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Original Article

Potential neuroprotective of trans-resveratrol a promising agent tempeh and soybean seed coats-derived against beta-amyloid neurotoxicity on primary culture of nerve cells induced by 2-methoxyethanol

Potencial neuroprotetor do agente trans-resveratrol em cascas de sementes de soja e tempê derivadas da neurotoxicidade beta-amiloide na cultura primária de células nervosas induzidas pelo 2-metoxietanol

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Abstract

Resveratrol, a natural polyphenol found in tempeh, has not been investigated especially in vitro as a neuroprotective agent against 2-methoxyethanol (2-ME)-induced beta-amyloid cytotoxicity. Beta amyloid peptides (Aβ) could initiate neurotoxic events and neuron-inflammatory response via microglial activation. However, it remains unknown whether the neurotoxic effect of beta-amyloid and/or associated with the potential of 2-ME to induce neurotoxic effects on primary culture of nerve cells induced by 2-ME. This study investigated potential neuroprotective of trans-resveratrol a promising agent tempeh and soybean seed coats-derived against beta-amyloid cytotoxicity on primary culture of nerve cells induced by 2-methoxyethanol. Biotium and MTT assays were used to analyze neurons, which were isolated from the cerebral cortex of fetal mice at gestation day 19 (GD-19). A standard solution of 2-methoxyethanol was dosed at 10 μL. The cultured cells were randomly divided into the following groups: (1) 2-ME group + resveratrol standard, (2) 2-ME group + resveratrol isolated from tempeh, (3) 2-ME group + resveratrol isolated from soybean seed coats, and (4) the control group, without the addition of either 2-ME or resveratrol. Exposure of the primary cortical neuron cells to beta-amyloid monoclonal antibody pre-incubated for 24 h with 10 μL of 4.2 μg/mL resveratrol and 7.5 mmol/l 2-methoxy-ethanol additions. Here, we report that the addition of 2-ME and resveratrol (standard and isolated from tempeh) of cell culture at concentrations of 1.4, 2.8 and 4.2 μg/mL showed that the majority of neurons grew well. In contrast, after exposure to 2-ME and Beta-amyloid, showed that glial activated. These findings demonstrate a role for resveratrol in neuroprotective-neurorescuing action.

Keywords: 2-methoxyethanol, resveratrol, neurotoxic, neuroprotective, neurorescuing.

Resumo

O resveratrol, um polifenol natural encontrado em tempê, não foi investigado apenas in vitro como agente neuroprotetor contra a citotoxicidade beta-amiloide induzida por 2-metoxietanol (2-ME). Os peptídeos beta-amiloides (Aβ) podem iniciar eventos neurotóxicos e resposta inflamatória dos neurônios via ativação microglial. No entanto, permanece desconhecido se o efeito neurotóxico do peptídeo beta-amiloide associado ao potencial do 2-ME causa efeitos neurotóxicos na cultura primária de células nervosas induzidas pelo 2-ME. Este estudo investigou o potencial neuroprotetor do agente trans-resveratrol em cascas de sementes de soja e tempê derivadas da citotoxicidade beta-amiloide na cultura primária de células nervosas induzidas pelo 2-metoxietanol. Ensaios de biotium e MTT foram utilizados para analisar os neurônios isolados do córtex cerebral de camundongos fetais no dia da gestação 19 (GD-19). As células cultivadas foram divididas aleatoriamente nos seguintes grupos: (1) grupo 2-ME + padrão de resveratrol; (2) grupo 2-ME + resveratrol isolado de tempê; (3) grupo 2-ME + resveratrol isolado de cascas de sementes de soja; e (4) grupo controle, sem a adição de 2-ME ou resveratrol. Houve exposição das células primárias dos neurônios corticais ao anticorpo monoclonal beta-amiloide pré-incubado por 24 horas, com 10 μL de 4,2 μg/mL de resveratrol, e adições de 7,5 mmol/l de 2-metoxietanol. A adição de 2-ME e resveratrol (padrão e isolado do tempê) da cultura de células nas concentrações de 1,4, 2,8 e 4,2 μg/mL mostrou que a maioria

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dos neurônios cresceu bem. Por outro lado, após a exposição ao 2-ME e beta-amiloide, a glia foi ativada. Esses achados demonstram um papel do resveratrol na ação neuroprotetora e de neurorresgate.

