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Master's Thesis

**Neuroprotective effects of *Carpinus  
tschonoskii* leaves extract against  
glutamate-induced oxidative stress in  
HT22 hippocampal cells**

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Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

July, 2020

HT22 해마 세포의 글루타메이트  
유발 산화 스트레스에 대한  
개서어나무(*Carpinus tschonoskii*)잎  
추출물의 신경 보호 효과.




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이 논문을 이학 석사학위 논문으로 제출함

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2020 년 07 월

**Neuroprotective effects of *Carpinus  
tschonoskii* leaves extract against  
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hippocampal cells.**

**Ji-Woon Hwang**

(Supervised by Professor Jae Hoon Kim)

A thesis submitted in partial fulfillment of the requirement

For the degree of Master of Science

July, 2020

This thesis has been examined and approved.

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## ABSTRACT

The present study investigated the neuroprotective effect of the intracellular protection mechanism using *C.tschonoskii* leaves extract (CTE) against glutamate-induced oxidative damage in HT22 cells. We found that pretreatment of HT22 cells with CTE significantly inhibited the cell death induced by glutamate in a dose dependent manner. CTE reduced ROS production and apoptosis induced by glutamate. In addition, CTE inhibited phosphorylation of p38 and Erk, transcription factors important for neuronal survival, and reduced the expression of Bax and AIF, apoptosis-related proteins. The result is that CTE in HT22 cells can prevent glutamate-induced apoptosis through ROS production, phosphorylation inhibition of oxidative stress related proteins, and reduction of expression. Therefore, it suggests that it can be applied to the development of therapeutic agents for diseases of the brain and nervous system.

## 1. INTRODUCTION

It is expected that the number of patients with degenerative cerebral nervous system disease will increase rapidly due to the prolonged aging of humans, and it lead development of treatment urgently.

Dementia is not a disease caused by a specific diagnosis, but by various causes such as a defect of memory, cognitive dysfunction and other loss of a function indication. There are Alzheimer's dementia, Vascular dementia, dementia with lewy bodies and frontotemporal lobar dementia.

In the Korea, Alzheimer's disease(AD) is estimated 50-60% of whole dementia patients and vascular dementia is estimated 15-20% of them, so the study of prevention and treatment of AD is a very important issue.

Although there is no clear cause for the major causes of AD, various causative hypotheses are being argued[29]. several studies, especially neuropathological studies, have emphasized the neuron oxidation of AD[30-31].

Injury of neuron by oxidative damage results in oxidative damage of major component such as lipid, protein and nucleic acid and eventually causes fatal cell death or necrosis.

*Carpinus* is composed of 40 species and is distributed in temperate zone of the northern hemisphere. Only 5 species of *C. tschnoskii*, *C. lausiflora*, *C. cordata*, *C. turcjaninowi*, and *C. Coreana* grow in the Korea. Recent research has shown that *Carpinus* leaves contain flavonoid compounds such as Tannin, Myricetin, Kaempferol, Apigenin and Luteroline[13], and have strong antioxidant and anti-inflammatory effects[14].

Glutamate plays an important role in learning and memory in neurons and is a major excitability neurotransmitter associated with acute and chronic neurodegenerative diseases as well as Alzheimer's and Parkinson's disease[1][15].

Physiologically, as brain glutamate concentration increase, intercellular ROS increases and neurotoxicity occurs. Recently, studies have been reported that cytotoxicity by glutamate causes mitochondria functional disorder and destroys mitochondrial dysfunction[3].



Abnormal  $\text{Ca}^{2+}$  flow in the cell activate Calpaine to activate apoptosis protein Bax[4-5].

In addition, mitochondrial functional disorder by oxidative stress translocated the pro-apoptosis factor (AIF) to the nucleus, eventually activating the apoptosis pathway[6].

Reactive oxygen species (ROS) are associated with tissue complication and factor that causes neurological diseases such as inflammation, aging, cancer, arteriosclerosis, hypertension, diabetes, ischemic stroke, Alzheimer's disease and parkinson's disease[7-8].

Over produced ROS cause apoptosis by activating MARK pathway[9]. Recent studies have shown that nerve damage from various cytokines, inflammation-inducing agents, heat shocks and etc phosphorylate the p38[10] and ERK is sensitive to oxidative stress in neuronal cell death. Therefore, according to important role of these MARK pathways, materials by suppressing phosphorylation of MARK pathways may be a candidate treatment for protecting neuronal cells.

This study was conducted to neuroprotective effect mechanism of *C.tschnoskii* extract by glutamate-induced oxidative stress in HT22 mouse hippocampal cell which has been widely used as a successful in vitro model.

