Master's Thesis

The neuroprotective effects of *Archidendron jiringa*(Jack)I.C.Nielsen extract inhibit glutamate-induced apoptosis in hippocampal HT22 cells.

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GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

July, 2020

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The neuroprotective effects of *Archidendron jiringa*(Jack)I.C.Nielsen extract inhibit glutamate-induced apoptosis in hippocampal HT22 cells.

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ABSTRACT

Archidendron Jiringa is a leguminous tree growing mainly in Southeast Asia. A recent study found that *A. jiringa* has the effect of anticancer, anti-inflammatory, anti-diabetic and insect repellents. However, the neuroprotective effect of the *A. jiringa* extract on glutamate induced cell apoptosis was not studied. Glutamate activates the formation of reactive oxygen species(ROS) and the pathways of the mitogen-activated protein kinase (MAPK). MAPK is the oxidative stress pathway, and simultaneously regulates Calpain 1, Caspase 12, Bax, Bcl-2 and AIF which are the Endoplasmic Reticulum(ER) stress pathway. In this study, the effects of *A. jiringa* extract on glutamate induced oxidative stress and ER stress in HT22 mouse hippocampal cells were investigated.

It has been found that pretreatment of *A. jiringa* extract reduces ROS production and recovers HT22 cells from glutamate induced cell apoptosis. In addition, our study confirmed that *A. jiringa* extract inhibited MAP kinase signaling pathway, activated Calpain 1, Caspase 12, Bax, AIF and inhibited Bcl-2. Our results suggest that the *A. jiringa* extract prevents damage from glutamate induced oxidative stress and ER stress. It provides experimental evidence that it can be used as a potential phytochemical for the treatment and prevention of neurodegenerative diseases due to the neuroprotective effects of *A. jiringa*.

1. INTRODUCTION

Recently, the temperature has been rising every year due to global warming. If the current condition is maintained, the average temperature will rise by more than 5℃ and most regions of South Korea, except for mountainous areas, will change to a subtropical climate. The average temperature rise in South Korea is 2.4 times steeper than the global average. As a result, the existing crop plantations are moving northward, and new crops are being introduced in the southern regions of the country[1, 2]. In preparation for this situation, there is a need to collect and develop subtropical genetic resources that are valuable as future income crops. We are studying overseas plant resources that were difficult to study due to technical or capital reasons, and was conducted to establish a sustainable bio-economic foundation by securing resources to cope with the changing climate.

The *Archidendron Jirninga* is a Southeast Asian leguminous tree traditionally used to treat toothache, chest pain and skin conditions, as well as to clean up blood and induce urination [3, 4].

A study on *A. jiringa* extract reported that increasing SOD, an enzyme important for protecting gastrointestinal mucous membranes, has an antimicrobial effect, an antioxidant effect by a strong DPPH radical scavenging ability, and an anti-cancer, an insect repellent and an anti-diabetic effect[3, 5, 6]. Despite many positive effects, the *A. jiringa* extract has not yet been studied for potential cell protection of the hippocampal nerve cells. This led to the possibility of *A. jiringa* as a candidate for brain disease treatment.

Glutamate is a major neurotransmitter for nerve stimulation and synapse formation[7, 8], and Glutamate-induced toxicity is a major cause of serious brain diseases such as ischemic stroke, epilepsy, Parkinson's disease, Alzheimer's disease, etc.[9, 10]. Glutamate is tightly regulated in the central nervous system, but when the extracellular glutamate level increases rapidly, reactive oxygen species (ROS) are generated, which increases the formation of intracellular Ca^{2+} levels necessary for

oxidative glutamate toxicity, leading to neurodegenerative disorders and neuron cell death[11].

In neurons, glutamate toxicity occurs in two main ways. One is glutamate receptor-mediated excitatory toxicity, which causes N-methyl-D-aspartate (NMDA) receptors to become overactive, where Ca^{2+} is excessively introduced into cells to form free radicals, thereby expressing various enzymes leading to cell death[12]. Also, another pathway does not pass through the glutamate receptor, and the content of glutathione which is the precursor of the cysteine in the cell is reduced by suppressing the inflow of the cysteine by the glutamate/cystine antiporter. Consequently, the ROS product is increased by the oxidative stress, and a vicious cycle causing redox imbalance and leading to ER stress is repeated to form a self-amplified loop to further activate the cell death[13]. As a result, in the process of killing neuron cells, an increase in ROS concentration in cells stimulates oxidation stress and ER stress, and plays an important role in developing neurological diseases[12].