Palavras-chave: 2-metoxietanol, resveratrol, neurotóxico, neuroprotetor, neurorresgate.

1. Introduction

Phthalates is one of the compounds used in the manufacture of fragrances, cosmetics, lotions and plastics processing. These compounds are embedded in the plastic structure as "plasticizers" and can be breakdown from the plastic structure, if they are exposed to heat (Johanson, 2000). More than 18 million pounds of phthalates have been used annually and poisoning of these compounds can occur every day, through breathing air and exposure through the skin (Adibi et al., 2008). Phthalic esters in the body are metabolized become methoxyacetic acid (MAA) and 2-methoxyethanol (2-ME). 2-ME is an industrial solvent produced by the reaction of ethylene oxide with anhydrous methanol (Kirk and Othmer, 1980; WHO, 2009). This compound is a colorless, volatile liquid, with high water solubility and used in chemical intermediate, solvent coupler of mixtures and water-based formulations. Although, in recent years 2-ME was declined in some countries by other substances due to risk management procedures, it is still in use in some areas. Several studies have shown that 2-ME is to induce neoplastic effects caused corticogenesis disorder. The disorder caused by the existence of these proteins as a result of 2-Methoxyethanol (WHO, 2009).

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is found in grape skins, berries, and red wine as well as peanuts and other nuts (Baur and Sinclair, 2006). These compounds is increase antioxidant defenses and decrease proinflammatory cytokines in hippocampal astrocyte cultures (Bellaver et al., 2015), as alternative treatment options in Alzheimer's disease (AD), can decrease the toxicity, aggregation of Aβ peptides in hippocampus of AD patients, promote neurogenesis, and prevent hippocampal damage (Gomes et al., 2018). The role of resveratrol as a neuroprotective agent can be mediated by astrocytes (Daverey and Agrawal, 2018), and the cAMP response element binding (CREB) protein. CREB is a protein in the neurotrophin family that is involved in neuroprotection, including neuronal survival, synaptic plasticity, and neurodevelopment, and is also involved in the pathophysiology of nerve degeneration (Kapczinski et al., 2008). Other studies also showed that resveratrol can inhibit beta-amyloid in microglia and astrocytes also has anti-inflammatory functions (Lu et al., 2010).

It is clear that the health and proper functioning of astrocytes are important for protecting neurons from various cellular insults (Okada et al., 2018). Oxidative stress as direct response has effect on the damage to astrocyte and neuron function that occurs during several neurodegenerative diseases (Molofsky et al., 2012; Phatnani and Maniatis, 2015). Thus, alleviating oxidative stress is a potential therapeutic approach to halt and reduce the inflammation and apoptosis in astrocytes.

Resveratrol may act as an antioxidant by modulating intracellular signaling pathways. One important intracellular signaling system is protein kinase C (PKC), a family of 12 serine/threonine kinases.

PKC has been found to modulate cell viability, resulting in the protection of various neuronal cells (Behrens et al., 1999; Cordey et al., 2003; Doré et al., 1999; Maher, 2001; Xie et al., 2000). This pathway is assumed to be involved in the neuroprotective effects of resveratrol against Aβ (Beta Amyloid)-induced neurotoxicity (Han et al., 2004). Resveratrol treatment against Aβ-related oxidative stress was examined in a cell culture model (human-derived SH-SY5Y neuroblastoma cell cultures), was shown to maintain cell viability and exert an anti-oxidative effect by enhancing the intracellular free radical scavenger glutathione (Lange and Li, 2018). Moreover, resveratrol treatment was cultured hippocampal to improve learning and memory (Zhao et al., 2013). Perhaps that is the reason that the addition of resveratrol effectively increased cell survival and decreased cell apoptosis (Sun et al., 2010). This observation, together with the fact that 2-ME and its metabolites can damage in cerebral cortex of mice (Irnidayanti et al., 2013), led us to investigate the potential of neurotoxicity of 2-ME and neuroprotective of resveratrol isolated from tempeh and soybean seed coats in primary cortical neuron cultures.

2. Materials and Methods

2.1. Preparation of resveratrol and 2-methoxyethanol (2-ME)

The trans-resveratrol standard (cat no. R5010) was obtained from Sigma Aldrich. Trans-resveratrol was also isolated from tempeh and soybean seed coats (Irnidayanti and Sutiono, 2019a, b). This compound was dissolved in distilled water at concentrations of 1.4 µg/mL, 2.8 µg/mL and 4.2 µg/mL. 2-ME (cat no. 284467) was obtained from Sigma Chemical Aldrich and dissolved in distilled water at a concentration of 7.5 mmol/L. In this study, the highest concentration of 2-ME in culture medium was less than 0.1% and it did not affect cell viability.

