Research Article

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American Journal of Phytomedicine and Clinical Therapeutics
ISSN 2321-2748

2017
Vol. 5 No. 1: 8

DOI: 10.21767/2321-2748.100321

Isolation and Neuroprotective Effect of [4(3,4-Dihydroxybenzoyloxymethyl)Phenyl-B-D Glucopyranoside] from *Origanum micranthum* on *In Vitro* Glutamate Toxicity Model of Sprague Dawley Rat Cortex Neurons

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Citation: Esen SK, Ali T, Ufuk O, et al. Isolation and Neuroprotective Effect of [4(3,4-Dihydroxybenzoyloxymethyl)Phenyl-B-D Glucopyranoside] from *Origanum micranthum* on In Vitro Glutamate Toxicity Model of Sprague Dawley Rat Cortex Neurons. Am J Phytomedicine Clin Ther. 2017, 5:1.

Abstract

Glutamate toxicity is a common neurological disorder characterized by an increase in the glutamate (Glu) concentration leads to excitotoxicity which cause cellular damage and death in the brain. *Origanum micranthum* is an endemic species in Turkey. *Origanum* species have many polyphenolic compounds. Rich polyphenol containing material is known to be highly neuroprotective effect. Based on this subject, we aimed to evaluate the neuroprotective effect of **Origanum micranthum** against glutamate-induced toxicity in the primery neuron culture of rat brain cortex. For modeling glutamate toxicity the cells cultures were exposed to 10^{-5} M glutamate for 20 min. Later, glutamate-induced cell cultures were treated with methanol, chloroform, ethyl acetate and aqueous extracts of aerial parts of **Origanum micranthum** for 24 h. Cell viability was evaluate by using MTT assay. MTT assay showed that ethyl acetate extract in 10^{-4} and 10^{-5} mM concentration have greater neuroprotective effect. [4(3,4-dihydroxybenzoyloxymethyl) phenyl-β-D-glucopyranoside] was isolated majority from ethyl acetate extract of **Origanum micranthum** by using chromatographic method and evaluated by ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz) and ESI-MS. MTT assay and Anexin V apoptose assay kit show 25, 50 and 100 µgr/ml concentrations of [4(3,4-dihydroxybenzoyloxymethyl) phenyl-β-D-glucopyranoside] exhibited a high neuroprotective activity at microgram concentrations.

Keywords: *Origanum micranthum*; Brain hypoxia; Glutamate toxicity; 4(3,4-dihydroxybenzoyloxymethyl) phenyl-β-D-glucopyranoside

Received: March 24, 2017; **Accepted:** March 28, 2017; **Published:** April 03, 2017

Introduction

Glutamate is main excitatory neurotransmitters in the mammalian central nervous system (CNS) which functionally contributes in all interaction between the neurons [1,2]. However, glutamate has the potential to induce neuronal dysfunction and degeneration when present in abnormally high extracellular concentrations [3,4]. This process is referred to as excitotoxicity, a term coined by John Olney [5], who posited that excessive stimulation by glutamate has the capacity to vastly increase intracellular calcium, affecting calcium homoeostatic mechanisms and triggering a cascade of events that ultimately result in cell death [6]. Recent studies have revealed that excitotoxicity was caused in Alzheimer, Parkinson's disease [7,8]. Huntington's disease [9] amyotrophic lateral sclerosis (ALS) [10,11] depression, ischemic and brain hypoxia [12-15]. Brain hypoxia is a common neurological disorder defined by an increase in the glutamate concentration leads to excitotoxicity is the pathological process by which nerve cells are damaged [16,17]. Prevention of the neuron ischemic/ hypoxic damage is the main problem in neurolog [18]. Hence, it is highly important to investigate novel effective plant ingredient to tolerate toxic level of L-glutamate rising in many ischemic and neurotoxic disease. *Origanum* plants spice and tea widely used all over the world under the vernacular name ''oregano''. They have great economic importance and medicinally used as antimicrobial, antifungal, antioxidant, antibacterial, antithrombin, antiparasetic