In the HT22 cell, toxicity due to glutamate is eliminated due to lack of an ionotropic glutamate receptor functionally identical to that of the NMDA receptor. So, it is a model that can study the cell death caused by oxidative stress by suppressing the cellular uptake of cysteine caused by excessive accumulation of glutamate.

In order to determine whether the *A. jiringa* extract is applicable to diseases associated with it by showing a neuroprotective effect function on the hippocampal nerve cells, the neuroprotective effect was verified by using HT22 cells through cell survival, cell apoptosis analysis, intracellular ROS formation and western blot analysis.

Figure 1. The picture of *Archidendron jiringa*.

2. MATERIALS AND METHODS

2.1 DPPH radical scavenging assay

The DPPH radical scavenging activity was measured by the method of Shimada et al., 1992. Sample extract was dissolved in 80% MeOH to a final concentration of 50 μg/ml. 0.2 mM DPPH in MeOH was added to 96-well plates. The absorbance was measured at 525 nm after 30 min. MeOH was used as control. The scavenging activity of DPPH radicals by the sample was calculated according to the following equation: DPPH radical scavenging activity (%) = (1-absorbance of sample/absorbance of control) × 100.

2.2 Cell culture and reagents

We obtained a HT22 cells line (mouse hippocampal neuronal cells) from Jae-ran Lee of Korea Research Institute of Bioscience and Biotechnology (KRIBB). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin-streptomycin (Gibco-BRL, Gaithersburg, MD, USA) and 37℃ in 5% CO_2 humidified atmospheres. Each cell suspension was subcultured by treatment with trypsin-EDTA(Gibco-BRL) every 2 days in order to maintain exponential growth.

2.3 Preparation of sample

The dry extract of *Archidednron jiringa* was provided by International Biological Material Research Center(KRIB, Korea). The sample was dissolved in 80% EtOH and used for the experiment.

2.4 Cell viability assay

HT22 cells were seeded into the 24-well plates at a density 5×10^4 cells/well, and pre-treated with *A. jiringa* extract from 0 to 20 ug/ml for 2 h, and then 4 mM of L-Glutamate(Sigma-Aldrich, MO, USA) were added. After incubation for 24 h, Cell viability was measured by using the WST-1(2-(4-iodophenyl)-3-(4-nitrophenyl)-5-

(2,4-disulfophenyl)-2H-tetrazolium) assay (EZ-Cytox, DoGen, Korea). Each well was added to a final concentration of 10% WST-1 solution after 30 min incubation at room temperature. The absorbance was measured at 450 nm by using the microplate reader.

2.5 Flow cytometry analysis

For apoptosis analysis, HT22 cells were seeded in 6-well plates. After 24 h, they were pre-treated with *A. jiringa* extract at doses of 5 and 15 ug/ml for 2 h, and then added 4 mM L-Glutamate for 12 h. After collecting the cells, we incubated the cells with Annexin V-FITC and PI (FITC Annexin V apoptosis detection kit, BD pharmigen). The apoptotic cells were detected by flow cytometry (LSRFortessa, BD).

2.6 Measurement of intracellular ROS

To evaluate the levels of intracellular ROS, HT22 cells were pre-incubated with *A. jiringa* extract at doses 5 to 15 ug/ml for 2 h, and added 4 mM L-Glutamate. After 12 h, Fluorescent probe dye was diluted with basic medium and warmed up in a water bath at 37℃. Cells were stained with 10 mM of 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, MO, USA) for 15 min in darkness at 37℃, and then harvested and washed with PBS three times. The green fluorescence, which reflects the intracellular ROS level, was measured by flow cytometry (LSRFortessa, BD).

2.7 Western blot

Cells were lysed in M-PER lysis buffer (Thermo science, Bonn, Germany) containing protease inhibitor cocktail(Sigma-Aldrich, MO, USA), 2 mM sodium vanadate, 30 mM sodium pyrophosphate, and 100 mM sodium fluoride. After total protein quantification, proteins were separated by 10 - 12% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred to nitrocellulose membranes (Amersham Biosience, Little Chalfont, Buckinghamshire, UK). Membranes were blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween 20 (TBST). Primary antibodies, such as, Calpain 1, Bax, , Bcl-2, CHOP,

Caspase12, GAPDH (Cell signaling technology, USA), phosphory-p38, p38, phosphory-ERK, ERK, phosphory-JNK, JNK were diluted 1:1000 in TBST and incubated overnight at 4°C. Secondary antibodies (Merck Millipore, Germany) were diluted in TBST 1:4000 and incubated for 1h. The immune complexes were detected by the ECL detection kit (Biosesang, Korea) according to the manufacturer's protocols.

