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Research Article

Neuroprotective effect of *Hypericum perforatum* extract against aluminum-maltolate induced toxicity in SH-SY5Y cells

Rabia Yaren Akkuş[1], Barış Bitmez[1], Seda Kuşoğlu Gültekin[1], İrem Gülfem Albayrak[1], Fatih Özen[1], Yiğit Deveci[2], Yusuf Sıcak[3], Emine Akalın Urusak[4], Ademi Fahri Pirhan[5], Belkıs Atasever Arslan[1*]

[1]Department of Bioengineering, Faculty of Engineering and Natural Sciences, Üsküdar University, Istanbul, Turkey
[2]Department of Chemistry, Institute of Science Mugla Sitki Koçman University, Muğla, Turkey
[3] Medicinal and Aromatic Plants Program, Köyceğiz Vocational School, Mugla Sitki Koçman University, Muğla, Turkey
[4]Department of Pharmaceutical Botany, Faculty of Pharmacy, İstanbul University, İstanbul, Turkey
[5]Department of Biology, Faculty of Science, Ege University, İzmir, Turkey

Abstract

Alzheimer's disease is multi-component neurodegenerative disorder. Oxidative stress disrupts regular functioning of metabolism in early-onset Alzheimer's disease. It causes Tau phosphorylation, formation of neurofibrillary tangle and neuron reduction. Due to intense binding of phosphorylated amino acids to aluminum, it induces self-assembly and deposition of high degree of phosphorylated cytoskeletal proteins, such as microtubule and neurofilament-associated proteins. In this study, it is aimed to consider the antioxidant potential of *Hypericum perforatum* extract against neurotoxicity caused by Aluminum-maltolate ($Al(mal)_3$) and its effects on APP gene expression. Four different groups were determined to observe the impact of *H. perforatum* extract. After the incubation of the cells for 24 hours, only the medium was placed in the first group as control. 500 µM $Al(mal)_3$ was added to the second group of cells. 20 µg mL^{-1} *Hypericum perforatum* extract was added to the third group. For the fourth group, 20 µg mL^{-1} *Hypericum perforatum* extract and 500 µM $Al(mal)_3$ were added. While $Al(mal)_3$ increased total antioxidant status levels in SH-SY5Y human neuroblastoma cells, *H. perforatum* extract significantly inhibited $Al(mal)_3$ induced oxidative stress. On the other hand, *H. perforatum* extract significantly decreased APP gene expression levels depending on $Al(mal)_3$ toxicity in SH-SY5Y cells. According to these results, *H. perforatum* extract significantly inhibited $Al(mal)_3$ neurotoxicity against SH-SY5Y cells. To determine

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[1]Correspondence: belkis.arslan@uskudar.edu.tr

synergistic and antagonistic effects of *H. perforatum* extract content is important to examine their specific effects of together with hyperforin, which is a phytochemical produced by some of the members of the plant genus Hypericum, to discover new therapeutic agents against neurodegeneration.

1. Introduction

Alzheimer's disease (AD) is multifactorial chronically progressive neurodegenerative disease (Atasever-Arslan et al., 2020; Ozer et al., 2020). Dementia affects approximately 50 million individuals in worldwide and it is estimated by researchers that this number will reach another 152 million individuals by 2050 (Fleszar et al., 2019). Two main neuropathological features of this disease are β-Amyloid plaques (Aβ) formed by extracellular aggregation of Aβ and construction of neurofibrillary tangles (NFY) by hyperphosphorylated Tau protein (Guan et al., 2021; Vaz and Silvestre, 2020). Aβ aggregation causes lipid peroxidation and apoptosis in neurons (Abdpour et al., 2021).

Although neurotoxic impacts of Aluminum (Al) in the central nervous system are well known, its effect in aluminum-induced neuronal fate is yet uncertain (Exley, 1999; Rebai and Djebli, 2008). Several studies in the literature shown an ascending ratio of AD or AD mortality in areas with high quantity of aluminum in the drinking water (Altmann et al., 1999). Aluminum can affect brain processes like axonal transport, phosphorylation or dephosphorylation of proteins, neurotransmitter synthesis, inflammatory responses, synaptic transmission, protein degradation, and gene expression (Kawahara and Kato-Negishi, 2011). Binding of aluminum to highly phosphorylated cytoskeleton proteins leads to their self-aggregation and accumulation (Muma and Singer, 1996). Maltolate (Mal) is a general ingredient of the human nutrition found in baked grains, soybeans, coffee, and, caramelized and browned foods. Aluminum-maltolate ($Al(mal)_3$) is a potent inducer of apoptosis that has been widely reported as an etiologic factor in AD (Menzies et al., 2017).

