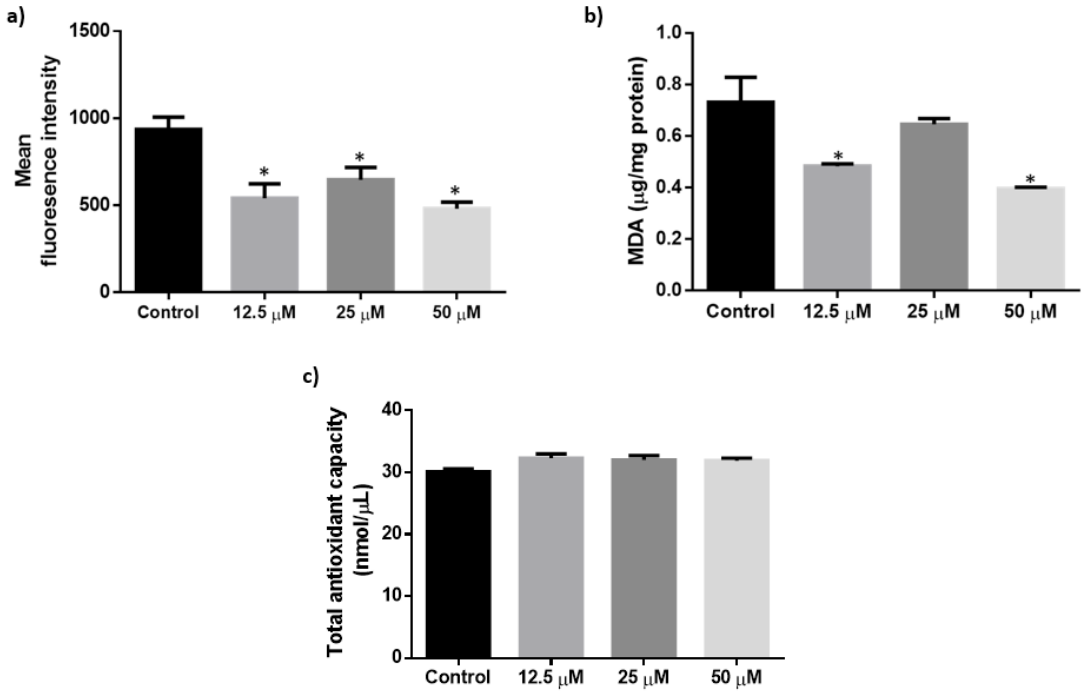
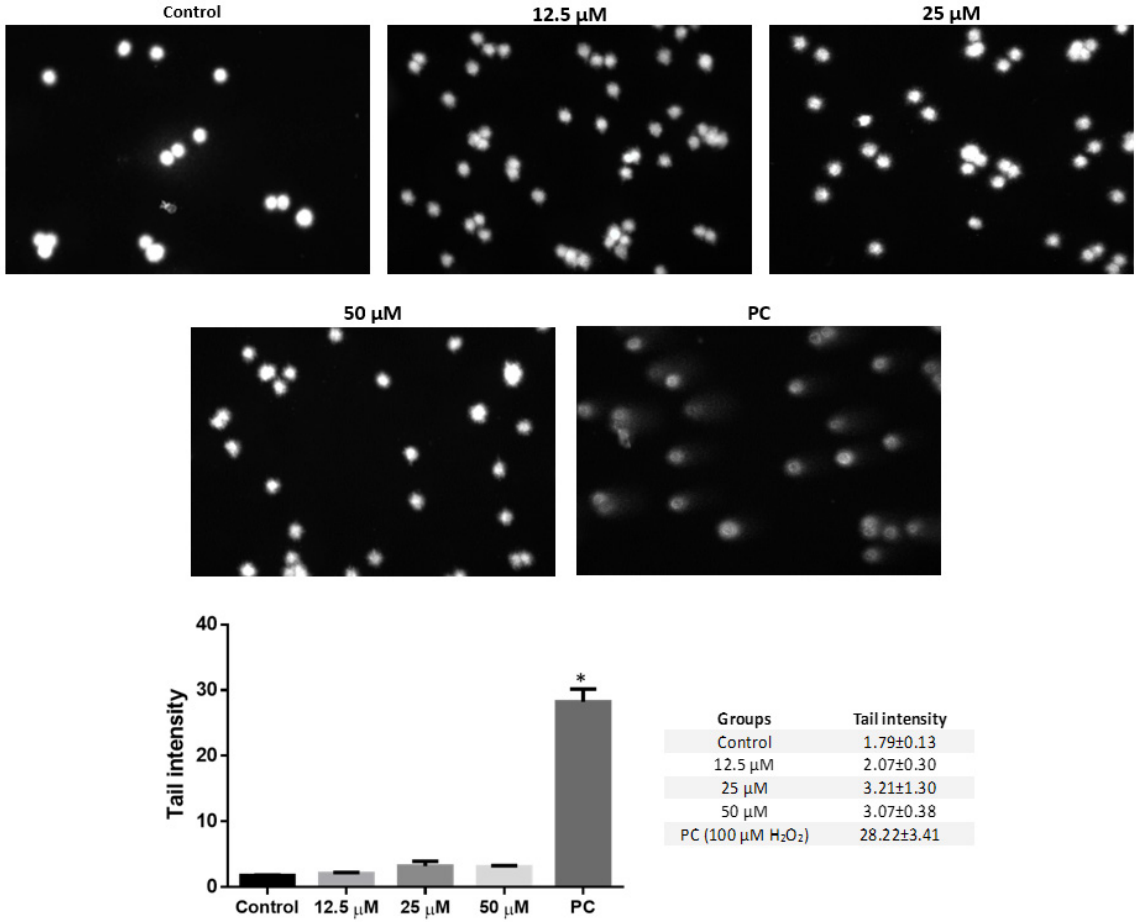