2.8 Statistical analysis

Error bars represent ± SD. The statistical analysis was conducted using GraphPad Prism 5 to identify significant differences based on the one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons (Turkey's test). The value of $p < 0.05$ was considered to indicate significant differences.

3. RESULTS

3.1 Subtropical natural product screening for radical scavenging

We screened various subtropical natural products based on DPPH radical scavenging ability. In Fig. 2, *Archidendron jiringa* extract showed the highest scavenging activity (94.6%). Next, *lophopetalum fimbriatum* extract showed the second highest scavenging activity (56.1%). In other extracts, the scavenging ability was less than 40%. Also, in Table.1, the value of IC50 of *Archidendron jiringa* is the lowest (36.41 μg/ml). Therefore, we selected *Archidendron jiringa* and used it for experiments.

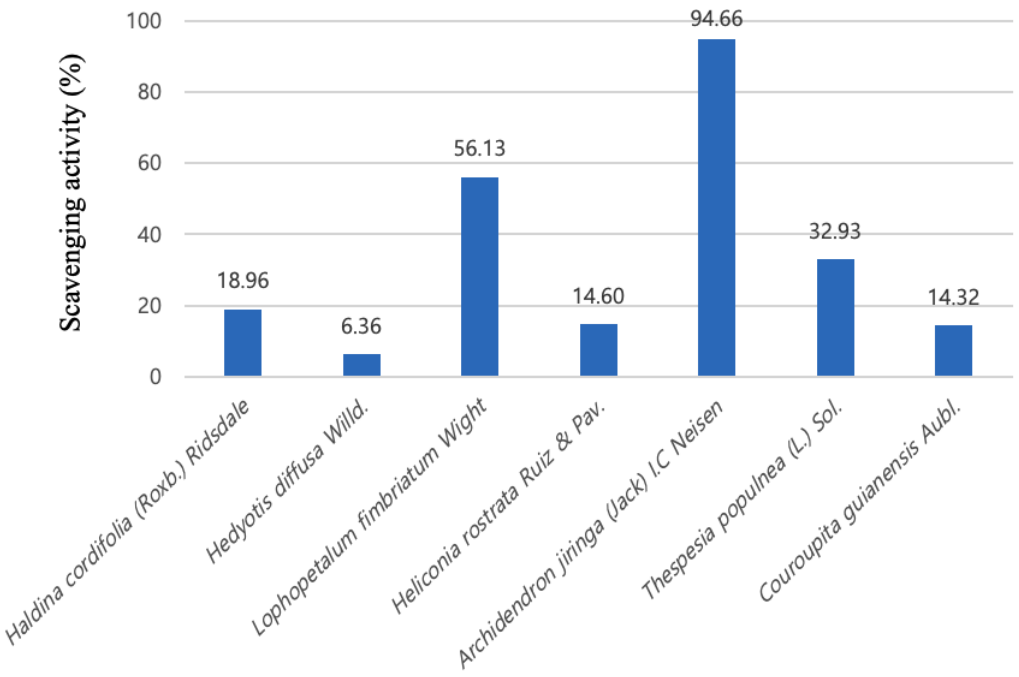

Figure 2. Subtropical natural product screening for radical scavenging activity

Country	Sample name	inhibition (%)	IC50 (μg/ml)
Bangladesh	*Haldina cordifolia* (Roxb.) Ridsdale	18.96	159.18
Bangladesh	*Hedyotis diffusa Willd.*	6.36	365.5
Bangladesh	*Lophopetalum fimbriatum Wight*	56.13	38.25
Bangladesh	*Heliconia rostrata Ruiz & Pav.*	14.6	234.19
Myanmar	*Archidendron jiringa* (Jack) I.C Nielsen	94.66	36.41
Myanmar	*Thespesia populnea* (L.) Sol.	32.93	73.86
Myanmar	*Couroupita guianensis Aubl.*	14.32	208.99

Table 1. Subtropical natural products list about country, inhibition, value of IC50.

3.2 *A. jiringa* extract protects glutamate-induced HT22 neuronal cell death

we examined the neuroprotective effect of *A. jiringa* extract against glutamate-induced apoptosis in hippocampal HT22 cells by WST-1 assay.

Before evaluating the neuroprotective effect of the *A. jiringa* extract on the HT22 cells, the cell viability was measured after treatment with glutamate for each concentration for 24 h in order to establish an appropriate concentration of the glutamate on the HT22 cells. A survival rate of the cell exposed to glutamate is remarkably reduced by 60% at 4 mM (Fig.2A).