2.2. Culture media

Neurobasal Medium (cat no.12349-015,Gibco), 1% GlutaMAX (cat no. 35050-061, Gibco), 1% penicillin-streptomycin (P4333-20 mL, Sigma), and 2% B-27 supplemented (cat no. 175054-044 Gibco) with 10 mL of 10% fetal bovine serum (cat no. S181H-500, Biowest) was used as complete media. Nine hundred microliters of trypsin-EDTA (cat no. L0930-100), 10 mg DNase 100 µg/mL (cat no.A3778,0010, Applichem) in 3 mL of cold Dulbecco's phosphate-buffered saline (cat no. P0750-N1L, Biowest). FBS 10% in DMEM (cat. no. L0104-500, Biowest).

2.3. Preparation of the cell suspension for the cell culture assay

The cerebral cortex was dissected from fetal mice (*Mus musculus*, Swiss Webster) brains on the 19th gestations day. The cerebral cortex was placed in 3 mL of cold PBS solution containing 900 µL of trypsin-EDTA solution and 100 µg/mL DNase and 10% FBS–DMEM was added, followed by incubation for 15 minutes with an AUTOFLOW IR Water-Jacketed CO2 Incubator (NUARE) and centrifugation (Hitachi Centrifuge Type CT6EL cat no. 905601) at 300 × g for 3 minutes. The cells were grown at 37 °C, 5% CO_2 and 90% relative humidity until the sub confluent stage. The cells were then harvested by treatment with trypsin-EDTA solution. The number of cells was counted in a hemocytometer, and the cell density was adjusted to 10,000 cells/mL in complete media. Neurons were plated on poly-D-lysine-coated dishes in neurobasal containing 1% GlutaMAX, 1% penicillin-streptomycin, 2% B-27 supplement, and 10% fetal bovine serum, maintained in this manner for confluent stages at 37 °C, 5% CO_2 and 90% relative humidity. The use and care of animals were in accordance with the statements of the Guide for Animal Experimentation of the Medical School of the University of Indonesia.

2.4. The MTT assay was carried out in triplicate in 96-well microtiter culture plates

The MTT assay was carried out for determining mitochondrial dehydrogenase activity in living cells. (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a tetrazole, is reduced to purple formazan by NADH in the mitochondria of living cells. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 540 and 570 nm) by an ELISA. The IC50 and R^2 values were calculated using graphs generated from Microsoft Excel, 2007 edition.

A total of 100 µL of the cell suspension (7500 cells) was added to each well of the 96-well plates and incubated at 37 °C, 5% CO_2 and 90% relative humidity for 7 days. The cells were washed with PBS and the medium changed to neurobasal medium without supplemented material for 2 h. Cells were treated with three different concentrations (1.4, 2.8 and 4.2 µg/mL) resveratrol extract (standard, tempeh and soybean seed coats). The plates were incubated for an additional period of 60 minutes in the CO_2 incubator. In control wells, only medium was added. Ten microliters of 5 mg/mL MTT was added to each well, and the plates were incubated for 3 h. At the end of the incubation period, the culture media was carefully removed, and 100 µL of MTT solvent was added to each well. After covering the plates with aluminum foil, the plates were incubated at room temperature for 18 h. The absorbance was measured at 540 nm with a reference filter of 570 nm with Microplate Elisa Reader.

2.5. Cytotoxicity of beta-amyloid incubated with resveratrol

Cell viability was examined by the Biotium assay. In brief, 100 µL of the cell suspension (7,500 cells) was added into each well of the 3 mL sterile culture plates and incubated at 37 °C, 5% CO_2 and 90% relative humidity for 7 days. Cell fractions were washed with PBS and the medium was changed to neurobasal medium without supplemented material for 2 h. Cells were treated with three different concentrations (1.4, 2.8 and 4.2 µg/mL) resveratrol (standard, tempeh and soybean seed coats), and the plates were incubated for an additional period of 60 minutes in the CO_2 incubator. Cells were washed again with PBS, the medium was exchanged for 500 µL of Biotium solution (2 mL of casein, 10 µL of EtBr and 5 mL of PBS) per sterile petri dish and incubated for 30 minutes. Biotium assay was employed here as an indicator of the primary cortical neuron cells proliferation and neural cytotoxicity, which is extensively used as a qualitative and reliable Fluorescent Imaging for cell viability. Exposure of the primary cortical neuron cells to 5 µg/mL of Beta-Amyloid monoclonal Antibody (LN27) Thermo Fisher pre-incubated for 24 h with 10 µL of 4.2 µg/mL Resveratrol and 7.5 mmol/L 2-Methoxyethanol additions in sterile 12-well plate. Cells were observed with a fluorescence microscope (ZOE, Bio-Rad).

