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ORIGINAL ARTICLE

Neuroprotective Effect of Compounds Isolated from *Euonymus alatus* on Glutamate-Induced Oxidative Stress in HT22 Hippocampal Cells

Youn Sik Jung[1], Hyeon Woo Lee[1], Choong Je Ma[1,2]

[1]Department of Medical Biomaterials Engineering, College of Biomedical Science, Kangwon National University, [2]Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon, South Korea

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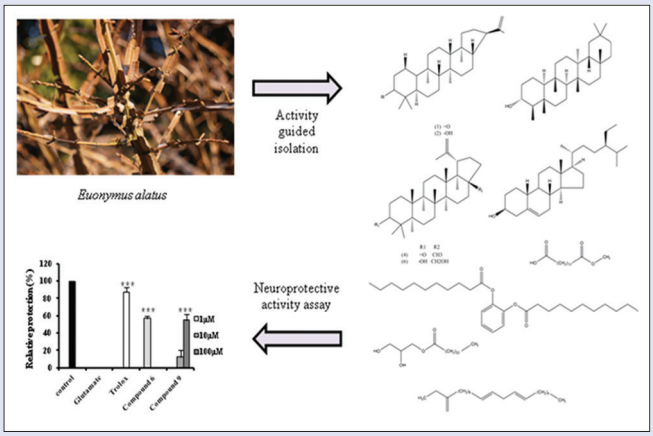
ABSTRACT

Background: *Euonymus alatus* is used to treat diabetes in China. It is also known to have antioxidant and anti-inflammatory effects. **Objectives:** In this study, we isolated 10 compounds from *E. alatus* and confirmed that compounds whether protected neuroral cell (HT22) against glutamate induced toxicity. **Materials and Methods:** The *n*-hexane fraction of *E. alatus* was significantly protected HT22 cells injured by the excitotoxic amino acid, L-glutamate. We isolated ten compounds from n-hexane fraction of *E. alatus*, and they were identified as moretenone (1), moretenol (2), friedelanol (3), lupenone (4), β-sitosterol (5), betulin (6), undecanoic acid, 1,2-phenylene ester (7), glycerol 1-tetracosanoate (8), methyl hydrogen tetradecanedioate (9), and 10,13-nonadecadienoic acid, methyl ester (10) by spectroscopic data such as UV, IR, NMR, Mass spectroscopy. **Results:** Their neuroprotective activity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Betulin (6) and methyl hydrogen tetradecanedioate (9) had significant neuroprotective activity against glutamate-injured HT22 cells. Furthermore, reactive oxygen species level and intracellular Ca^{2+} was decresed by betulin (6) and methyl hydrogen tetradecanedioate (9) and these compounds increased mitochondrial membrane potential, total glutathione (GSH) level, GSH reductase activity and GSH peroxidase activity. **Conclusion:** Our results suggest that betulin (6) and methyl hydrogen tetradecanedioate (9) significantly protect HT22 cells against glutamate-induced oxidative stress, through anti-oxidative activities.

Key words: Alzheimer's disease, antioxidant, *Euonymus alatus,* HT22, neuroprotection

SUMMARY

- Ten compounds were isolated from *Euonymus alatus*. Among them, betulin (6), methyl hydrogen tetradecanedioate (9) exerted significant neuroprotective activity against neuronal cell death in glutamate injured HT22 cells through the antioxidative pathway.



Abbreviations used: AD: Alzheimer's disease; Aβ: Amyloid beta plaque deposit; NFT: Neurofibrillary tangles; ROS: Reactive oxygen species.

Correspondence:

Prof. Choong Je Ma,
Department of Medical Biomaterials Engineering, College of Biomedical Science, Kangwon National University, Hyoja-2 Dong, Chuncheon 200-701, South Korea.
E-mail: cjma@kangwon.ac.kr

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INTRODUCTION

Because of human life-span increase, incidence rate of various degenerative diseases is steeply increasing these days. Among them, neurodegenerative disease such as Alzheimer's disease resulted in very serious problem to patients themselves and also made their society pay huge expenses. It was well known that glutamate was endogenous and had an important roles as a neurotransmitter under the normal concentrations.[1] However, excessive glutamate in the central nervous system made high risk of neuronal dysfunction and resulted in neurodegenerative diseases including Alzheimer's and Parkinson's disease.[2-4] Glutamate neurotoxicity is occurred by glutamate receptor, N-methyl-D-aspartate or AMPA/KA, mediated excitotoxicity and reactive oxygen species (ROS)-mediated oxidation. As a result, neuronal cells were died through apoptosis or necrosis.[1] The HT22 cell lines were originated from mouse hippocampus and did not have glutamate receptors. In this reason, HT22 has been famous for evaluation of mechanisms of glutamate-injured neuronal cell death. High concentration of glutamate decreased the glutamate/cystine antiporter and incurred reduction of glutathione (GSH), an endogeneous anti-oxidative compound. Continuously, glutamate increased intracellular ROS level and Ca^{2+} concentration. Increased Ca^{2+} caused to lipid peroxidation by lipoxygenase and mitochondrial dysfunction by change of mitochondrial membrane potential. Through these processes,