Hypericum perforatum plant, with a prevalent distribution around the world, belongs to the order Malpighales, contains more than 16,000 species (Rizzo et al., 2020). It has been widely used for conventional medicine for the treatment of some kind of illnesses, like minor burns, anxiety, and depression. In the literature, there are researches on the chemical structure, pharmacological effects and drug interactions of *H. perforatum* extract and its components. It is known that *H. perforatum* extract and active ingredients of major directly affect

neuroprotective mechanisms and indirectly affect antioxidant mechanisms (Oliveira et al., 2016).

The SH-SY5Y (ATCC® CRL-2266™) cell line, originating from malignant neuroblastoma and showing epithelial morphology (Kitlinska, 2007), was chosen as the cell line to be used to investigate the effects of *H. perforatum* on neurodegeneration induced with $Al(mal)_3$.

The amyloid precursor protein (APP) is a type I single pass transmembrane protein (Collin *et al.*, 2004) which plays an important role in the pathogenesis AD (Aydin *et al.*, 2012).

In this research, it was aimed to determine the antioxidant potential of *H. perforatum* extract towards neurotoxicity which caused by $Al(mal)_3$ and its effects on APP gene expression.

2. Material and Methods

2.1. Preparation of Hypericum perforatum Extract

The flower parts of the *H. perforatum* plant was used for the extract. Plants have been collected from Ege University, Bornova Campus, Izmir Province, Turkey. Identification of species was done by Assistant Professor Ademi Fahri Pirhan, from Ege University, Faculty of Science, Department of Biology. The flowers were dried at room temperature. The extraction method suggested by Deveci et al., 2021 was developed and used (Deveci et al., 2021). Then dried flowers were extracted using a Soxhlet apparatus with methanol. Methanol extracts were dissolved in distilled water using an ultrasonic bath and filtered. Filtration was made acidic with 3% H_2SO_4 at pH 3-4 and extracted with chloroform. The prepared chloroform extracts were dried over anhydrite Na_2SO_4 filtered and concentrated under vacuum.

2.2. SH-SY5Y human neuroblastoma cells

SH-SY5Y cells were subjected to analyze effects of *H. perforatum* on neurodegeneration induced with $Al(mal)_3$. The cells were purchased from "American Type Culture Collection (ATTC)". SH-SY5Y cells were incubated and resuspended according to the research of Arslan et. al., 2017 (Arslan et al., 2017).

2.3. Cell Viability Assay

MTT ("3-(4,5-dimethylthiazole-2yl)-2,5-diphenyl tetrazolium bromide") cytotoxicity assay was performed to detect the suitable dose range of the prepared *H. perforatum* for experiments on SH-SY5Y cells (Arslan et al., 2017).

SH-SY5Y cells were counted and adjusted to 100,000 cells per mL. Ten µL of 6 different stock solutions (1000, 500, 200, 100, 50, and 10 µg mL^{-1}) of the extract were mixed with 90 µL of medium including cells to make the final concentrations as 100, 50, 20, 10, 5, and 1 µg ml^{-1}. Plates were incubated for 48 hours in a humidified environment at 37˚C in a 5% CO_2 incubator. After incubation 10 µL MTT (5 mg mL^{-1}) was added to each well for 4 hours. Then, 80 µL of the supernatant in the wells was withdrawn and 100 µL of 50% solution of sodium dodecyl sulfate (SDS) dissolved in isopropyl alcohol was added (pH 5.5). The prepared SDS mixture destroys the formazan crystals formed by MTT. The resultant color was measured at 570 nm on Multiskan™ GO Microplate Spectrophotometer. As controls, the cells incubated only with the medium were used. The cell viability effect of the extract was compared to control and was calculated (Kaya et al., 2016).