and antihyperglyacemic [19,20]. *Origanum* genus represented by 21 endemic taxa in Turkish flora. [21,22]. Phytochemical analyzed showed *Origanum* species are rich in the phenolic monoterpenoid, various phenolic, lipids and fatty acids, flavonoids and anthocyanin's [23]. Various studies showed that polyphenols could play a preventive role in neurological disorders [24-26]. In this study, neuroprotective effect of chloroform (OMC), methanol (OMM), ethyl acetate (OME), and aqueous (OMA) extracts and one of the major components [4(3,4-dihydroxybenzoyloxymethyl) phenyl-β-D-glucopyranoside] of *Origanum micranthum* Vogel (OM) were evaluated on glutamate toxicity for the first time. Firstly, OMC, OMM, OME and OMA extracts was studied. OME extract showed the highest activity. Then, OM1 which is the major component of OME was evaluated. Several and repeated chromatographic methods were used for the isolation of OM1. The structure of the compound was elucidated by means of spectral analysis (^{1}H NMR, ^{13}C NMR, 2D NMR and ESI-MS). In vitro MTT test and Anexin V assay kit were employed to quantify the cell viability and apoptosis pathway on rat primary brain cortex neuron cells cultures.

Results and Discussion

The methanol extract of the aerial parts of *O. micranthum* was suspended in water an extracted with $CHCl_3$ and EtOAc, consecutively. The neuroprotective effect of methanol, chloroform, ethyl acetate and aqueous extracts were tested against glutamate-induced brain hypoxia in the brain cortex neuron cell cultures. Cell viability and activated apoptosis pathway were measured by using MTT assay kit and flow cytometry assay kit. MTT assay showed that ethyl acetate extract in 10^{-4} and 10^{-5} mM concentrations have greater neuroprotective effect. Because of this high activity, we decided to try the major component (OM1) of the ethylacetate extract. OM1 was found significantly effective. OM1 was isolated by various chromatographic techniques from EtOAc extract. The structure of [4(3,4-dihydroxybenzoyloxymethyl)phenyl-β-D-glucopyranoside] was elucidated by ^{1}H NMR, ^{13}C NMR, 2D NMR and ESI-MS.

Compound OM1

White precipitate ESI-MS M/Z ($[M+Na]^+$ $[C_{20}H_{22}O_{10}]^+$; 445). ^{1}H NMR (400 MHz, DMSO-d6): δ 7.35 (d, 2H, H-2', H-6', J= 8.4 Hz), 7.34 (d, 1H, H-2, J= 2.2 Hz), 7.30 (dd, 1H, H-6, J= 2.2, J= 8.4 Hz), 7.02 (d, 2H, H-3', H-5', J= 8.4), 6.78 (d, 1H, H-5, J= 8.4), 5.17 (s, 2H, C-7'), 4.86 (d, 1H, 1'', J= 7.3 Hz), 3.11-3.67 (sugar protons). ^{13}C NMR (100 MHz, CD_3OD): δ 166.2 (C-7), 157.9 (C-4'), 151.2 (C-4), 145.7 (C-3), 130.4 (C-1'), 130.3 (C-2', C-6'), 122.6 (C-6), 121.2 (C-1), 116.9 (C-2, C-3', C-5'), 116.1 (C-5), 101.0 (C-1''), 77.7 (C-3''), 77.3 (C-5''), 73.9 (C-2''), 70.4 (C-4''), 66.1 (C-7'), 61.4 (C-6''). ^{1}H-NMR and ^{13}C-NMR ^{1}H-NMR and ^{13}C-NMR data agree with data given in the literature for 4(3,4-dihydroxybenzoyloxymethyl)phenyl-β-D-glucopyranosid [27]. OM1 compound is a whitish precipitate. When considered İTK analyses, it is dark under UV254 nm and colorless under UV366 nm. It was reported to give yellowish – brownish colour after the injection of vanillin/sulfuric acid reactive and heated at 110 °C for 1-2 min. In $CHCl_3$: MeOH: H_2O (70:30:3) solvent system, Rf was found to be 0.44. When the results of ^{1}H-NMR, ^{13}C-NMR and 2D-NMR spectrum were taken into consideration, compound was decided to be 4(3,4-dihidroksibenzoiloksimetil) phenyl-β-D- glucopyranoside. When considered the results of ^{1}H-NMR, protons of A2B2 system were observed at δH= 7.02 (2H, d, J= 8.4 Hz) and δH= 7.35 (2H, d, J= 8.4 Hz) ppm. Such signals supported the structure of 1,4- disubstitute benzene in the molecule. Proton signals of other aromatic ring were observed at δH= 6.78 (1H, d, J= 8.4 Hz) ppm, δH= 7.34 (1H, d, J= 2.2) ppm and δH= 7.30 (1H, dd, J= 8.4, J= 2.2 Hz) ppm. The signal observed at δH= 5.17 (2H, s) ppm, implied a benzylic methylene bearing acetoxy group. At δH= 4.86 (1H, d, J= 7.3 Hz) ppm, anomeric proton signal of glucose was observed. Other proton signals of glucose were observed at δH= 3.11-3.33 (5H, m) ppm and δH= 3.67 (1H, d, J= 11.0 Hz) ppm. When considered the results of ^{13}C-NMR, characteristic signals of glucopyranosyl part at δC= 101.0, 77.7, 77.3, 73.9, 70.4 and 61.4 ppm. One methylene (δC= 66.1 ppm), one ester (δC= 166.2 ppm), 5 quaternary aromatic carbons (δC= 157.9, 151.2 145.7, 130.4 and 121.2 ppm) and 7 tertiary carbons (δC= 130.3 (2C), 122.6, 116.9 (3C) and 116.1 ppm) in aglycone part of the molecule were in agreement with literature [27].