Next, WST-1 assay was performed to evaluate the cell viability of the *A. jiringa* extract for glutamate induced HT22 cells. After pre-treatment of *A. jiringa* extract by concentration, glutamate was treated and then cell viability was measured. We confirmed that the *A. jiringa* extract recovered 46.7%, 67.3%, 96% and 92.5% at 5, 10, 15, 20 μg/ml , respectively, and showed a values similar to the the control group at 15 μg/ml. Additionally, at concentration 20 μg/ml, it was confirmed that it did not adversely affect cell viability (Fig.2B).

A morphological change of the HT22 cells are measured by a microscope for the glutamate induced cell death inhibition effect, and compared with the result of Figure 2B, it is determined that the cell viability and the morphological results are consistent (Fig.2C). These results suggest that treatment with A. *jiringa* extract can protect HT22 cells in glutamate-induced toxicity.

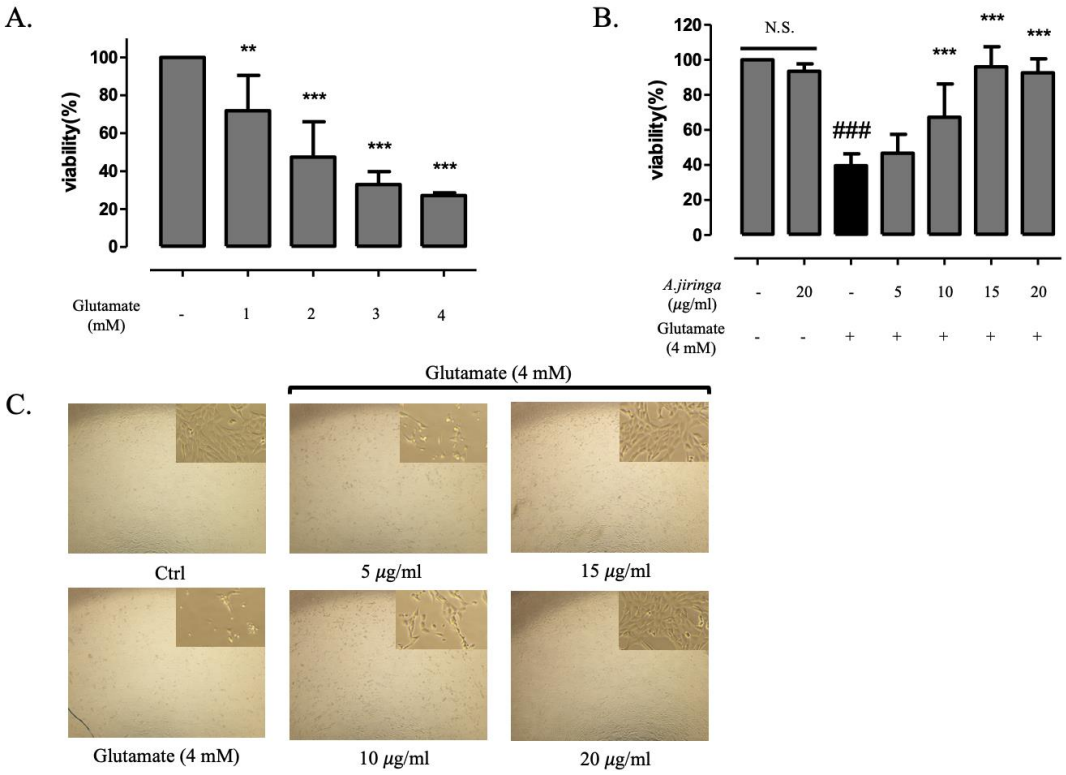

Figure 3. Pretreatment with *A. jiringa* extract protected HT22 cells against glutamate-induced cell death. (A) WST-1 assay was performed after treatment with glutamate (0 – 4 mM). (B) WST-1 assay was performed after treatment with *A. jiringa* extract (0 – 20 μg/ml) for 2 h, followed by glutamate (4 mM) treatment on HT22 cells. (C) The cells of pretreatment with *A. jiringa* extract morphology was detected by microscopy. Each bar represents the mean ± SD (n=3) from three independent experiments per group. ##p<0.01 and ###p<0.001 vs. Ctrl. *p < 0.05, **p < 0.01, ***p < 0.001 vs. glutamate treated cells.

3.3 *A. jiringa* extract reverses glutamate-induced apoptotic HT22 cell death.