## 2. MATERIALS AND METHODS

### 2.1. Materials.

EtOH extract (80 %) and several solvent fractions from *C. tschonoskii* were purchased from Jeju Biodiversity Research Institute of Jeju Technopark (JBRI), which has professional facilities and provides extracts from plants growing on Jeju Island for research purposes in biotechnology and related industrial fields.

### 2.2. Cell culture.

Mouse HT22 hippocampal cells were gifted by Jae-ran Lee from Korean Research Institute of Bioscience and Biotechnology (KRIBB). The HT22 cells were maintained in DMEM supplemented with 10 % fetal bovine serum (Gibco-BRL, Gaithersburg, MD, USA), 100 µg/ml streptomycin (Invitrogen), 100 U/mL penicillin at 37 °C.

### 2.3. Cell viability assay.

We measured cell viability using the WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) solution (Boehringer Mannheim, Mannheim, Germany). Cells were seeded  $2.5 \times 10^4$  in 24-well plates. After cells were incubated for 24 h, we were treated with CTE for 24 h at 37 °C. Each well was added to a final concentration of 10 % WST-1 solution after 15 min incubation at room temperature. The absorbance was measured at 450 nm using the microplate reader.

### 2.4. Cell ROS analysis.

Intracellular ROS levels were determined using the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Rosenkranz et al., 1992). This molecule is cleaved intra-cellularly by esterases to form non-fluorescent 2',7'-dichlorofluorescein (DCFH), which is transformed to the fluorescence compound 2',7'-dichlorofluorescein (DCF) upon oxidation by ROS. HT22 cells were seeded  $5.0 \times 10^4$  in 6-well plates for 12 h. The cells were pretreated with 20 µg/ml of CTE and 4mM glutamate for treatment for 12 h. After collecting the cells, we were treated 10

$\mu$ M DCFH-DA for 15 min at 37°C under 5% CO<sub>2</sub> in the dark. The apoptotic cells were detected by flow cytometry (LSRFortessa, BD)

## **2.5. Cell apoptosis analysis.**

For apoptosis analysis, HT22 cells were seeded in 6-well plates. After 24 h, they were treated with CTE for 24 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. Western blot assay**

Cells were lysed in M-PER lysis buffer (Thermo science, Bonn, Germany) containing protease inhibitor cocktail (Roche), 2 mM sodium vanadate, 30mM sodium pyrophosphate, and 100mM sodium fluoride. After total protein quantification, proteins were separated by 10 % SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to nitrocellulose membranes (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK). Membranes were blocked with 5 % skim milk in TBST. Primary antibodies, such as phospho-Erk(44/42) (Thr202/204), phospho-p38 (Thr180/Tyr182), Bax, AIF and GAPDH (Cell signaling technology, USA), 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 protein bands were detected by the ECL kit (Biosesang).

## **2.7. GC-MS MS analysis.**

The sample was eluted in ethanol. The analysis was performed using a gas chromatograph (GC 2010, Shimadzu Corporation). The GC was equipped with an AOC-20i auto-injector and a triple quadrupole tandem mass spectrometer (TQ8040, Shimadzu Corporation). One  $\mu$ l volume of the sample was injected in splitless mode on Rtx-4MS (30 m x 0.25 mm, 0.25- $\mu$ m film thickness) column (restek). The carrier gas and the collision gas were helium and argon, respectively. Oven temperature started from 80 °C (3 min hold time) to 310 °C (3 min hold time). The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. The data acquisition was started at 20 min.