3. Results and Discussion

3.1. Results

Administration of a 2-ME dose of 10 mM and resveratrol at doses of 1.4, 2.8 or 4.2 µg/mL indicated that the resveratrol could increase the viability of neuronal cells. Following the increase of concentrations of resveratrol (standard, tempeh and soybean seed coat), the percent live cells increased gradually up to 100% (Tables 1 and 2). In the control group, the cells grew well and formed neuronal networks after 7 days of in vitro culture (Figure 1). When exposed to 4.2 µg/mL all resveratrol, the majority of the cells grew well (Figures 2, 3 and 4). In contrast, after exposure to 2-methoxyethanol, the neurons began to die (Figure 5). The susceptibility of cells to 2-ME+resveratrol exposure was characterized by IC50 values. IC50 values refer to the classification of Gessler et al. (1994); the inhibitory activity of test substances in vitro is divided into three categories: (a) the best activity is when the IC50 value is less than 10 µg/mL, the activity is good if the IC50 value is between 10-50 µg/mL, and the activity is not good if the IC50 value is more than 50 µg/mL. This interpretation of the IC50 value illustrates the ability of a resveratrol concentration to inhibit nerve cell growth in vitro by 50%. The lower the IC50 value, the higher the effectiveness of resveratrol inhibition on the growth of nerve cells. Table 3 shows that the cytotoxic activities of resveratrol from the soybean seed coats (IC50: 17.43 µg/mL) and standard resveratrol (IC50:17.64 µg/mL) were lower than that of resveratrol from tempeh (IC50: 32.22 µg/mL). Table 4 indicated that cytotoxic activities of resveratrol from the soybean seed coats (IC50: 13.40 µg/mL) and standard resveratrol (IC50:17.38 µg/mL) were also lower than that of resveratrol from tempeh (IC50: 31.14 µg/mL). Nevertheless, all of the resveratrol samples tested had IC50 values between 10-50 µg/mL (good activity), and can therefore serve as a good source of bioactivity.

Result of viability cell assay was found from the resveratrol isolated from tempeh, soybean seed coat and

Table 1. Effect of Various concentrations of Resveratrol and 2-Methoxyethanol against Primary Neuron cell viability (540 nm).

Concentration of 2-Methoxyethanol and Resveratrol	% Cell Viability		
	2-ME (10mM)	2-ME (10mM)	2-ME (10mM)
	Resveratrol (Standard)	Resveratrol (Tempeh)	Resveratrol (Soybean Seed Coat)
1.4 (µg/mL)	88.2	100	92.05
2.8 (µg/mL)	94.73	100	85.59
4.2 (µg/mL)	100	100	100

Table 2. Effect of Various concentrations of Resveratrol and 2-Methoxyethanol against Primary Neuron cell viability (570 nm).

Concentration of 2-Methoxyethanol and Resveratrol	% Cell Viability		
	2-ME (10mM)	2-ME (10mM)	2-ME (10mM)
	Resveratrol (Standard)	Resveratrol (Tempeh)	Resveratrol (Soybean Seed Coat)
1.4 (µg/mL)	87.43	100	88.02
2.8 (µg/mL)	95.41	98.25	82.97
4.2 (µg/mL)	100	100	100

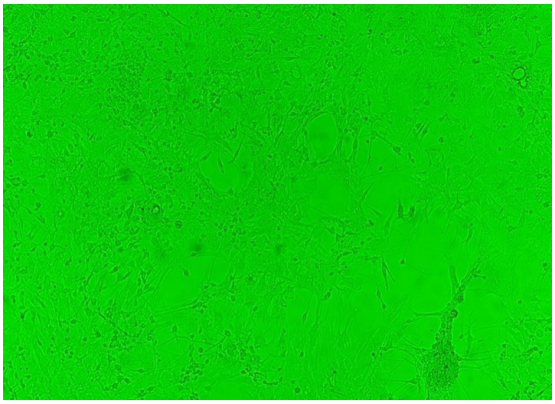

Figure 1. Control Group (10 x10).