Neuron culture

The neuroprotective effect of OME (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} mM), OMM (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} mM), OMC (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} mM), OMA (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} mM) by inducing glutamate 10^{-5} mM in cultured brain cortex neuron were investigate. The MTT assay confirmed that 10^{-4} and 10^{-5} mM concentration of OME extract show any significantly differences compared to the glutamate 10^{-5} mM control groups and effectively related to neuroprotective effect. But different concentration of OMM, OMC and OMA extracts did not show any promise compared to the glutamate 10^{-5} mM control groups **(Figure 2).** Duo to the high neuroprotective effect of OME extract at 10^{-4} ($p<0.001$ significant difference) and 10^{-5} ($p<0.05$ significant difference) concentrations, it was decided to work on major component in this extract**.** OM1 is one of the major compound of OME extract which has a phenolic glycoside structure. Various studies showed that polyphenols could play a preventive role in neurological disorders [24-26]. For detecting the effective dose of OM1, different concentrations were applied to cultured neuron plate. The results exhibited that low dose of OM1 by tolerating free radicals increased safety and also viability to neuron cells. There is need for new molecules that has a therapeutic effect in the pharmaceutical industry. OM1 shows promise as neuroprotective agent for treatment brain disease in relation to glutamate toxicity.

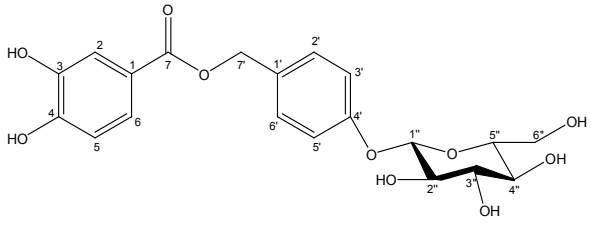

Figure 1 OM1 structure.

Experiment

Chemicals and reagents

Neurobasal medium (NBM), fetal calf serum (FCS), antibiotic antimycotic solution (100×), L glutamine and trypsin–EDTA were obtained from Sigma-Aldrich® (St. Louis, MO, USA). ^{1}H and ^{13}C NMR spectra were recorded on Varian Mercury plus 400 MHz for proton and 100 MHz (Varian Inc., Calif, USA) for carbon by using tetramethylsilane as internal standard. The solvents were DMSO-d6 and CD_3OD. Silica gel (0.040-0.063 mm Merck and 0.063-0.200 mm, Merck, Darmstadt, Germany) were used for column chromatographic separation. TLC analyses were applied on pre-coated Kieselgel 60 F254 (Merck, Darmstadt, Germany) aluminum sheets and compounds were detected under UV lamp and 1% vanillin/H_2SO_4 reagent as revelator by heating at 100 °C.

Plant material

The aerial parts of *O. micranthum* were collected from Kozan (Adana Province, 1300 m, Turkey) in August 2009 and identified by Dr. Ufuk ozgen. A voucher specimen was deposited at the Herbarium of Ankara University Faculty of Pharmacy (AEF 25873).