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neuronal cells were damaged by oxidative stress and finally died by necrotic and apoptotic process in HT22 cells.[5-8] Therefore, protection of these necrotic and apoptotic cell death induced by oxidative stress by glutamate might be a very highly recommended strategy to development of medicine to prevent or treat neurodegenerative disorders.[9]

Euonymus alatus has been known as the burning bush in Korea. This medicinal plant used as traditional medicine. It prevented the progress of atherosclerosis, reduced dysmenorrhea, regulated blood circulation, relieved pain, and eliminated stagnant blood.[10-12] Its wing shaped cork cambium of stem of *E. alatus* has been used to treat cancer in Korean traditional medicine.[13] Major components of *E. alatus* are flavonoids including quercetin, kaempferol and rutin and alkaloids including alatamine, eunymine, and evorine.[14,15] According to recent pharmacological studies of *in vitro* and *in vivo* assay, it has been reported that *E. alatus* showed anti-tumor activities.[16] However, neuroprotective activity of *E. alatus* has not yet been published in our knowledge. In the course of search for neuroprotective activities in various natural product extracts, the total methanolic extract of the *E. alatus* was exerted neuroprotective activity in our assay system.

We presently isolated 10 compounds from methanolic extract of *E. alatus* based on activity guided isolation and identified their structures by spectroscopic data. Moreover, we also evaluated the neuroprotective effect of these compounds against glutamate-induced neurotoxicity in HT22 cells.

MATERIALS AND METHODS

Plants

The herbs of *E. alatus* were obtained from Kyungdong traditional herbal market (Seoul, Korea). The voucher specimen (No. CJ090M) has been deposited at the Natural Product Laboratory, Kangwon National University (Chuncheon, Korea).

Isolation and analysis reagents

All solvents such as methanol, n-hexane, chloroform, ethyl acetate, and n-Butanol for extraction and fraction were purchased from DAE JUNG in Korea. Silica gel 60-F_{254} plates (Merck, Germany) were used for thin-layer chromatography (TLC) analysis. The spots on TLC were visualized by applying to Anisaldehyde-H_2SO_4 followed by heating. Silica gel (70–230 mesh, Merck, Germany) and sephadex LH-20 (bead size 25–100 μm, Sigma, USA) were used for stationary phase of column chromatography. High-performance liquid chromatography (HPLC) grade solvents, such as water, methanol, and acetonitrile were purchased from J. T. Baker (USA). trifluoroacetic acid was purchased from DAE JUNG in Korea.

Cell culture and *in vitro* test regents

Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, Hank's balanced salt solution and trypsin-EDTA for cell culture were purchased from Gibco BRL Co, (U.S.A). Glutamate, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO, sodium bicarbonate ($NaHCO_3$), HEPES, 2',7'-dichlorofluorescin, L-GSH reduced, L-GSH oxidized, hydrogen peroxide solution, rhodamine 123, 5',5'-dithiobis (2-nitrobenzoic acid) and triton X-100 were obtained from sigma (U.S.A). Fura 2-AM was purchased from Dojindo (U.S.A). 0.2 μm membrane filter was purchased from ADVANTEC in Japan.

Extraction and partition of *Euonymus alatus*

Dried herbs of *E. alatus* (6.57 kg) of MeOH extract was obtained by ultrasonication-assisted extraction. The solvent of extract was removed by rotary vacuum evaporator and 175.85 g of residue was obtained. We added distilled water to this residue to make water suspension. After that, this suspension was fractionated with organic solvent based on solvent polarity such as n-hexane, $CHCl_3$, EtOAc and n-BuOH. As a result, we obtained four fractions including n-hexane (33.14 g), $CHCl_3$ (15.4 g), EtOAc (6.13 g) and n-BuOH (53.02 g) fractions, respectively.