To analyze the type of cell apoptosis, Annexin V / PI staining was measured with a flow cytometer. Concentrations of 5, 10 and 15 ug/ml, which showed significance in cell viability, were used. Apoptotic cell death increased significantly to 61.6% in the glutamate induced group compared to 4% in the control group. However, the treatment of *A. jiringa* extract decreased to 54.7% and 24.9%, 7.9% at 5,10, 15 μg/ml, respectively. The above results confirmed that the *A. jiringa* extract exhibits neuroprotective effects by inhibiting apoptotic cell death by glutamate.

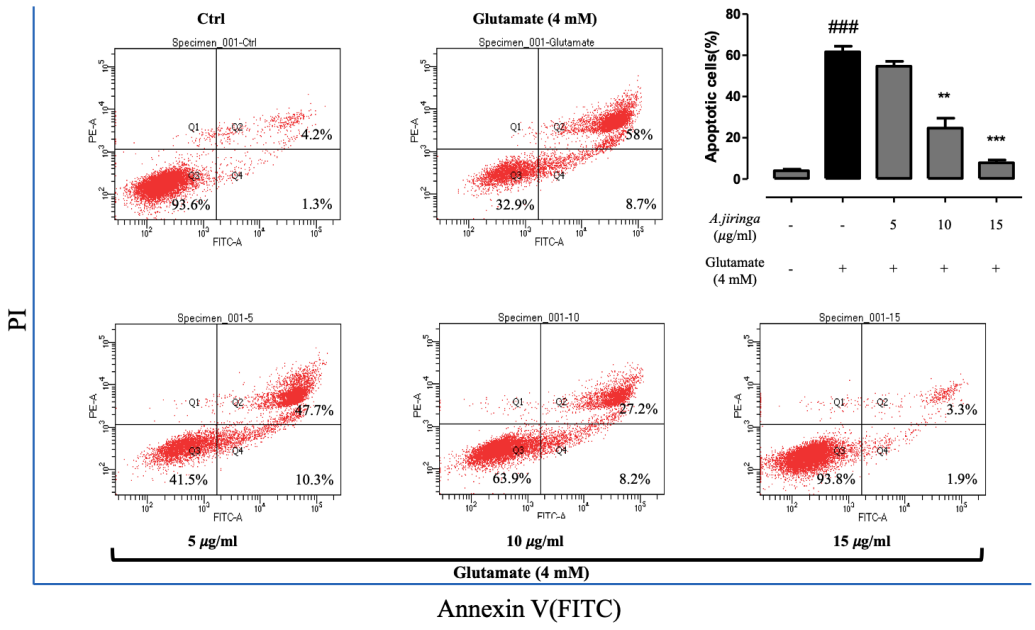

Figure 4. Glutamate-induced apoptotic cell death was inhibited by *A. jiringa* extract. *A. jiringa* reduced apoptosis of glutamate-induced HT22 cells, as determined by flow cytometry. Each bar represents the mean ± SD (n=3) from three independent experiments per group. ##p<0.01 and ###p<0.001 vs. Ctrl. *p < 0.05, **p < 0.01, ***p < 0.001 vs. glutamate treated cells.

3.4 *A. jiringa* extract inhibits glutamate-induced ROS production.

Changes in ROS in the early stages of glutamate induced cell death are important indicators. It is well established that glutamate toxicity is mediated by ROS production and cell death induced by oxidative stress. And excessive production of ROS can be an important mediator that damaged cell structures [14]. A recent study also found that ROS is involved in ER stress [15, 16, 17].

The effect of glutamate on the ROS was measured using flow cytometry. Already, another study reported there was a high increase in the ROS at the time of 12 h exposure, and we conducted experiments using this as a reference [9].

An N-acetylcysteine (NAC) is treated as a positive control in order to compare the effect of *A. jiringa* extract on the accumulation of ROS in glutamate induced HT22 cells. As shown in Fig.3A and 3B, the amount of ROS production in HT22 cells increased by about 2.2 times when glutamate was treated alone. In contrast, the *A. jiringa* extract is reduced from 5, 10, 15 μg/ml to 2.1 times, 1.4 times, 0.7 times, respectively, compared to the glutamate treated group and showed similar effects to the *A. jiringa* extract (15 μg/ml) during NAC (1mM) treatment. The results suggest that the *A. jiringa* extract effectively protects HT22 cells from cell apoptosis caused by ROS generated by glutamate.