Figure 3. Effects of UR-144 on a) ROS level, b) MDA level, and c) TAC level in SH-SY5Y after 24 hours. *$p<0.05$ versus control group.

Koller et al. (2015) used SCGE assay and micronucleus assay on human lymphocyte cells and salmonella microsome assay to evaluate the genotoxicity potential of some SCs. Their results show inhibition of the cell division after treatment to 0.1 to 1 mM UR-144 for three hours. Additionally, an increase in the DNA migration and induction of micronucleus and chromosomal aberrations were noticed. Those results confirm the WHO's concerns about its genotoxicity (WHO 2014). Conversely, the results of the present study show no significant DNA damage in the exposed cells compared to the negative group.

In the present study, the genotoxicity potential of UR-148 was evaluated by the Comet assay after the treatment with UR-144 for 24 hours. The results showed no significant differences between the groups UR-144- treated and the negative group (Figure 4.).



Groups	Tail intensity
Control	1.79±0.13
12.5 µM	2.07±0.30
25 µM	3.21±1.30
50 µM	3.07±0.38
PC (100 µM H_2O_2)	28.22±3.41

Figure 4. DNA damage induction by UR-144 in SH-SY5Y cell line. *p<0.05 versus the control group. PC: positive control (100 µM H_2O_2)

Besides the possible toxicity of UR-144, Nielsen, Holm, Olsen, & Linnet (2015) mentioned the metabolism of UR-144 by CYP 450 enzymes as CYP3A4, CYP1A2, and CYP 2C enzymes; this indicated the possibility of drug/chemical-UR144 interaction, the uses of UR-144 in combination with other synthetic cannabinoids, herbs, and drugs increase the chance of UR-144 induced toxicity by the synergism effect or by decreasing the metabolism of that drugs. Similarly, Ashino, Hakukawa, Itoh, & Numazawa (2014) showed a weak inhibitory effect of UR-144 on CYP1A enzymes, which also could increase the risk of interaction. While the lab studies were done on highly pure chemicals, the street chemicals could contain high levels of organic and inorganic impurities that also increase the risk of toxicity (Abdin, Yeboah, & Jacob 2020).

In conclusion, there is an increase in the use of SCs. UR-144 is an example of these SCs. The reported cases show the toxicity of UR-144 in the neuronal system that could be lethal. However, there are very few studies evaluating its toxicity. The negative results in this study and some previous works could draw a pink view for this chemical, especially since UR-144 was developed as a drug and passed some tests. However, the limited information, conflicting findings, and reports on the toxicity of other synthetic cannabinoids highlight the urgent need for further research to evaluate and better understand the toxicity of UR-144.

AUTHOR CONTRIBUTION STATEMENT

M.A. & T.B. designed and wrote the manuscript; M.A. & T.B. performed the experiments; M.A.

analyzed the data; M.A. performed the computational analyses; M.A. & T.B. assisted with the manuscript modification; M.A. supervised the study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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