Isolation of ten compounds from hexane fraction of *Euonymus alatus* extract

The n-hexane fraction was loaded in a silica gel column (95 cm × 9 cm, 70–230 mesh) and applied as mobile phase of gradient condition of $CHCl_3$: MeOH (100:1→0:1, v/v). After that we obtained seven fractions (A to G). Fraction B was evaporated *in vacuo* and dissolved in MeOH. And then compound 1 (346.0 mg) was obtained as a crystal form. Fraction D solution was evaporated *in vacuo* and suspended in MeOH. And then compound **2** (455.3 mg) was obtained as a precipitation form. To obtain compound 3, fraction C was loaded to medium pressure liquid chromatography (MPLC) in the condition of mobile phase of $CHCl_3$: MeOH = 100:0→0:1, v/v and three fractions (C1-C3) were obtained. Fraction C3 solution was evaporated *in vacuo* and suspended in MeOH. And then, compound **3** (14.2 mg) was obtained as a precipitation form. Fraction B was subjected to by Silica gel column chromatography with condition of mobile phase of hexane: Ethyl acetate = 100:1→1:1, v/v (B1-B8). Fraction B5 solution was evaporated *in vacuo*. And compound 4 (324.7 mg) was obtained as a crystal form. Fraction F was loaded to silica gel column chromatography in the condition of $CHCl_3$: MeOH = 200:0→0:1 (v/v) and 19 of subfractions were obtained (F1-F19). Fraction F5 solution was evaporated *in vacuo*. Moreover, compound **5** (64.7 mg) was obtained as a crystal form. Fraction F7 was loaded to MPLC with the mobile phase condition of $CHCl_3$: MeOH = 100:0→0:1 (v/v) and three subfractions were obtained (F7a-F7c). Fraction F7c solution was evaporated *in vacuo*. Compound 6 (447.1 mg) was obtained as a powder form. Fraction F6 solution was evaporated *in vacuo*. Compound **7** (245.6 mg) was obtained as a powder form. And compound 8 (15.4 mg) was obtained as a powder form in fraction F10. Fraction B was loaded to silica gel column chromatography with the mobile phase condition of hexane: Ethyl acetate = 100:1→1:1 (v/v) and eight subfraction were obtained (B1-B8). Fraction B3 was loaded to preparative HPLC. The mobile phase condition is acetonitrile (ACN): Water = 20:80→100:0 (v/v) and four subfractions were obtained (B3a-B3d). Compound 9 (10.3 mg) was obtained from fraction B3a by preparative HPLC (ACN: Water = 20:80→100:0, v/v). Compound 10 (9.6 mg) was obtained from fraction B3c by preparative HPLC (ACN: Water = 20:80→100:0, v/v).

Cell culture

The HT22 cell, mouse hippocampal cell line, was provided by Seoul National University, Korea. The cells were cultured in DMEM media. DMEM media were used after filtered through 0.25 μm membrane filters. We added 10% FBS, 1% penicillin/streptomycin, 2 mg/ml $NAHCO_3$ and 15 mM HEPES to DMEM media as supplements. Cells were incubated at 37°C in a humidified incubator with 5% CO_2.

Measurement of cell viability

MTT assay is applied for the evaluation of cell viability. The cells were loaded in a 48-well plate (concentration of cells: 2×10^4 cells/well). And, cells were incubated for 23 h at 37°C in a humidified incubator with 5% CO_2. After 1 h of treatment of test samples and positive control, trolox (50 μM), glutamate (2.5 mM) was added in each wells. After 24 h,

150 µl MTT solution (concentration of 1 mg/ml in PBS) was treated into each well. After 3 h, culture media were removed with suction. The insoluble dark blue formazan product was dissolved in 250 µl DMSO to purple solution. Absorbance (Ab) of purple solution was at 570 nm by a microplate reader. The neuroprotective activity of samples was determined by relative protection (%); Relative protection = (Ab of glutamate-treated with sample group – Ab of only glutamate-treated group)/(Ab of control group – Ab of only glutamate-treated group) ×100.

Measurement of reactive oxygen species

2', 7'-dichlorofluorescin diacetate (H_2-DCF-DA) was used for the measurement of glutamate-induced ROS changes in HT-22 cells. The cells were incubated for 24 h at 37°C in a humidified incubator with 5% of CO_2. Glutamate (2.5 mM) was added in each wells after 1 h of treatment of test samples and positive control, trolox (50 µM). After 8 h, 40 µl H_2-DCF-DA was treated into each well. After 1 h, the culture media were removed with suction. The fluorescence product was dissolved with 200 µl triton x-100. The fluorescence was measured at 530 nm of emission wavelength and 488 nm of excitation wavelength.

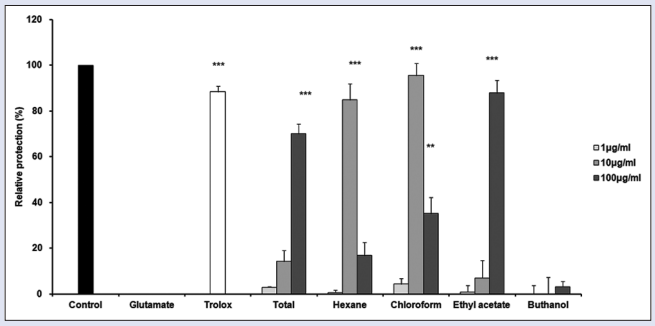

Figure 1: The neuroprotective effects of *Euonymus alatus* fraction against glutamate-induced cytotoxicity in HT22 cells. Cell was pretreated with 1, 10, and 100 µg/ml of the fractions and trolox (30 µM) and then 30 µl of 2.5 mM glutamate was treated after 1 h. Each bar represents the mean ± standard deviation of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 versus glutamate-injured cells (ANOVA)

Measurement of intracellular Ca^{2+} level

Excessive glutamate induced increase of intercellular Ca^{2+} concentration. Increased Ca^{2+} concentration leads to neuronal cell death through the malfunction of the mitochondrial membrane. Cytosolic Ca^{2+} concentration was measured with Fura-2AM fluorescent dye in HT22 cells. HT22 cells were cultured for 24 h at 37°C in a humidified incubator with 5% CO_2. Glutamate (2.5 mM) was added in each well after 1 h of treatment of test samples and positive control, trolox (50 µM). After 1 h, 40 µl Fura-2AM (2 µM) was treated into each well. After 20 min, the cells culture media were changed to HEPES buffer saline and put on the table for 1 h. The fluorescence was measured at 510 nm of emission wavelength and 380 nm of excitation wavelength.