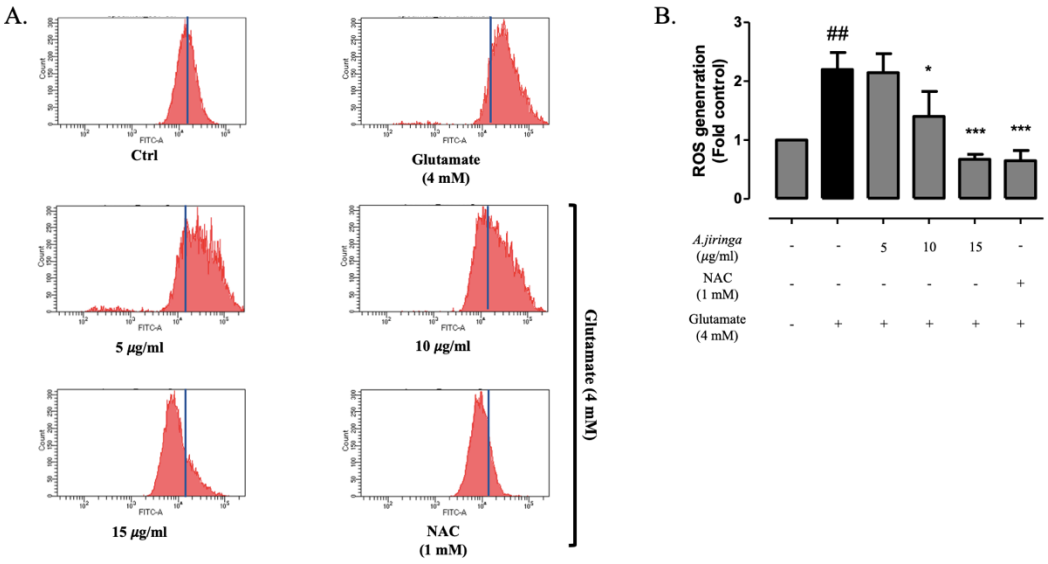

Figure 5. *A. jiringa* extract reduced glutamate-induced ROS production. HT22 cells were pretreated with different concentrations of *A. jiringa* extract(5, 10, 15 μg/ml) for 2 h, and then followed by glutamate (4 mM). after 24 h, cells were treated with 10 μM DCF-DA for 15 min, and DCF fluorescence, as an indicator of the amount of ROS, was measured by flow cytometry. Each bar represents the mean ± SD (n=3) from three independent experiments per group. ##p<0.01 and ###p<0.001 vs. Ctrl. *p < 0.05, **p < 0.01, ***p < 0.001 vs. glutamate treated cells.

3.5 *A. jiringa* extract regulates signal pathway related MAPK and ER stress.

Another study suggests that MAP kinase activation and oxidative stress contribute to pathology of neurodegenerative diseases[8, 15, 18]. Accordingly, the effect of *A. jiringa* extract on the activation of ERK, p38, JNK by glutamate was investigated.

As a result, ERK, p38 and JNK phosphorylation levels of *A. jiringa* extract treated group were reduced (Fig.4). The above results indicate that the *A. jiringa* extract shows a neuroprotective activity by regulating oxidative stress-related proteins.