Isolation and extraction studies

The air-dried and powdered aerial parts (410 g) of *O. micranthum* (OM) were extracted by refluxing with methanol (2 L × 3) on a mantle. Methanol extract was concentrated and dried under reduced pressure to get a residue (98 g). Methanol extract (95 g) (OMM) was dissolved in 300 mL H_2O: MeOH (9:1) and partitioned with chloroform (OMC) and then ethyl acetate (OME). The remaining extract was the aqueous phase (OMA). OMA (12 g) was subjected to silica gel column by eluting $CHCl_3$: MeOH: H_2O (80:20:2, 70:30:3, and 50:50:5, respectively) solvent system. Fractions 18-20 gave compounds 1 (**OM1**) **(Figure 1).**

Neuron cell cultures

This study was conducted at the Medical Experimental Research Center in Ataturk University (Erzurum, Turkey). The ethical committee of Ataturk University approved the study protocol (42190979-000-E.1600112628). Neuron cell cultures were prepared using nine new-born *Sprague-Dawley* rat fetuses as described previously All cerebral cortex of rats were removed and minced by lancets in sterile petri dish. The chemical decomposition was achieved added 5 mL Dulbecco Modified Eagle Medium (DMEM) plus 2 mL Trypsin-EDTA (0.25% trypsin- 0.02% EDTA) and 8 µL of DNase type 1 (120 U/mL) for 25-30 min in 5% CO_2 at 37°C and then centrifuged at 800 rpm for 3 min. The single cell that was obtained after physical and chemical decomposition was divided into poly-D-lysine four 48 well plate 150 µl to each well (costar, USA). The plates were left in the incubator including 5% CO_2 at 37°C. The plates were then changed with a fresh Neurobasal medium (NBM) of half of their volumes every three days until the cells were branched and had reached certain maturity and *in vitro* experiments were performed ten days later **(Figures 2 and 3).** By the 10th day O. ME (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} mM), O. MM (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} mM), O. MC (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} mM), O. MA (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} mM) and Glutamate (10^{-5} mM) were prepared and added to cultures plate. 150 µL NBM was added to the neuron cell cultures control group. The same procedure was carried out for OM1 (25, 50, 100, 200, 400 and 800 µgr/ml).

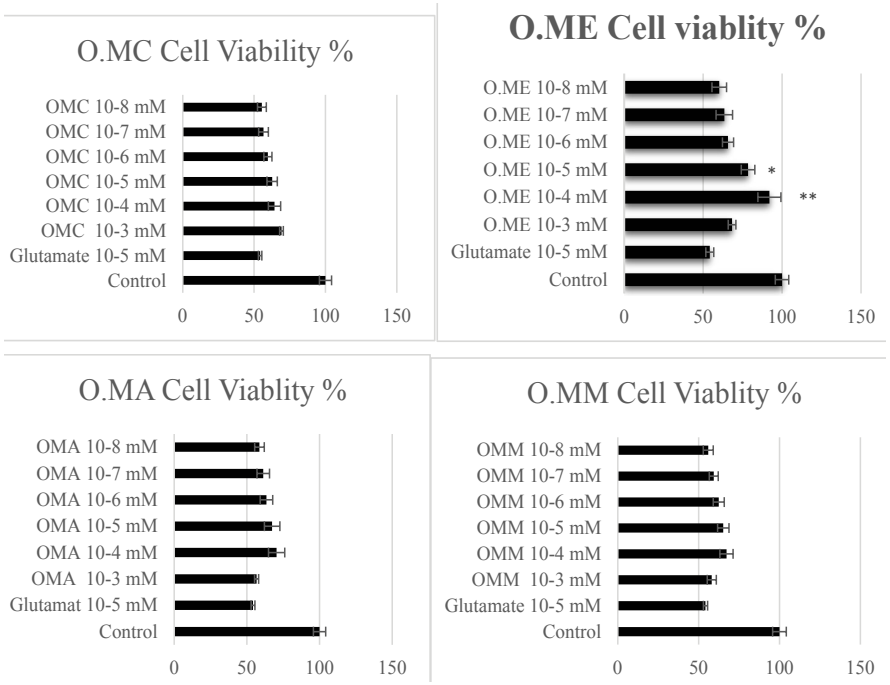

Figure 2 Graphics shows in vitro neuroprotective effects of OMC, OME, OMA and OMM ingredient of OM (extract; 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} mM), Glutamate 10^{-5} mM and Control groups on neuron cells. *Significant differences at the P<0.05 level from the glutamate 10^{-5} mM control group. **Significant differences at the p<0.001 level from the glutamate 10^{-5} mM control group.