Measurement of mitochondrial membrane potential

Rhodamine 123, the fluorescent dye, was used for the measurement of mitochondrial membrane potential, as reported previously. HT22 cells were cultured for 24 h at 37°C in a humidified incubator with 5% CO_2. Glutamate (2.5 mM) was added in each well after 1 h of treatment of test samples and positive control, trolox (50 µM). After 24 h, 30 µl Fura-2AM (10 µM) was treated into each well. After 30 min, the cells culture media were removed with suction and washed with PBS two times. The fluorescence product was dissolved with 200 µl triton x-100 (1%, w/v). The fluorescence was measured at 525 nm of emission wavelength and 488 nm of excitation wavelength.

Measurement of total glutathione content and activities of antioxidant enzymes

HT22 Cells were loaded into 6-well plates (concentration of cells: 3.4×10^4 cells/well). The cells were incubated for 24 h at 37°C. Glutamate (2.5 mM) was added in each well after 1 h of treatment of test samples and positive control, trolox (50 µM). After 24 h, the cells culture media were removed with suction and washed with PBS two times. To obtain cell lysates, cells were homogenized with homogenizer. The cell lysate was centrifuged for 30 min at 10,000 g at 4°C. Antioxidative enzyme activity and GSH contents were measured with supernatant of the cells after centrifuge.

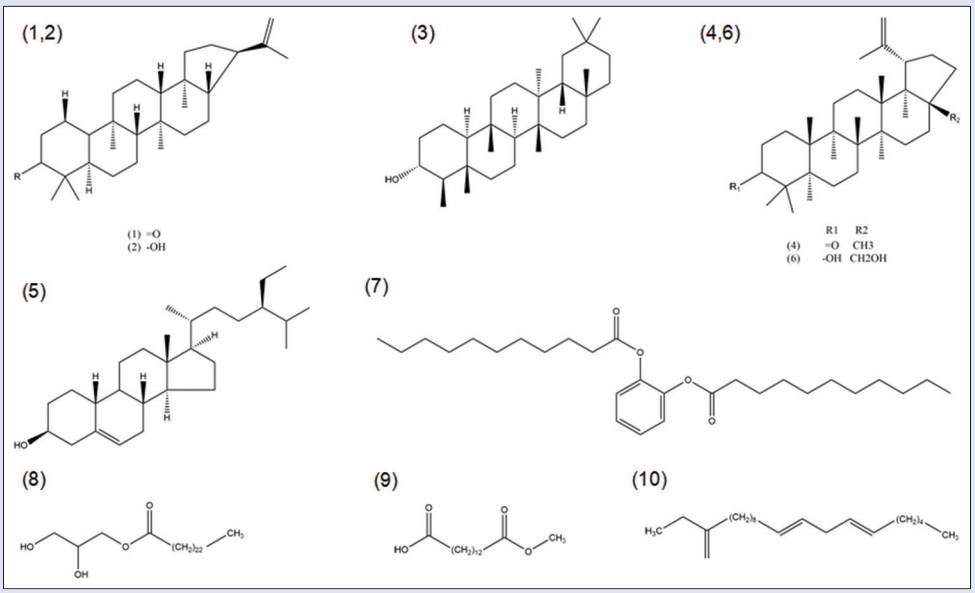

Figure 2: Chemical structure of compounds 1–10 isolated from *Euonymus alatus*

RESULTS AND DISCUSSION

Isolation of compound 1–10

6 kg of *E. alatus* were extracted with 80% methanol and total methanolic extract (200 g) was obtained. Methanolic extract was fractionated sequentially according to solvent polarity with n-hexane, $CHCl_3$, EtOAc, and n-BuOH solvent. Among them, n-hexane, chloroform, and EtOAc fraction were showed significant neuroprotective activities against glutamate-induced toxicity in HT-22 cells [Figure 1]. Therefore, we tried to isolate active compounds from n-hexane fraction by using various of chromatographic techniques such as open-column chromatography with stationary phase of silica gel or, sephadex LH-20, MPLC and preparative HPLC. As a results, we isolated moretenone (1), moretenol (2), friedelanol (3), lupenone (4), β-sitosterol (5), betulin (6), undecanoic acid, 1,2-phenylene ester (7), Glycerol 1-tetracosanoate (8), Methyl hydrogen tetradecanedioate (9), and 11,14-Nonadecadienoic acid, methyl ester (10) [Figure 2]. Their structures were elucidated by comparison of various spectroscopic data such as ^1H and ^{13}C NMR.