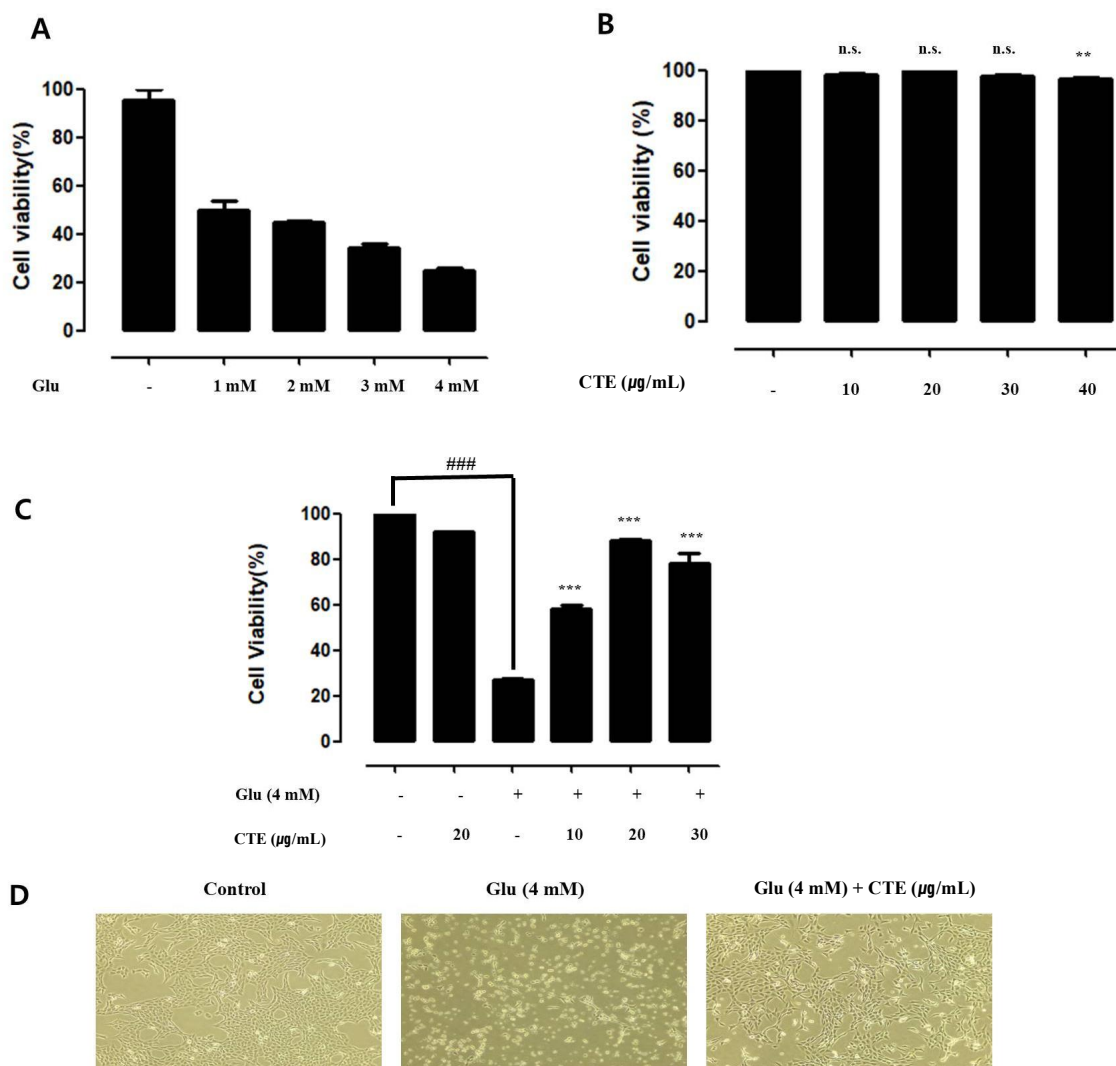
## **2.8. Statistical analysis.**

Data were presented as average with standard deviation (SD). The differences between multiple groups were analyzed using turkey's *post hoc* method.  $P < 0.05$  was regarded statistically significant.

### 3. RESULTS

#### 3.1. Measured of cell viability by WST-1 Assay.

It was measured cell viability from CTE by Glutamate-induced cytotoxicity in HT22 cells. Through the analysis, it can be figured out the effect to cytotoxicity by glutamate treatment group and CTE pre-treated. HT22 cells were treated by Various concentration of glutamate and CTE. After 24hour, cell viability were measured by WST-1 assay. Cell viability was reduced to 75% in 4mM glutamate treatment group (Fig1. A and B). When various concentrated CTE treated in HT22 Cell, there isn't any significant difference. HT22 cells were pre-treated with 10, 20, 30  $\mu\text{g}/\text{mL}$  of CTE and 4 mM glutamate for 24 hr. Glutamate treatment group reduced cell viability to 72 %. Cell viability was increased to 92 % In twenty  $\mu\text{g}/\text{mL}$  of CTE and 4mM glutamate pretreated.