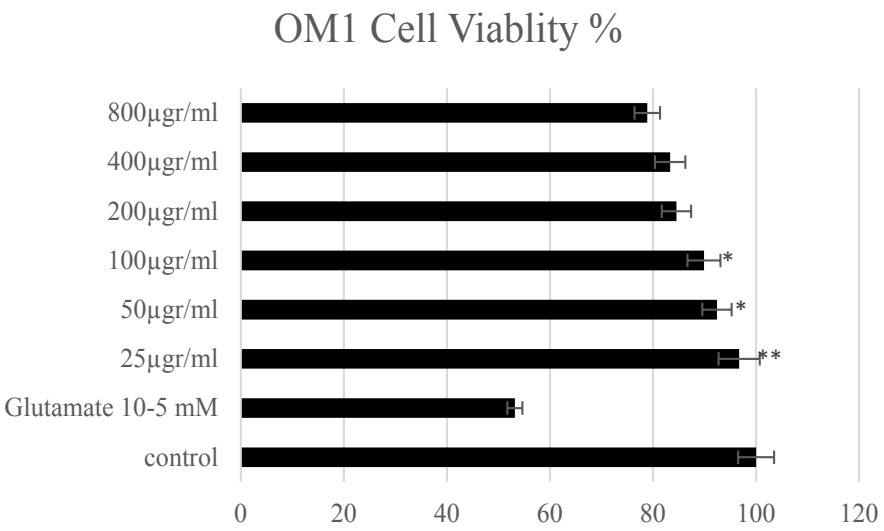

Figure 3 *In vitro* neuroprotective effects of OM1 (pure compound; 25, 50, 100, 200, 400 and 800 µgr/ml) and Glutamate 10^{-5} mM and Control groups on neuron cells by using MTT assay kit. *Significant differences at the P<0.05 level from the glutamate control group. **Significant differences at the p<0.001 level from the glutamate control group.