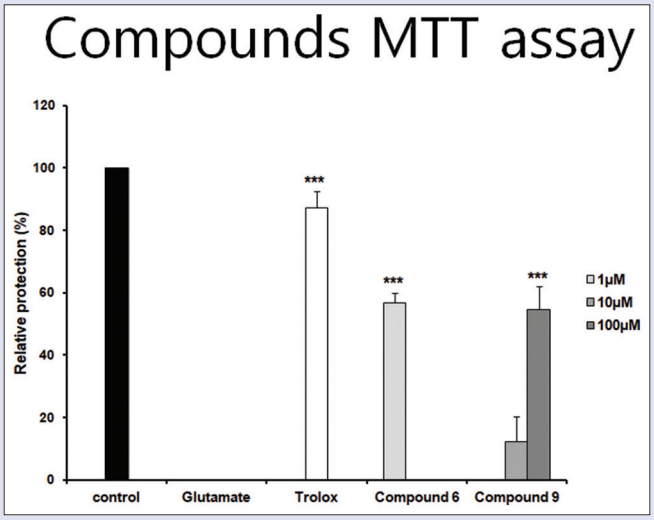

Figure 3: The neuroprotective effects of compounds 1–10 on glutamate-induced cytotoxicity in HT22 cells. Cell was pretreated with 1, 10 and 100 μM of the isolated compounds 1–10 and trolox (30 μM) and then 30 μl of 2.5 mM glutamate was treated after 1 h. Each bar represents the mean ± standard deviation of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 versus glutamate-injured cells (ANOVA)

Neuroprotective activity assay

Neuroprotective activity against glutamate injured neurotoxicity was evaluated by MTT assay. Neuroprotective activities of partitioned fractions and isolated compounds (1–10) were evaluated. All partitioned fractions except BuOH fraction showed significant neuroprotective effect [Figure 1]. Also, Betulin (6) and methyl hydrogen tetradecanedioate (9) significantly reduced glutamate-induced cytotoxicity in HT22 cells [Figure 3]. Moreover, compound **6** showed most potent neuroprotective effect at 0.1 μM [Figure 4a] and compound 9 showed most potent neuroprotective effect at 100 μM [Figure 4b]. These data showed that *E. alatus* total methanolic extract, fraction, and betulin (6) and methyl hydrogen tetradecanedioate (9) had potent neuroprotective activities in excitotoxic glutamate-injured HT22 cells.

Inhibition of reactive oxygen species accumulation

Glutamate-treated cells increased ROS formation by 140.01% compared to the control group. Betulin and methyl hydrogen tetradecanedioate were effectively suppressed ROS production in the dose dependant manner. Betulin (6) decreased ROS formation at the concentration of 0.1 μM by 116.92% of untreated control group [Figure 5a]. And, methyl hydrogen tetradecanedioate (9) also diminished ROS accumulation at the concentration of 100 μM by 121.21% of untreated control group [Figure 5b]. According to these data, we suggested that betulin and methyl hydrogen tetradecanedioate inhibited death of glutamate-injured HT22 cell by suppress of ROS production.

Inhibition of intracellular Ca^{2+} level

Elevated levels of Ca^{2+} in cells generally make an induction of ROS overproduction and as a result, induce lipid peroxidation. After treatment of two active neuroprotective compounds, their effect of glutamate-induced intracelluar Ca^{2+} concentration were evaluated with Fura 2-AM fluorescent dye. Intracellular Ca^{2+} concentration increased by glutamate treatment by 130.42% of control treated group. Betulin and methyl hydrogen tetradecanedioate significantly decreased intracellular Ca^{2+} concentration that increased after glutamate treatment. 0.1 μM

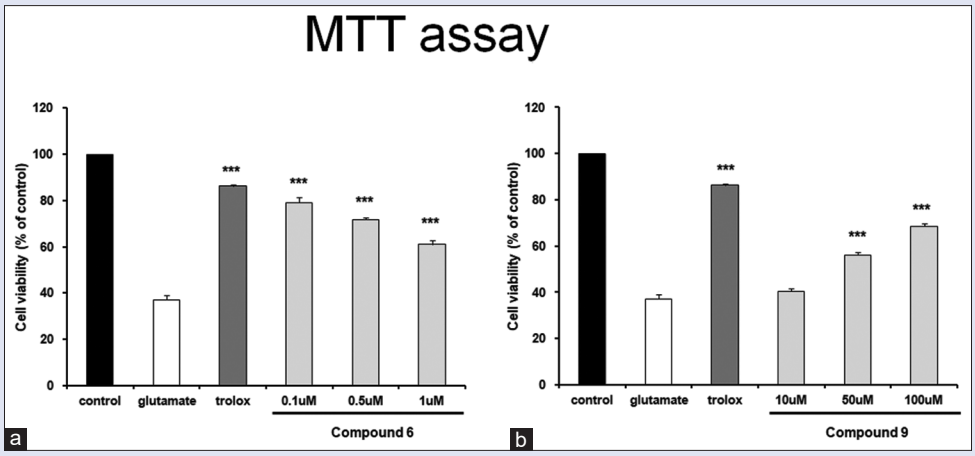

Figure 4: The neuroprotective effects of compound 6 (a) and compound 9 (b) on glutameate-induced cytotoxicity in HT22 cells. Cell was pretreated with 0.1, 1, and 10 μM of compound 6 and 10, 50, and 100 μM of compound 9 and trolox (30 μM). Then 30 μl of 2.5 mM glutamate was treated after 1 h. Each bar represents the mean ± standard deviation of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 versus glutamate-injured cells (ANOVA)