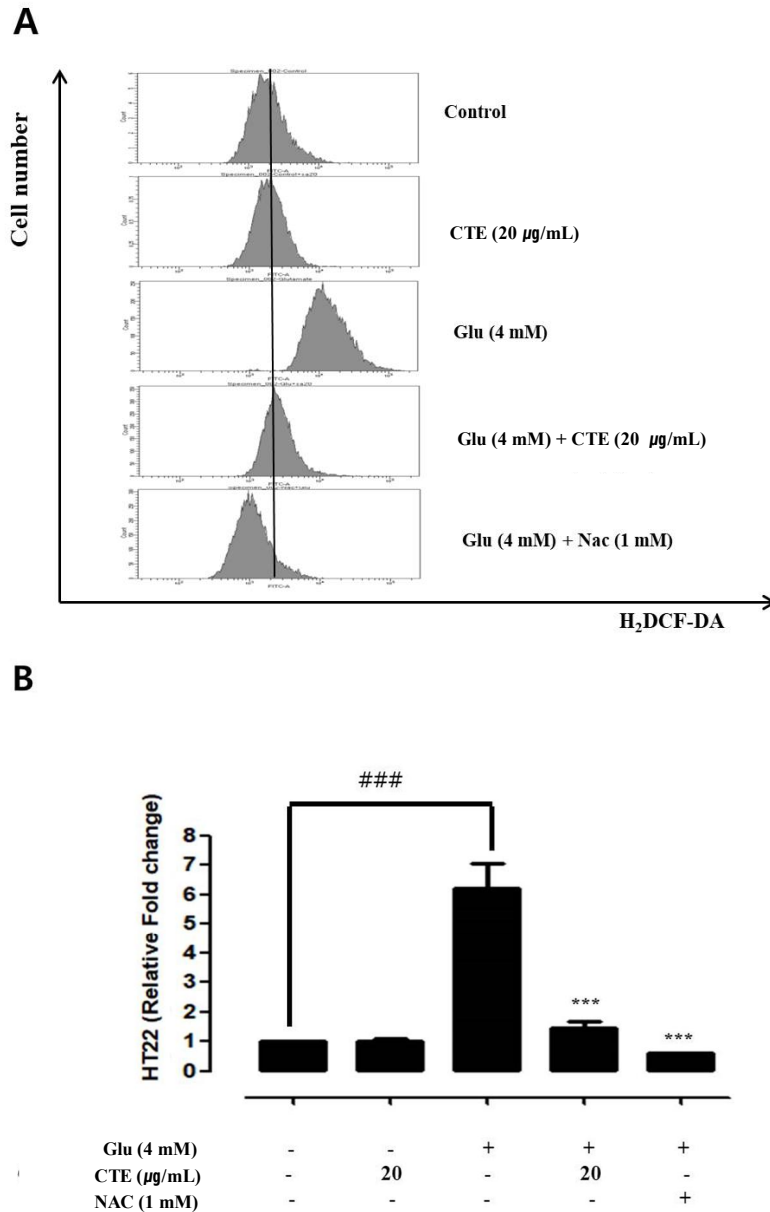
**Figure 1.** Protective effects of CTE against glutamate-induced cytotoxicity in HT22 cells.

Cytotoxicity was assessed after 24 hr incubation period with various concentrations (1, 2, 3 and 4 mM) of glutamate (A). Mouse HT22 hippocampal cells was treated with various concentrations (0, 10, 20, 30 and 40 µg/ml) of CTE for 24hr (B). Cells were pretreated with glutamate (4 mM) for 24 hr, followed by treatment with various concentrations for 24 hr. (C). Glutamate-induced toxicity in HT22 cells as assessed by phase contrast microscopy (100x) (D). Cell viability was determined by WST-1 assay. Graphs showed mean  $\pm$ SEM, \*\* $P < 0.01$  vs. control group.(A and B). ### $P < 0.001$  vs. control group, \*\*\* $P < 0.001$  vs. glutamate-treated group.(C and D)

### **3.2. CTE inhibits glutamate-induced ROS production.**

It was measured ROS effect of CTE by glutamate-induced oxidative stress in HT22 cells.

Fig 2. A and B show that production result of intercellular ROS by glutamate-induced. Through the analysis, it can be figured out the effect of glutamate-induced increases of ROS by glutamate treatment group. Intercellular ROS were measured and increased 6 times to compared control group. Twenty  $\mu\text{g}/\text{mL}$  CTE and 4 mM glutamate pretreated reduced intercellular ROS as 5 times compare 4 mM glutamate treated group in HT22 cells. NAC were used positive control group. This result of the analysis, twenty  $\mu\text{g}/\text{mL}$  of CTE effects neuroprotective by suppressing production of intercellular ROS.



**Figure 2.** Effect of CTE on glutamate-induced ROS production in HT22 cells. Cells were pretreated with twenty µg/ml of CT for 12 hr, followed by exposed to 4mM glutamate for 12 hr. Cells were stained with carboxy-H2DCFDA and production of ROS was measured using a flow cytometer (A). Quantitative analysis of the histograms expressed as the realtive fold change of ROS production in HT22 cells (B). Graphs showed mean ±SEM, ###P<0.001 vs. control group, \*\*\*P<0.001 vs. glutamate-treated group.