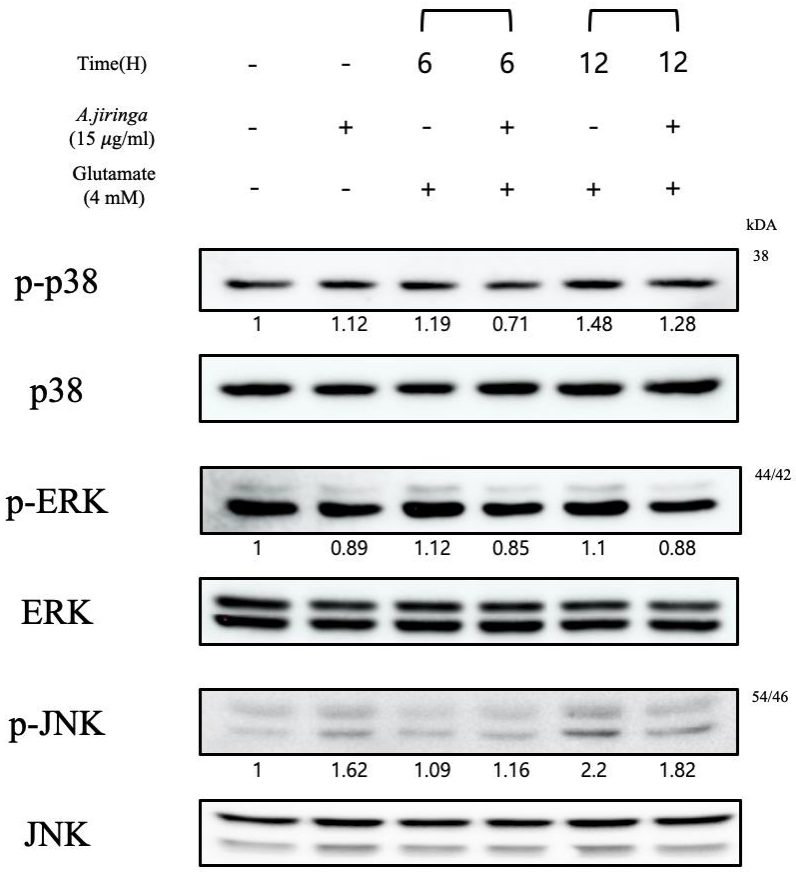

Figure 6. Pretreatment of *A. jiringa* extract inhibits the phosphorylation activities of ERK, p38, JNK activation. Cells were pre-treated for 2 h with 15 μg/ml *A. jiringa* extract, and then exposed for 12 h to 4 mM of glutamate.

In other studies, the claim that ROS produced by oxidative stress amplifies ER stress is involved in cell death [15, 16]. We investigated the expression of the ER stress-related proteins Calpain 1, Caspase 12, Bax, Bcl-2 and AIF.

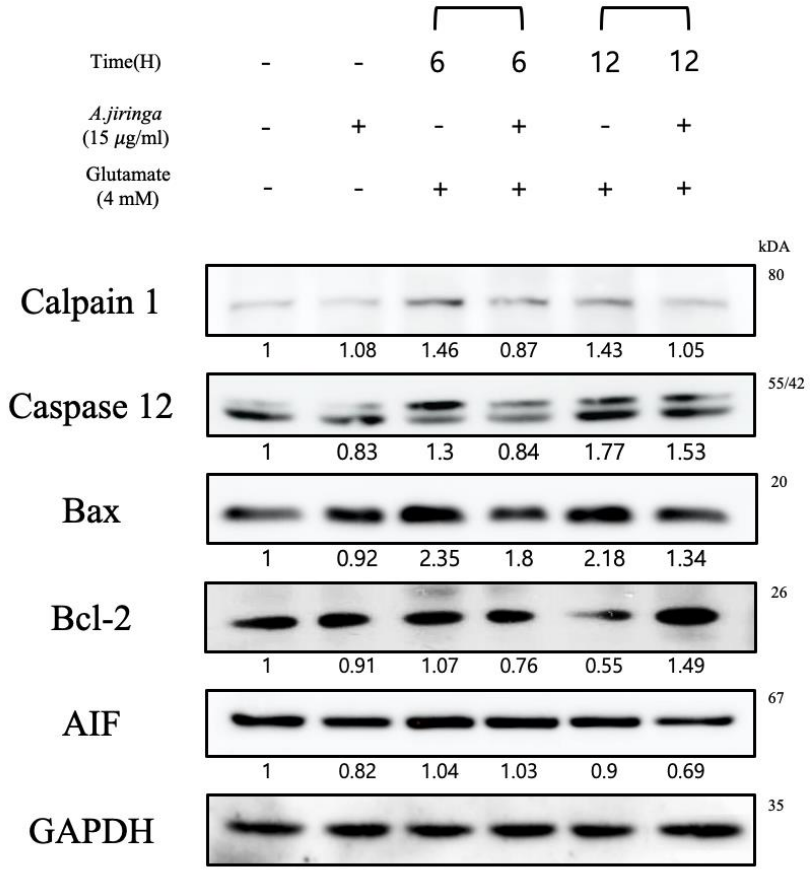

Figure 7. *A. jiringa* extract regulates the Calpain 1, Caspase-12, Bax, Bcl-2, AIF

Calpain 1 activated when Ca^{2+} homeostasis collapses and Caspase 12 activated only by ER stress have been confirmed to decrease the protein expression in the *A. jiringa* extract treatment group compared to the control group.

In addition, the activation of Calpain activates Bax and suppresses Bcl-2, thereby releasing AIF from the mitochondria[19, 20]. The *A. jiringa* extract treated group has decreased in Bax and AIF compared to the control group, and Bcl-2 increased the protein band at 12 h as compared with the control group(Fig.5). These results suggest that *A. jiringa* extract suppresses cell apoptosis pathway caused by ER stress.