of Betulin (6) decreased Ca^{2+} concentration to 106.91% of control group [Figure 6a]. And, methyl hydrogen tetradecanedioate (9) also diminished Ca^{2+} concentration at the concentration of 100 μM by 105.56% of untreated control group [Figure 6b]. These results

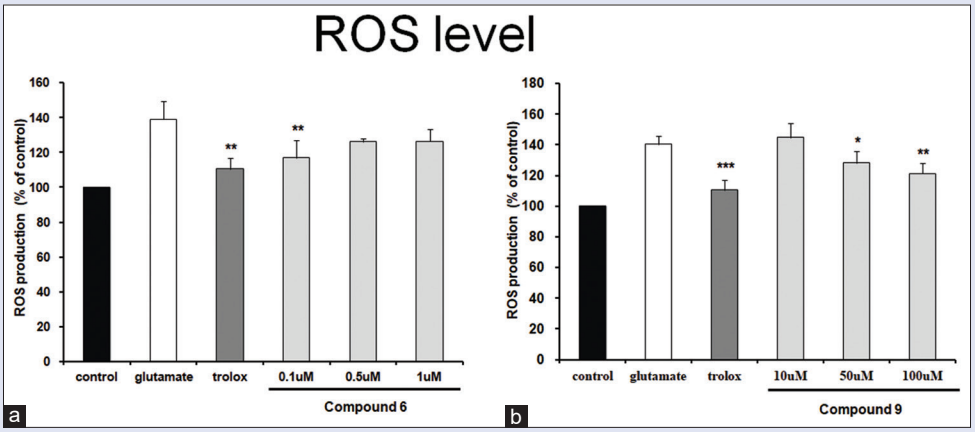

Figure 5: The reactive oxygen species level of compound 6 (a) and compound 9 (b) on glutamate-induced cytotoxicity in HT22 cells. Cell was pretreated with 0.1, 1, and 10 μM of compound 6 and 10, 50, and 100 μM of compound 9 and trolox (30 μM). Then 30 μl of 2.5 mM glutamate was treated after 1 h. Each bar represents the mean ± standard deviation of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus glutamate-injured cells (ANOVA)

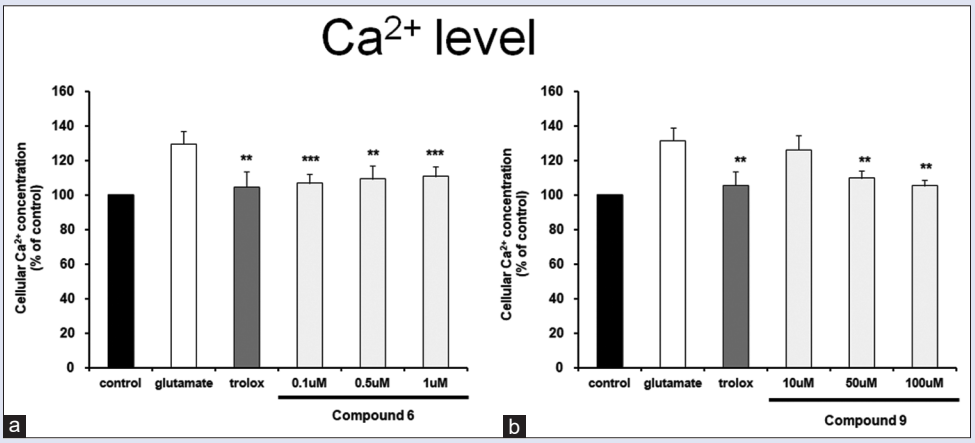

Figure 6: The Ca^{2+} level of compound 6 (a) and compound 9 (b) on glutamate-induced cytotoxicity in HT22 cells. Cell was pretreated with 0.1, 1, and 10 μM of compound 6 and 10, 50 and 100 μM of compound 9 and trolox (30 μM). Then 30 μl of 2.5 mM glutamate was treated after 1 h. Each bar represents the mean ± standard deviation of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus glutamate-injured cells (ANOVA)