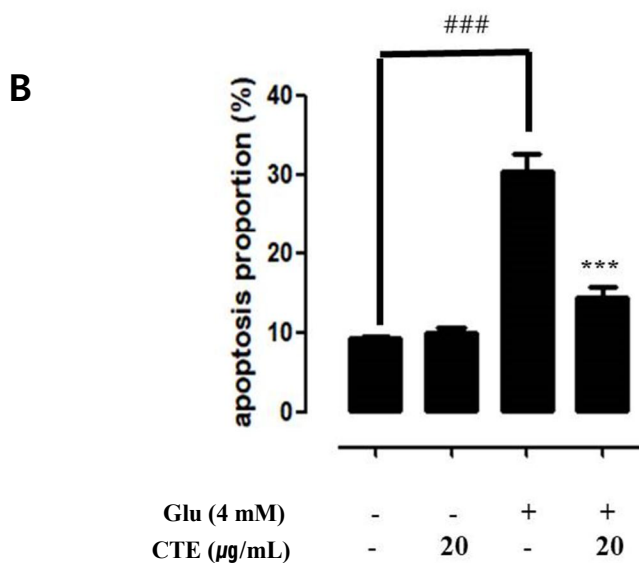
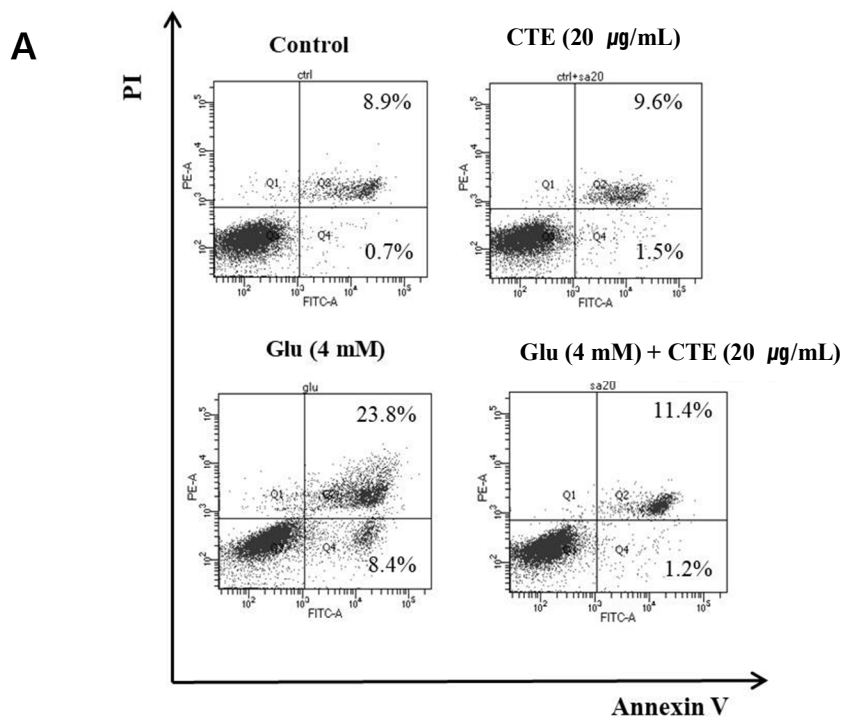
### **3.3. CTE inhibits glutamate-induced apoptotic cell death.**

Apoptosis reactivity from CTE by glutamate-induced production of apoptosis in HT22 cells was measured.

Fig 3. A and B show that production of apoptosis by glutamate-induced. Through the analysis, it can be figured out the effect to glutamate induced increases Apoptosis.

4mM glutamate treated group increased apoptosis to about 31 % compared control group.

Twenty  $\mu\text{g/ml}$  of CTE and 4 mM glutamate pre-treated was reduced Apoptosis to about 12 % compare 4mM glutamate treated group in HT22 cells. Twenty  $\mu\text{g/ml}$  of CTE effects neuroprotective by suppressing Apoptosis.



**Figure 3.** Effect of CTE on types of glutamate-induced apoptotic cell death in HT22 cells.

Cells were pretreated with twenty µg/ml of CTE for 24 hr, followed by exposure to 4mM glutamate for 24 hr. Cells were labeled with FITC-Annexin V and PI, and then measured by flow cytometry. Representative flow cytometry analysis scattergrams for Annexin V and PI staining (A). Quantitative analysis of the histograms expressed as percentage of apoptotic or necrotic cell death in total cells (B). Graphs showed mean ± SEM, ###P<0.001 vs. control group, \*\*\*P<0.001 vs. glutamate-treated group.