2.4. Preparation of Aluminum Maltolate

$Al(mal)_3$ used in this study is preferable to other aluminum salt forms used in in vitro mechanical studies since it does not form $Al(OH)_3$ precipitates that are insoluble at physiological pH (Zhou and Yokel, 2005). Aluminum was used as a water-soluble mole solution of $AlCl_3.6(H_2O)$. To obtain a 10 mM solution, 20 mM $AlCl_3.6(H_2O)$ was dissolved in distillate water and the pH was arranged to 3.20 mM Maltol (3-Hydroxy-2-Methyl-4-Pyrone) was dissolved in phosphate buffered saline (0.1 M PBS) and the pH was arranged to 7.4. An aluminum-maltol mixture was prepared by combining equal volumes of 20 mM stock aluminum and maltol solutions to give a final concentration of 10 mM for each substance. Then the pH was arranged to 7 with NaOH (Langui et al., 1990).

2.5. Experimental Groups

Four different groups were determined to observe the effect of *H. perforatum* extract. After the cells were incubated for 24 hours, only the medium was placed in the first group as control. 500 µM $Al(mal)_3$ was added to the second group. 20 µg mL^{-1} *H. perforatum* extract was added to the third group. For the fourth group, 20 µg mL^{-1} *H. perforatum* extract and 500 µM $Al(mal)_3$

were added. After 24 hours, RNA isolations of all groups were made for Real-Time PCR (qPCR) experiments.

2.6. RNA Isolation

RNA isolation was performed by using "RNeasy-Mini kit Qiagen Part 1 (74104)" according to the instructions of manufacturer. After concentrations of the RNAs were measured, they was stored at -80˚C.

2.7. Real Time PCR

Analysis of the expression of APP, and β-Actin genes was performed with the "GoTaq 1-Step RT-qPCR System Technical Manual (Cat no: A6020)" kit. β-Actin gene expression in cells was examined for normalization. To calculate alterations of gene expressions was used 2−ΔΔCT method (Livak and Schmittgen, 2001).

2.8. Total Antioxidant Status (TOS) Measurement

Total Antioxidant Status (TOS) levels of the experimental groups were measured a "Rel Assay Diagnostics (Cat no: RL0017, Gaziantep, Turkey)". The kit method is based on the reduction of the green-blue ABTS radical to the colorless ABTS form by antioxidant molecules in the sample. The total antioxidant level in the sample was measured at 660 nm absorbance. The assay was calibrated with an antioxidant standard solution known as Trolox equivalent, which is a vitamin E analog. The results obtained are expressed in mmol L^{-1}. TOS amount was calculated according to the formula:

$$\text{TOS amount} = \frac{[\Delta\text{Abs H2O} - \Delta\text{Abs Sample}]}{[\Delta\text{abs H2O} - \Delta\text{Abs Standart}]}$$

2.9.Total Oxidant Status (TAS) Measurement

Total Oxidant Status (TAS) level measurement was made with a "Rel Assay Diagnostics (Cat no: RL0024, Gaziantep, Turkey)". The experimental protocol is based on the oxidant molecules in the sample oxidize the iron ion-chelate complex to iron ion. The oxidation reaction is prolonged by the booster molecules present in large quantities in the reaction medium. In this

way, the ferric ion forms a colored complex with the chromogen in an acidic environment. The color intensity is related to the total amount of oxidant molecules present in the sample. An it can be measured spectrophotometrically at 530 nm absorbance The assay was calibrated with hydrogen peroxide standart solution. The results obtained are expressed in μmol L^{-1}.

$$\text{TAS amount} = \frac{[\Delta\text{Abs Sample}]}{[\Delta\text{Abs Standart}]} \text{x } 10^{*}$$

(*= Concentration of standart)

2.10. Statistical Analysis

For statistical analysis of results, "Statistical Product and Service Solutions (SPSS)" program was used. Statistical differences between the groups were analyzed by using "One-way analysis of variance (ANOVA)" in SPSS program and with the "Least significant difference (LSD) test" as post-hoc test.

3. Results and Discussion

Hypericum species generally have high antioxidant properties according to the diversity of phenolic compounds they contain. In regard with the results of a research investigating the in vitro free radical discharge impact of the *H. perforatum* plant, it was determined that the antioxidant effect of the extract was directly proportional to the concentration. It has been stated that the plant extract has strong hydroxyl and superoxide anion discharge impacts and also prohibits lipid peroxidation (Mir et al., 2019).