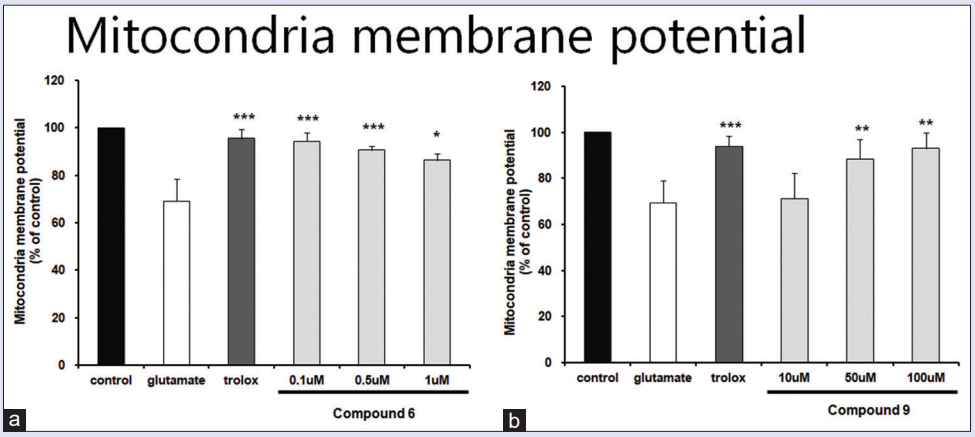

Figure 7: The mitochondrial membrane potential of compound 6 (a) and compound 9 (b) on glutamate-induced cytotoxicity in HT22 cells. Cell was pretreated with 0.1, 1, and 10 μM of compound 6 and 10, 50 and 100 μM of compound 9 and trolox (30 μM). Then 30 μl of 2.5 mM glutamate was treated after 1 h. Each bar represents the mean ± standard deviation of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus glutamate-injured cells (ANOVA)

indicated that betulin and methyl hydrogen tetradecanedioate inhibited death of glutamate-injured HT22 cell by suppress of intracellular Ca^{2+} concentration.

Inhibition effect glutamate-induced mitochondrial dysfunction

High amount of ROS is the one of the reason of mitochondrial injury. After mitochondrial injury by ROS, mitochondrial membrane potential consequently was disrupted. Then, we tried to evaluate effect of two neuroprotective compounds on mitochondrial membrane potential after glutamate treatment in HT22 cells. Rhodamine 123, a kind of fluroscent dye, was used for measurement of mitochondrial membrane potential in our assay system. The mitochondrial membrane potential was significantly decreased to 69.29% of control group after glutamate treatment in HT22 cells according to oxidative stress. However, 0.1 μM of Betulin (6) restored mitochondrial membrane potential to 94.34% of control group [Figure 7a]. And, methyl hydrogen tetradecanedioate (9) also significantly increased mitochondrial membrane potential in a dose dependent manner and showed maximal activity at the concentration of 100 μM by 92,26% of untreated control group [Figure 7b]. These results indicated that