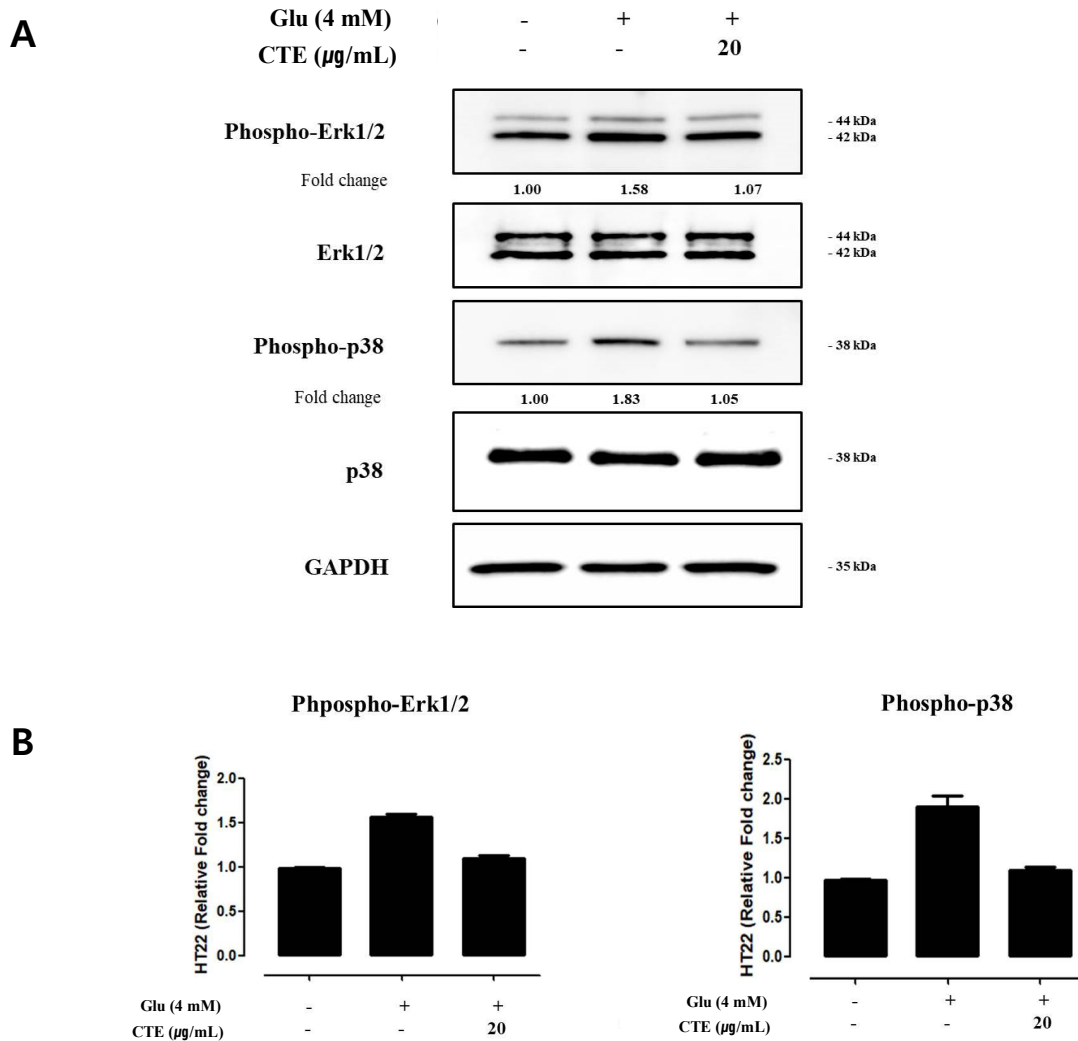
### **3.4. Measurement of phosphorylation in MAPK pathways.**

Protein phosphorylation from CTE by MAPK pathways protein of glutamate-induced oxidative stress in HT22 cells was measured.

Fig 4. A and B shows phosphorylation of protein by glutamate-induced. 4mM glutamate-induced increased phosphorylation of protein which is included p38 and ERK in MAPK pathways.

Twenty  $\mu\text{g/ml}$  of CTE and 4mM glutamate's pre-treated was reduced phosphorylation of protein compare 4mM glutamate treated group in HT22 cells.

Twenty  $\mu\text{g/ml}$  of CTE effects neuroprotective by suppressing MAPK pathways.