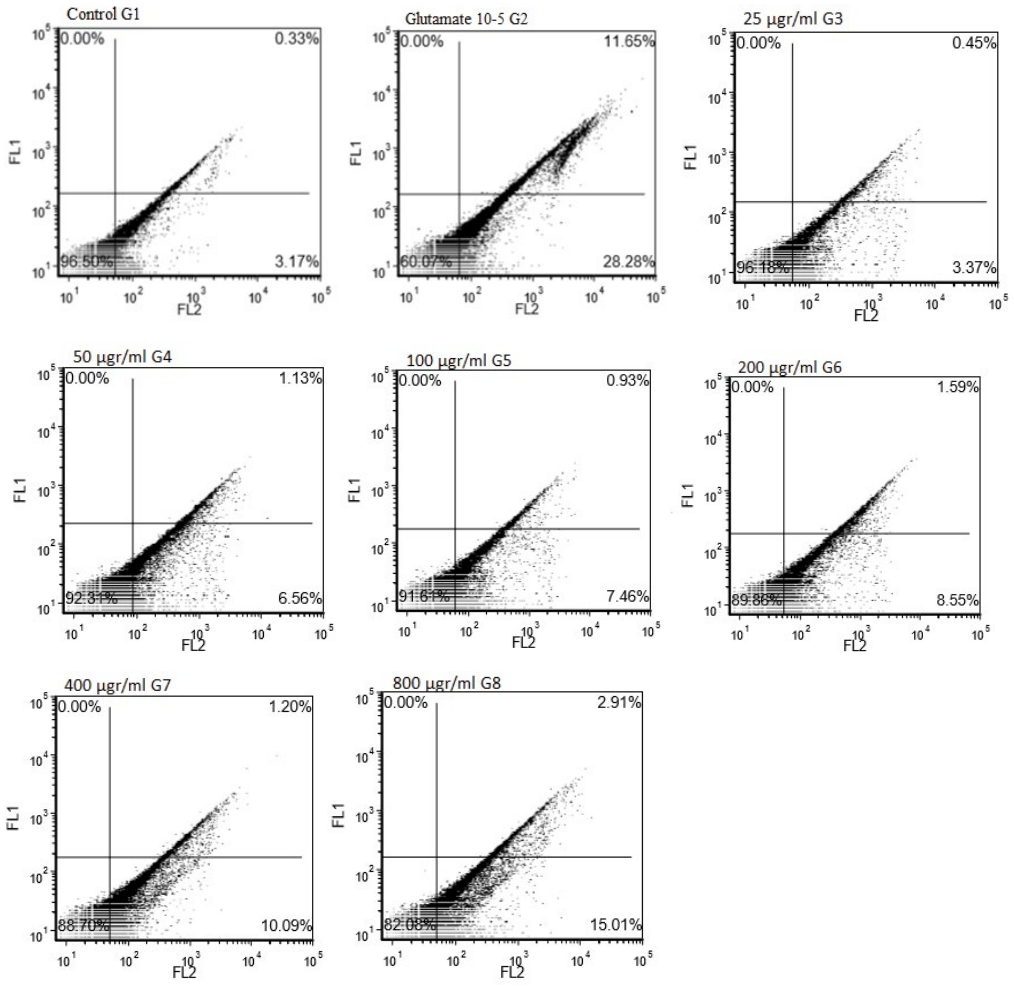

Figure 4 Shows the neuroprotective effects of OM1 (pure compound; 25, 50, 100, 200, 400 and 800 µgr/ml), glutamate 10^{-5} mM and control groups on primary neuron cell cultures by using Anexin V apoptosis assay kit. 25, 50 and 100 µgr/ml concentration of OM1 showed highly protective effect. The results showed that 25 µgr/ml is the best offered dose for protective effect.

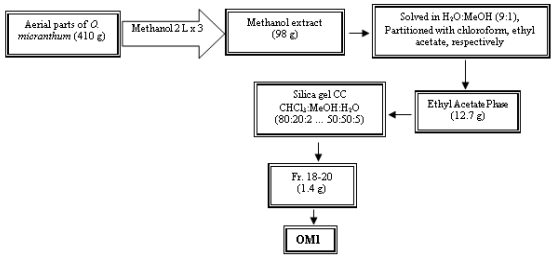

Figure 5 Scheme of Extraction and Isolation.

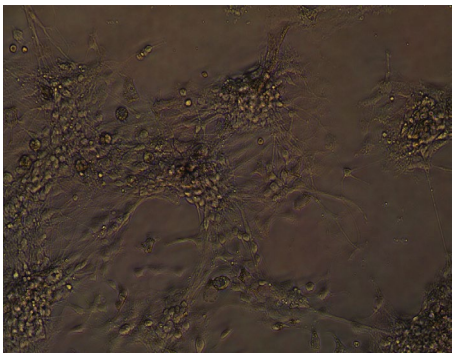

Figure 6 Invert microscopy image of neuron cells in 10th day (Leica microscope X20).

MTT assay

In this study, MTT assay was carried out by commercially available kit (Cayman Chemical Company, USA). The cells were seeded in 96-well plates. Cells were incubated at 37°C in a humidified 5% CO_2 and 95% air mixture 48 h and later, OME (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} mM), OMM (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} mM), OMC (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} mM), OMA (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} mM), Glutamate (10^{-5}) and Control groups were added to cultures for 24 h. 10 µL of MTT reagent was added to the cell cultures for 4h and the plate was incubated in CO_2 incubator at 37°C. 100 µL of crystalline solvent solution was added to each well **(Figures 3-6).** The intensity of the formazan was measured at 570 nm wavelengths with Multiskan™ GO Microplate Spectrophotometer reader (Thermo Scientific, Canada, USA). The same procedure was carried out for OM1 (25, 50, 100, 200, 400 and 800 µgr/ml).

Flow cytometry analysis

For evaluate the apoptosis pathway, Anexin V assay kit (Invitrogen) was obtained. The staining with the Annexin V-FITC were done according to the manufacturer methods. The nuclear were stained with propidium iodide for 5 min. The samples were read by flow cytometry device (CyFlow® Cube Flow Cytometer, Sysmex).

Statistically analysis

The statistical analysis was performed by one-way analysis of variance (ANOVA) using the SPSS 20.0 software. $p<0.001$ and $p<0.05$ were considered as statistically significant difference for all tests.

Acknowledgements

This work was supported by the Research Fund of Atatürk University (BAP-2016/108).

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