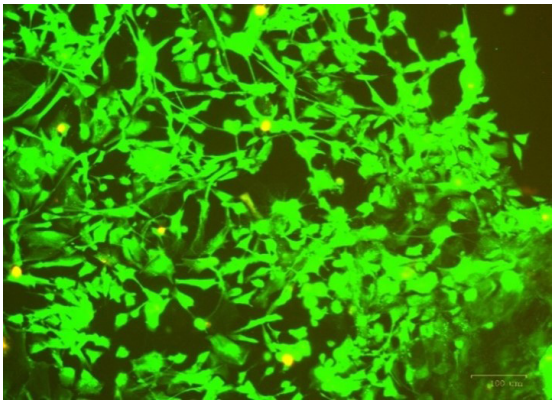

Figure 3. Treatment group : 2- ME + Resveratrol isolated from Tempeh (10 x10).

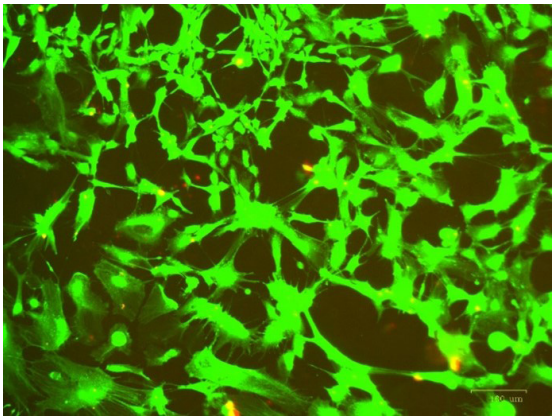

Figure 2. Treatment group : 2- ME + Resveratrol Standard (10 x10).

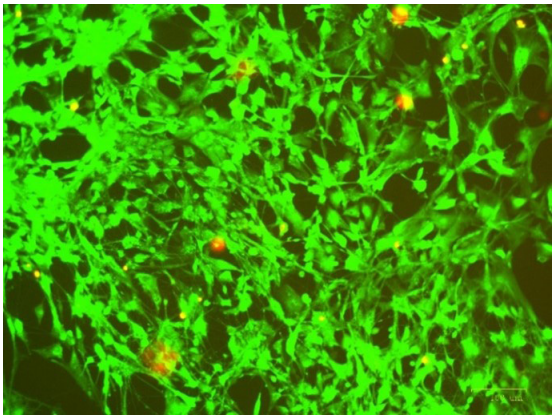

Figure 4. Treatment group : 2- ME + Resveratrol isolated from Soybean seed coat (10 x10).

standard, which shows all viability value are 100% and can be used as an anti-apoptotic agent. These data are supported by images of the neuronal culture cells. In the control group, the cells grew well and formed neuronal networks after 7 days of in vitro culture (Figure 1). Surviving cells are shown with green fluorescence, and apoptotic cells are shown with red fluorescence (Figure 2, 3, 4 and 5). More bright red cells were visible in the 2-ME group (Figure 5), while few apoptotic cells were detected in the 2-ME + resveratrol group (Figure 2, 3 and 4). When 1.4 µg/mL, 2.8 µg/mL or 4.2 µg/mL resveratrol was added to the culture medium, the number of cells with bright red nuclei decreased, showing that resveratrol attenuated neuronal apoptosis. This result indicates that the neuroprotective effect strengthens with an increase in the concentration of resveratrol.

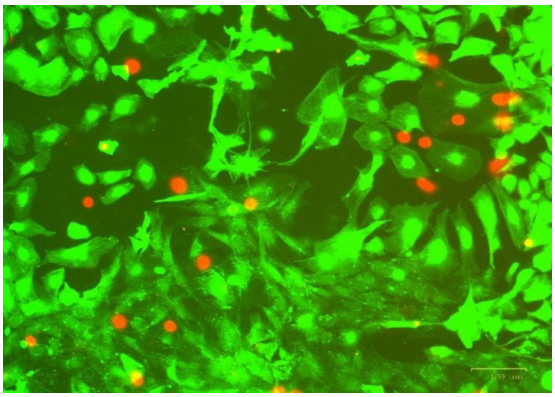

Figure 5. Treatment group : 2- ME (10 x10).