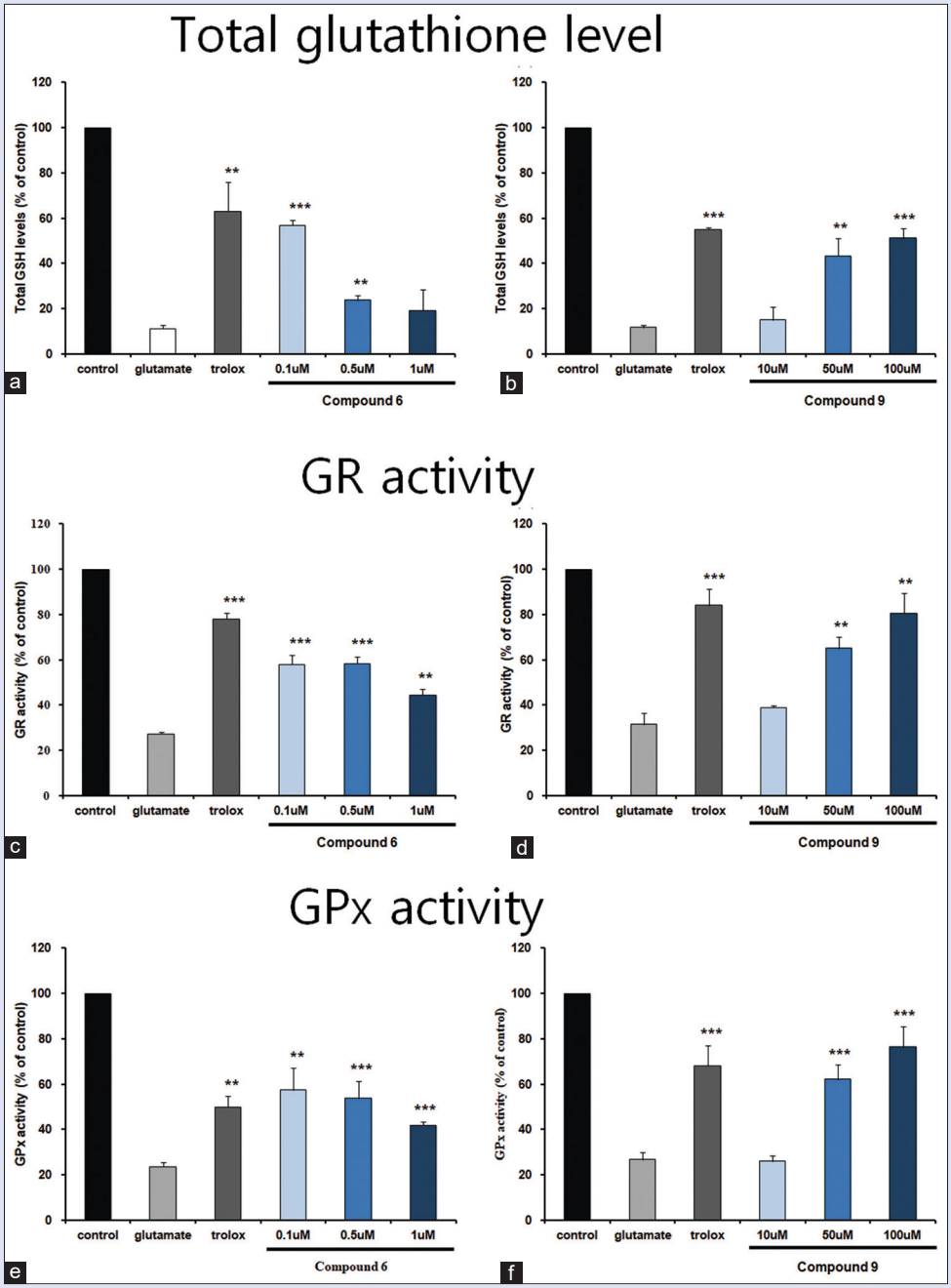

Figure 8: The glutathione level (a and b), glutathione reductase (c and d) and glutathione peroxidase activity (e and f) of compound 6 and compound 9 on glutamate-induced cytotoxicity in HT22 cells. Cells were pretreated with 0.1, 1, and 10 μM of compound 6 and 10, 50 and 100 μM of compound 9 and trolox (30 μM). Then, 30 μl of 2.5 mM glutamate was treated after 1 h. Each bar represents the mean ± standard deviation of three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus glutamate-injured cells (ANOVA)

betulin and methyl hydrogen tetradecanedioate inhibited death of glutamate-injured HT22 cell by inhibition of glutamate-induced mitochondrial disruption.

Restored total glutathione and antioxidant enzyme, glutathione reductase activity and glutathione peroxidase

GSH is a very important endogenous sulfur containing compound shows anti-oxidative activity in CNS. Also, GSH reductase (GR) and GSH peroxidase (GPx) have very important roles to produce GSH in cells. However, high concentration of glutamate was caused to diminish of GSH by declining of cysteine uptake into cells. Decreases of GSH and/or anti-oxidative enzyme such as GR and GPx consequently induce neuronal cell death. Then, we tried to evaluate effect of two neuroprotective compounds on activity of anti-oxidative enzyme and GSH content after glutamate treatment in HT22 cells. The amount of GSH was decreased to 11.38% of control group and activities of GR and GPx were inhibited to 28.95% and 24.76% to control group after 2.5 mM of glutamate treatment, respectively. However, the treatment of 0.1 μM of betulin (6) and 100 μM of methyl hydrogen tetradecanedioate (9) in HT22 cells elevated the GSH levels to 56.82% and 51.10%, respectively [Figure 8a and b]. Also, treatment of 0.1 μM of betulin (6) and 100 μM of methyl hydrogen tetradecanedioate (9) in HT22 cells increased the GR activity to 57.90% and 80.59% respectively [Figure 8c and d]. Finally, the activity of GPx decreased by glutamate was significantly recovered by betulin (6) and methyl hydrogen tetradecanedioate (9) by 57.35% and 76.30%, respectively [Figure 8e and f]. It was indicated that amount of GSH and activities of GR and GPx were preserved by betulin (6) and methyl hydrogen tetradecanedioate (9).

CONCLUSION

In this study, we isolated 10 compounds from n-hexane fraction of *E. alatus* and they were identified as moretenone (1), moretenol (2), friedelanol (3), lupenone (4), β-sitosterol (5), betulin (6), undecanoic acid, 1,2-phenylene ester (7), glycerol 1-tetracosanoate (8), methyl hydrogen tetradecanedioate (9), and 10,13-nonadecadienoic acid methyl ester (10) These compounds were identified with comparison of ^{1}H-NMR, ^{13}C-NMR and mass spectroscopic data to reference data.

We performed MTT assay of isolated compounds for evaluation of neuroprotective activity against cell death in glutamate-injured HT22 cells by oxidative stress. Among 10 compounds, betulin (6) and methyl hydrogen tetradecanedioate (9) showed neuroprotective activity. Also, two compounds restored ROS level and intracelluar Ca^{2+} level, mitochondrial membrane potential, total GSH amount, GSH reductase and GSH peroixdase activity to the control levels. According to these data, we concluded that betulin (6) and methyl hydrogen tetradecanedioate (9) were exerted neuroprotective activity in glutamate-injured HT22 cells via anti-oxidative effect.

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Conflicts of interest

There are no conflicts of interest.

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