Hypericum extracts show antidepressant properties through hyperforin. In addition, naphthodiantrone hypericin contained in the extract is a potential anticancer agent and there are studies where it has been identified as a potential treatment against neurodegenerative diseases, such as AD (Garg et al., 2012; Hofrichter et al., 2013). It has been determined that tetrahydrohyperforin increases the intracellular Ca^{+2} concentration, resulting in stronger synaptic responses, which prevents degeneration caused by Aβ oligomers (Ittner and Götz, 2011; Nussbaum et al., 2013).

In another study, it was found that the expression of APP gene increased with the gradual increase of $Al(mal)_3$ dose (Liang et al., 2013). Zhang et al., found that APP gene expression

enhanced in PC12 and PC12-ApoE4 cells treated with $Al(mal)_3$ depending on gradual increase depending on $Al(mal)_3$ concentration (Zhang *et al.,* 2019). Therefore, *H. perforatum* extract may have therapeutic effect on APP gene expression in the neurodegeneration induced by $Al(mal)_3$.

Apart from AD, studies evaluating the effects of hyperforin-rich standardized *H. perforatum* extract on Parkinson's disease are also available in the literature. An extract containing 6% hyperforin was administered intraperitoneally to rats with neurotoxicity at 4 mg kg^{-1} per day. Accordingly, it was determined that there was a decrease in nerve damage in the extract administered group (del Rio et al., 2013).

It has been shown that when 100 and 200 μM of $Al(mal)_3$ is applied to SH-SY5Y cells, there is an excessive increase in intracellular ROS levels and synthesis of proteins involved in neuroinflammation. Also it induces unfolded protein response (UPR). Stress by Endoplasmic reticulum (ER) causes many neurological disorders, including AD. In a study researching the impact of aluminum on ER stress, it was shown that aluminum potentially increases protein amounts such as caspase 3, caspase 9, EIF2α, PERK, and inflammatory biomarkers nitric oxide, HMGB1, NF-Kb and NLRP3. It was also determined that aluminum changed IL6, IL10, IL1β, and TNFα mRNA levels. Comprehensive findings demonstrated that aluminum regulates the UPR influence via ER stress resulting in activation of inflammatory pathway and apoptotic proteins in neuronal cells (Rizvi et al., 2016).

In an animal model research about the neuroprotective effect of *H. perforatum*, they concluded that alcoholic extract has neuroprotective activity, which may be related to the prevention of Aβ peptide neuronal degeneration observed in AD (Akiyama et al., 2000).

In order to understand whether *H. perforatum* extract has a protective effect against the neurotoxicity of $Al(mal)_3$, the effect of the extract on cell viability in SH-SY5Y cells was investigated. SH-SY5Y cells were incubated with different concentrations as 100, 50, 20, 10, 5, and 1 μg mL^{-1} of *H. perforatum* extract. The most appropriate dose of *H. perforatum* extract for all experiments in this research was determined as 20 μg mL^{-1} considering cell viability (Figure 1).

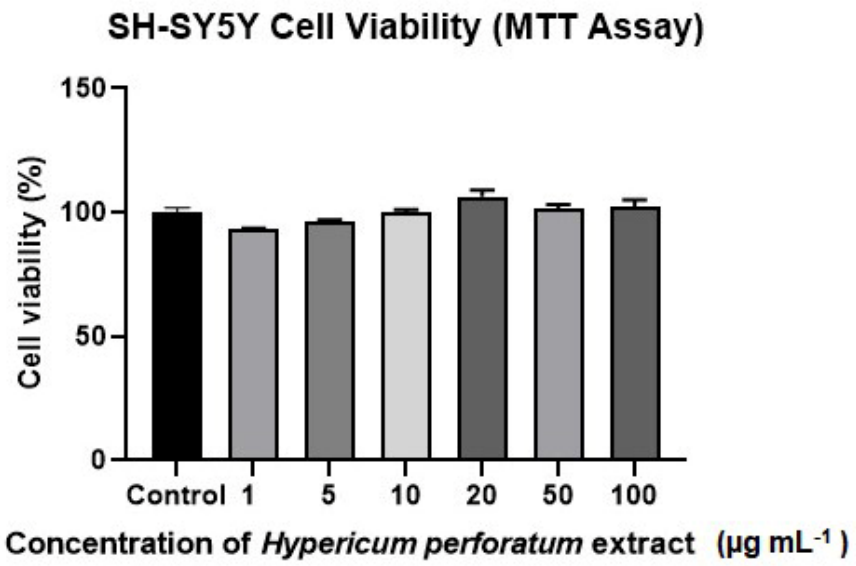

Figure 1. Cell viability % results of *Hypericium perforatum* extract on SH-SY5Y cells.