Although we cannot exclude the possibility that resveratrol 4.2µg/mL isolated from Tempe had some direct effects on neurons, since evidence suggest that such effects played only a minor. The administration of resveratrol 4.2µg/mL isolated from Tempe compared with resveratrol standard (Figure 6), indicating that resveratrol strongly attenuates Beta-amyloid by decreasing in the number of death cells examined by biotium assay (Figures 7 & 8). In the presence of 4.2µg/mL resveratrol, the levels of death cells of 2-Methoxyehanol induced were relatively marked lower. In cultures exposure with 2-Methoxyethanol alone, Beta-amyloid induced a significant increase in the number of death cells examined by Biotium assay, most of which were glia (Figure 9).

4. Discussion

In this study, we addressed whether 2-methoxyethanol-induced neurotoxicity can be inhibited by resveratrol. Our results showed that resveratrol isolated from tempeh and soybean seed coats increased neuronal viability and decreased cell apoptosis. Therefore, the neuroprotection of resveratrol against 2-methoxyethanol neurotoxicity is possibly mediated by induced NO (nitric oxide) production,

Table 3. Cytotoxic effect of various concentrations of Resveratrol and 2-Methoxyethanol Against Primary Neuron cell viability in MTT assay (540 nm).

Concentration of 2-Methoxyethanol and Resveratrol	% Inhibition		
	2-ME (10mM)	2-ME (10mM)	2-ME (10mM)
	Resveratrol (Standard)	Resveratrol (Tempeh)	Resveratrol (Soybean Seed Coat)
IC50	(17.64 ± 0.99 µg/mL)	(32.22 ± 1.18 µg/mL)	(17.43 ± 0.16 µg/mL)
1.4 (µg/mL)	11.8	0	7.95
2.8 (µg/mL)	5.27	0	14.41
4.2 (µg/mL)	0	0	0

Table 4. Cytotoxic effect of various concentrations of Resveratrol and 2-Methoxyethanol Against Primary Neuron cell viability in MTT assay (570 nm).

Concentration of 2-Methoxyethanol and Resveratrol	% Inhibition		
	2-ME (10mM)	2-ME (10mM)	2-ME (10mM)
	Resveratrol (Standard)	Resveratrol (Tempeh)	Resveratrol (Soybean Seed Coat)
IC50	(17.38 ± 0.99 µg/mL)	(31.14 ± 0.02 µg/mL)	(13.40 ± 0.30 µg/mL)
1.4 (µg/mL)	12.57	0	11.98
2.8 (µg/mL)	4.59	1.75	17.03
4.2 (µg/mL)	0	0	0

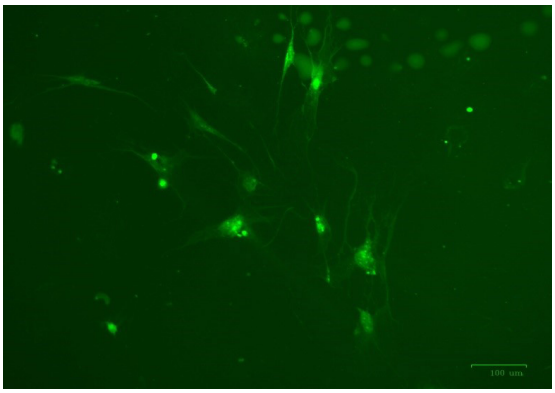

Figure 6. Treatment Group with resveratrol standard after induced by Beta-Amyloid (10 x10).

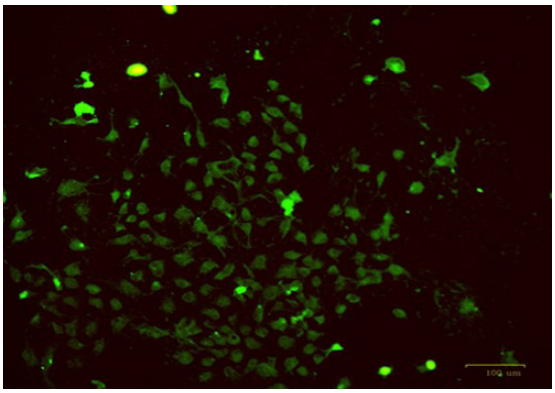

Figure 8. Treatment Group with resveratrol tempeh + 2-ME after induced by Beta-Amyloid (10 x10).