**Figure 4.** Effect of CTE on glutamate-induced oxidative toxicity relative proteins in HT22 cells.

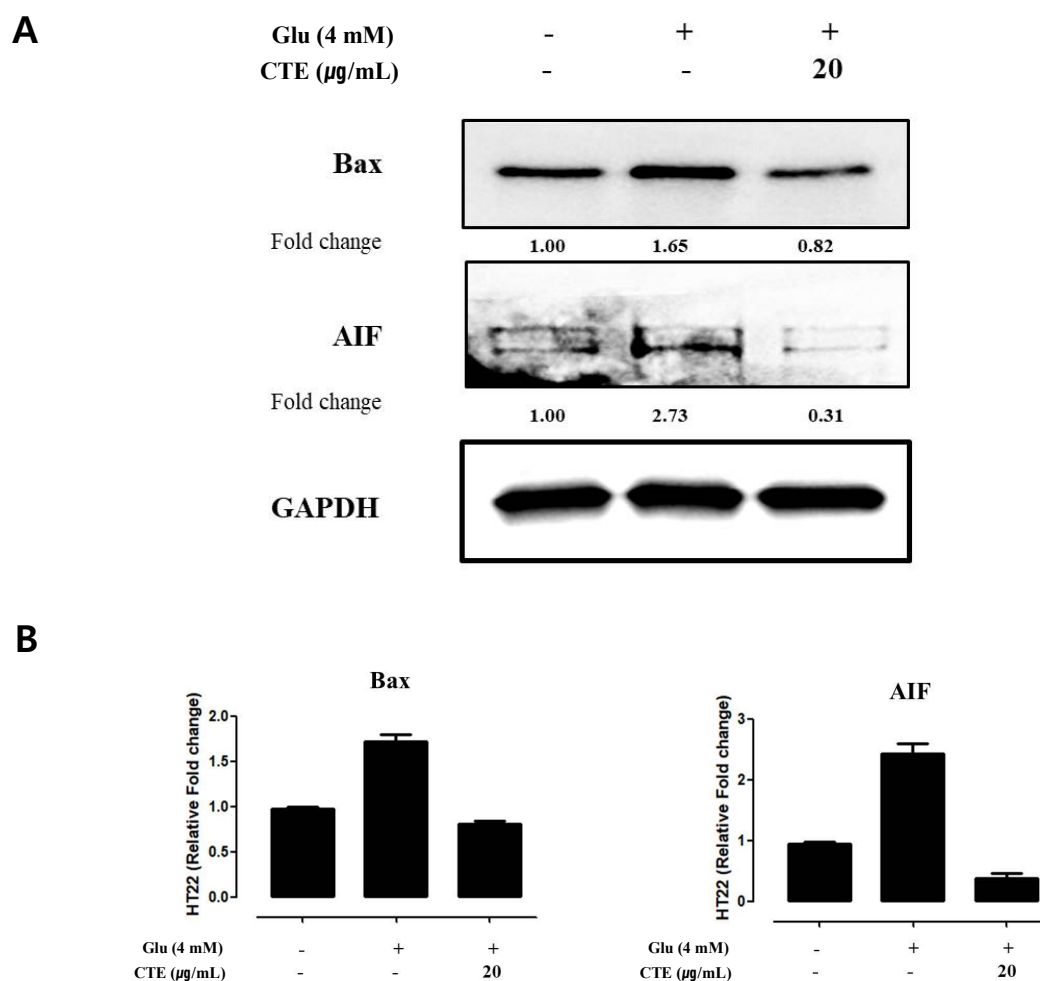
Cells were pretreated with twenty  $\mu\text{g/ml}$  of CTE for 12 hr, followed by exposing to 4 mM glutamate. After cell treatment, each groups were maintained in the original medium 12 hr. Equal amounts of proteins in each group were subjected to western blot using the indicated antibodies. Equal protein loading was confirmed by actin expression.

### **3.5. Measurement of protein expression in pro-apoptotic pathways**

Protein expression from CTE by pro-apoptotic pathways protein of glutamate-induced oxidative stress in HT22 cells was measured.

Fig 5. A and B shows protein expression by glutamate-induced. 4 mM glutamate-induced increased protein expression which is included pro-apoptotic protein (Bax) and pro-apoptotic factor (AIF). twenty ug/ml of CTE and 4mM glutamate's pre-treated was reduced protein expression compare 4 mM glutamate treated group in HT22 cells.

Twenty  $\mu\text{g/ml}$  of CTE effects neuroprotective by reducing Bax and AIF of protein expression in apoptotic pathways.



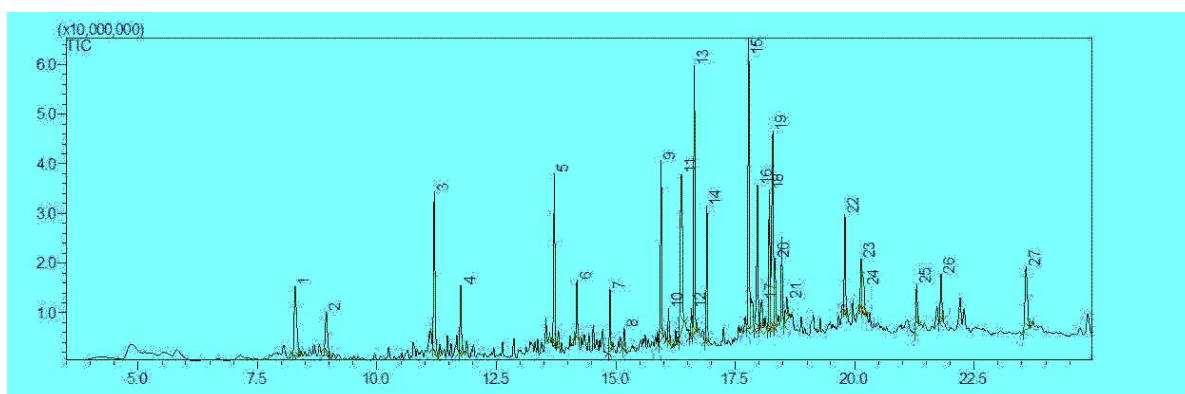
**Figure 5.** Effect of CTE on glutamate-induced pro-apoptotic relative proteins in HT22 cells.