$Al(mal)_3$ incubation of SH-SY5Y cells was significantly increased APP gene expression in those cells. Conversely, *H. perforatum* extract meaningfully decreased this effect of $Al(mal)_3$ in SH-SY5Y cells. In addition, gene expression level of APP gene is lower than control in the group 2 (only *H. perforatum* extract incubation) (Figure 2).

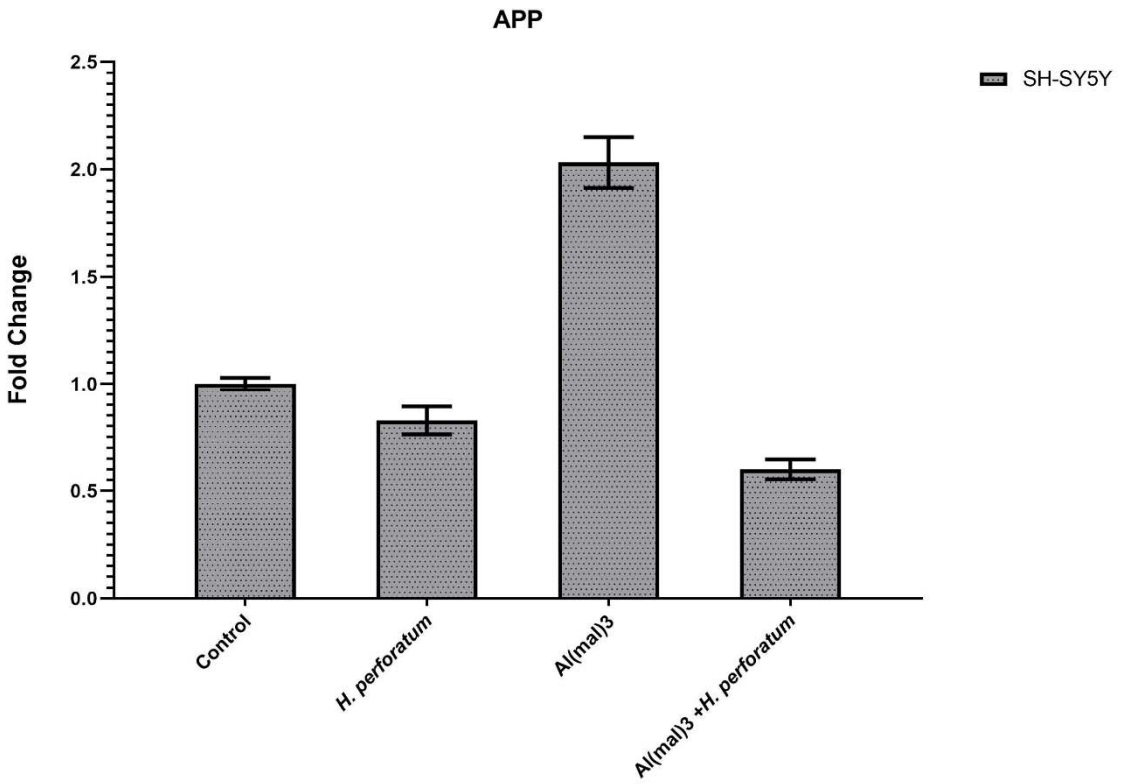

Figure 2. APP gene expression levels in control, H. perforatum, $Al(mal)_3$ and *H. perforatum* + $Al(mal)_3$ treated SH-SY5Y cells.

In this study, $Al(mal)_3$ significantly increased TOS level comparing with other experimental groups and control. Altough TOS levels of *H. perforatum* involved in groups 2 and 4 were higher than control group, it significantly decreased this effect of $Al(mal)_3$ in group 4 (Figure 3). On the other hand, *H. perforatum* and $Al(mal)_3$ did not change TAS levels in SH-SY5Y cells (Figure 4). It was shown in the literature that $Al(mal)_3$ significantly increased TOS level in SH-SY5Y and PC-12 cells (Wang *et al.,* 2019).