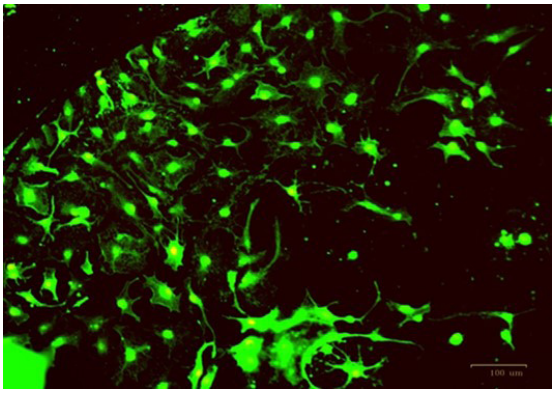

Figure 7. Treatment Group with resveratrol tempeh after induced by Beta-Amyloid (10 x10).

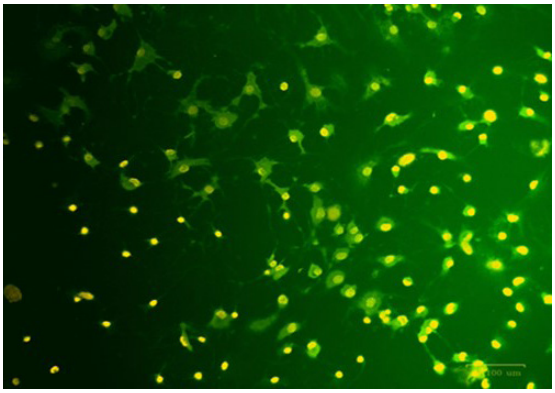

Figure 9. Treatment Group with 2-ME after induced by Beta-Amyloid (10 x10).

and resveratrol significantly protected cells from free radical overload induced neuronal death (Lu et al., 2006). The results of the MTT study of all resveratrol samples at a dose of 4.2 µg/mL showed 0% inhibition against neuronal cell culture in 100% viable cells, but resveratrol from soybean seed coats showed good activity (IC50: 10-50 µg/mL). The cytotoxicity of resveratrol may be due to the presence of flavonoids, which have been reported to have cytotoxic activity due to the presence of phenolic groups (Matsuo et al., 2005).

In our culture model, Resveratrol 4.2 µg/mL isolated from Tempe clearly prevented microglia-dependent Beta-Amyloid toxicity against 2-ME (Figure 6, 7 and 9). Resveratrol isolated from tempeh may facilitate the anti-apoptotic mechanism and/or inhibit the proapoptotic mechanism by regulating certain specific signaling pathways than resveratrol standard. In particular, the underlying mechanism and found that resveratrol shows better anti-apoptotic output and supports the fact that resveratrol is less toxic to astrocytes for longer periods of time (Daverey and Agrawal, 2018). It has been shown that resveratrol samples isolated from tempeh have a positive effect on glia's cells. Based on previous research (Almeida et al., 2009; Yeung et al., 2004) shown than resveratrol can overexpression SIRT1 and activated microglia induced by beta amyloid. The effects of resveratrol on increasing viability and preventing apoptosis are mediated by the Shh signaling pathway. Shh signaling may act on Sirt1 to exert these effects (Tang et al., 2017). It is obvious that glial are important for protecting neurons from various cellular offended and oxidative stress as direct response that occurs during 2-ME induced to cells culture of cerebral cortex. Therefore, resveratrol may be having neuroprotective effect to glia cells and we recommend that further research be conducted to substantiate the neuroprotective effects of resveratrol isolated from tempeh on glial cell cultures. Interestingly, resveratrol was mostly focused on the neuroprotective–neurorescuing properties against the neurotoxic effects (Jang and Surh, 2003). The neuroprotective–neurorescuing action of resveratrol is especially against beta amiloid-induced neurotoxicity (Hardy and Selkoe, 2002).

5. Conclusion

This finding shows the role of resveratrol in nerve protection. Resveratrol at a dose of 2.4 µg/mL can increase cell viability to 100% and inhibit cell death from reaching 0% in primary neuronal cell cultures. This research demonstrated that resveratrol, a natural product isolated from tempeh and soybean seed coats, protected neurons against 2-methoxyethanol-induced damage to cells of cerebral cortex

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