4. DISCUSSION

Archidendron jiringa has traditionally been used to purify blood and to treat dysentery. Recently, *A. jiringa* showed an increase in SOD, an important enzyme for gastrointestinal mucosa protection, and decreased the development of gastric lesions. Other studies have reported that phenol components, flavonoids, terpenoids and alkaloids are present and have strong DPPH radical scavenging activity. In addition, there are reports that it has anti-diabetic, anti-cancer, and antibacterial effects[3-6]. Based on this, we conducted a study to examine the potential of neuroprotection related to cell death induced by glutamate.

Several studies have shown that the initiation and progression of neurodegenerative diseases are associated with oxidative and ER stress[16, 17]. The mechanisms of glutamate induced oxidative toxicity disrupts glutamate/cysteine antiporter, depleting the cellular antioxidant GSH. After the depletion of GSH, the weakened antioxidant capacity causes a series of biological processes such as ROS accumulation and Ca^{2+} influx[10]. Ultimately, the accumulation of ROS induces oxidative stress and ER stress, leading to cell death[8, 16]. Although the mechanism between oxidative stress and ER stress has not been specifically identified, there have been reports that ROS generated by oxidative stress accelerates ER stress, which also leads to an increase in ROS cycle, and evidence has been shown that there are many connections between the two pathways[16, 17, 22]

We have successfully confirmed the protection effect against glutamate induced apoptosis, as previously suggested, by improving cell viability, reducing apoptosis rates, inhibiting ROS overproduction, and regulating protein expression using HT22 neuronal cells.

Through the WST-1 assay, it was confirmed that the *A. jiringa* extract had an effect of protecting from toxicity by glutamate. Additionally, the flow cytometer analysis by Annexin V and PI staining for cell apoptosis analysis showed that the *A. jiringa* extract significantly reduced cell apoptosis.

Mitochondria are damaged when the influx of Ca^{2+} into the cytoplasm increases due to glutamate. Ca^{2+} affects both ER stress and oxidative stress, and ROS is produced and released from the mitochondria to the cytoplasm, resulting in cell death [23]. Our results confirmed that the *A. jiringa* extract significantly reduces the production of ROS by glutamate.

Excessive ROS production activates the MAP kinase pathway, induces phosphorylation of ERK, p38, JNK to participates in the cell apoptosis pathway. MAP kinase protein is known as a representative oxidative stress-related protein[24]. In previous studies, it has been reported that sustained activation of ERK causes oxidative stress induced cell death[25]. In particular, a study found that ERK is sensitive to oxidation stress in nerve cell death [8, 9].

The JNK and p38 pathways have a variety of mechanisms that triggered by stress and are involved in cell differentiation and apoptosis [26].
Consistent with these studies, it was found that *A. jiringa* extract inhibits phosphorylation of ERK, JNK, p38, blocking the cell apoptosis pathway.
MAPKs have also been reported to be associated with ER stress[16, 17]. A recent study suggests that activation of the JNK pathway occurs before inducing ER stress[27].

Endoplasmic reticulum (ER) is an organ that synthesizes secretory organs and membrane proteins, and ER stress is generated by the homeostatic variation of Ca^{2+} [28]. Ca^{2+} overload is the main mediator of the mitochondria-related glutamate induced toxicity and activates Calpain 1. Many studies suggest that calpain plays an important role in apoptosis pathways[29, 30]. When the ER function is severely damaged, apoptosis is induced and caspase 12 is activated [31]. In addition, Ca^{2+} emitted from ER increase the ROS production of mitochondria, Bax is activated and Bcl-2 is suppressed, destroying the mitochondrial membrane. After then the apoptosis factor, AIF, is activated[32].

In this study, it was confirmed that the *A. jiringa* extract treatment suppresses Calpain 1 and Caspase 12 from the result of protein expression associated with ER stress. This can be inferred that the unbalanced Ca^{2+} influx has been restored. In addition, it was confirmed that both Bax and AIF, which are cell apoptotic factors activated by Calpain 1, were decreased by *A. jiringa* extract.

In total, it is found that glutamate induced oxidative stress and ER stress by ROS production in HT22 hippocampal neuron cells, and *A. jiringa* extract reduces ROS accumulation and suppresses expression of proteins related to apoptosis pathways such as Calpain 1, Caspase 12, Bax, AIF and MAPKs to prevent cell death. This indicates that the *A. jiringa* extract has neuroprotective activity and suggests potential as a natural neuroprotective agent for the treatment of neurodegenerative diseases.

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