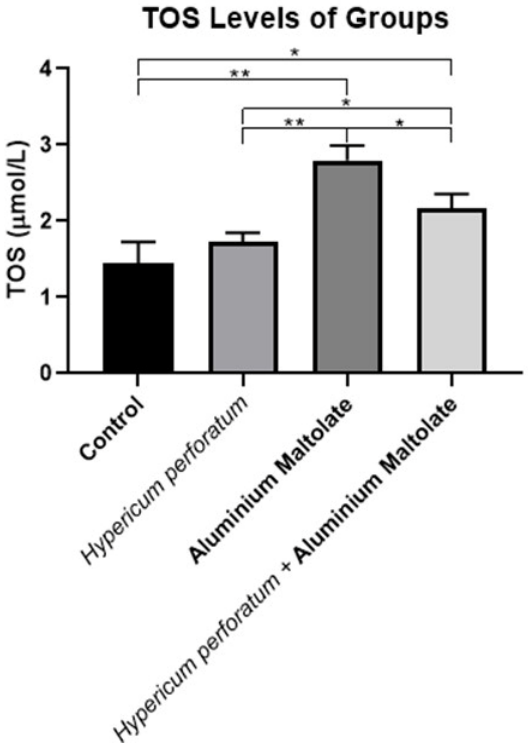

Figure 3. TOS levels in control, *H. perforatum*, $Al(mal)_3$ and *H. perforatum* + $Al(mal)_3$ treated SH-SY5Y cells.

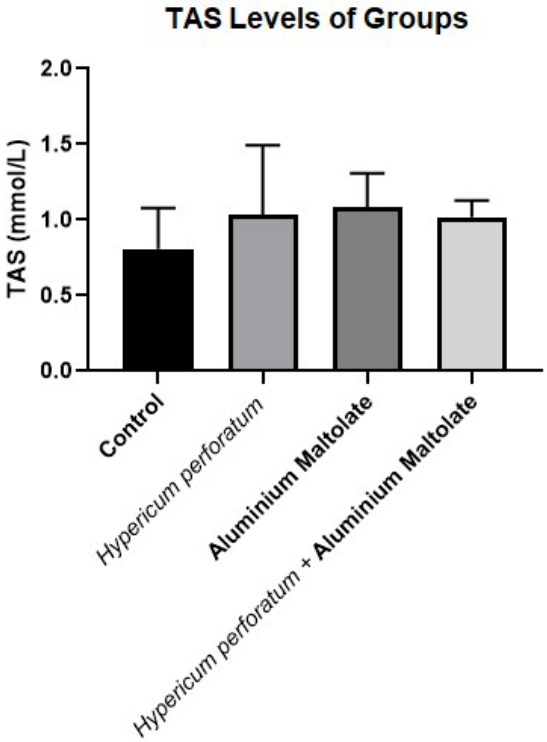

Figure 4. TAS levels in control, *H. perforatum*, $Al(mal)_3$ and *H. perforatum* + $Al(mal)_3$ treated SH-SY5Y cells.

It was shown that hyperforin, the major content of *H. perforatum*, reduces ROS production and prevents the formation of Aβ oligomers and Aβ fibrils in neurotoxicity induced by injecting amyloid fibrils into the hippocampus of rats (Dinamarca et al., 2006; Griffith et al., 2010). Huang et al., showed that hyperforin decreased $Al(mal)_3$ induced Tau phosphorylation and Aβ1-42 formation and increased cell viability. Degradation of APP are regulated by the inhibition of AKT/GSK-3β signaling pathway. It is thought that Hyperforin controls phosphorylation of this signaling pathway (Huang et al., 2017).

According to these results, *H. perforatum* extract significantly inhibited $Al(mal)_3$ neurotoxicity against SH-SY5Y cells. To determine synergistic and antagonistic effects of *H. perforatum* extract content is important to examine their specific effects of together with hyperforin to discover new therapeutic agents against neurodegeneration.

4. Conclusion

Various researches have revealed that *H. perforatum* extract has a neuroprotective effect, but it was shown for the first time in this study that it has a neuroprotective effect against $Al(mal)_3$ toxicity. According to these results, *H. perforatum* extract significantly inhibited $Al(mal)_3$ neurotoxicity against SH-SY5Y cells. To determine synergistic and antagonistic effects of *H. perforatum* extract content is important to examine their specific effects of together with hyperforin to discover new therapeutic agents against neurodegeneration. In the direction of fully elucidate the effect of *H. perforatum* on neurodegeneration, studies with this plant extract should be increased.

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