Cells were pretreated with twenty  $\mu\text{g/ml}$  of CTE for 12 hr, followed by exposing to 4 mM glutamate. After cell treatment, each groups were maintained in the original medium 12 hr. Equal amounts of proteins in each group were subjected to western blot using the indicated antibodies. Equal protein loading was confirmed by actin expression.

### **3.6. Analysis of GC-MS/MS from CTE.**

Fig 6 was analysis result of GC-MS/MS from CTE. 13,15 and 19's peak area among various peak is 8.4%, 9.5% and 9.0%. Table 1 shows compound name and activity about 3 peaks of Fig 6.

No.13 peak is anti-oxidant, no.15 peak is anti-cancer and no.19 is anti-inflammatory of activity.



**Figure 6.** Chromatogram of CTE by GC-MS/MS.

Number	RT	Name of the compound	Compound nature	Activity
13	16.642	Hexadecanoic acid, ethyl ester	Palmitic acid ester	Anti-oxidant, Anti-androgenic flavor
15	17.781	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R*,R*-(E)]-	Diterpene	Anti-microbial, Anti-cancer, Anti-inflammatory
19	18.284	Ethyl linoleolate	Linolenic acid	Anti-inflammatory

**Table 1.** Compound name and activity in the CTE.



### 3. DISCUSSION

When a people get older, the blood vessels of the brain cells and myocardial cells become narrow and clogged, resulting in a fatal heart attack or cerebral infarction.

It also brings many changes to the blood vessels that make up the vascular system. Accordingly, fatal cerebrovascular occur such as dementia and stroke [21].

One of the main causes of Alzheimer's is oxidative stress and Glutamate is known to cause oxidative stress in cells in the central nervous system[15].

CTE has strong antioxidant and anti-inflammatory effects[14], It is known to effect a neuroprotective effect in the Parkinson model[11].

This study was conducted the cell viability and neuroprotective effect mechanism by using HT22 mouse hippocampal cell by Glutamate which caused oxidative damage to measure the protective and antioxidant effects of CTE effect.

As a result of the WST-1 analysis in this study, glutamate cause cytotoxicity in HT22 cell, (Fig. 1A), and CTE doesn't cause cytotoxic in HT22 cell (Fig. 1B).

CTE and glutamate's pre-treated shows to increase cell viability (Fig 1. C and D). From the above results, CTE has neuroprotective effect by glutamate-induced cytotoxicity in HT22 cells.

Mitochondria plays an important role in the process of apoptosis and are important targets for oxidative toxicity by glutamate[16].

Mitochondria is damaged when the influx of calcium by glutamate in the cytoplasm increases[4], First, ROS is generated from the mitochondrial electron transport system, which is caused apoptosis[17].

From the ROS analysis of this study, it is shown that CTE significantly reduces ROS by glutamate (Fig 2. A and B).

Over glutamate secretion in the central nervous system outside the neurons forms typical excitotoxicity, it produces early apoptosis in the early stage and promotes late apoptosis in the late stage[18]. As a result of the flow cytometer analysis by Annexin V and PI staining for the type analysis of apoptosis, it was shown that CTE was significantly reduced early and late apoptotic cell death (Fig. 3 A and B).

Apoptosis is essential for normal brain development, but it is also important for a variety of degenerative neurological disorders that result from the death of certain parts of the neuron.

The mitogen-activated protein kinase (MAPKs) involved in signaling pathways are serine/threonine kinases involved in regulating various cellular responses, such as cell proliferation, differentiation, and apoptosis[19]

Among the MAPKs, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK/SAPK) and p38 kinase play an important role[20].

CTE suppress phosphorylation of ERK and P38 in signal mechanism study of CTE by glutamate-induced oxidative stress(Fig 4 A and B).

ERK phosphorylation increases apoptosis by the response to the stimulus such as hypoxia, growth factor deficiency and H<sub>2</sub>O<sub>2</sub>[11].

The abnormal Ca<sup>2+</sup> flow inside the cell by oxidative stress activates calpaine to activate the apoptosis proteine (Bax)[4-5].

Increased pro-apoptotic proteins(bax) expression forms pores in the mitochondrial membrane, causing cytochrome c and pro-apoptosis factor(AIF)[24-25].

Promotes the release of AIF is a flavoprotein located inside the mitochondria that plays an important role in ROS metabolism and has oxidoreductase activity. AIF is released from the mitochondria during apoptosis and migrates to the nucleus to induce DNA fragmentation, causing caspase-independent cell death[22-23]

CTE reduced the protein expression of Bax and AIF by glutamate-induced oxidative stress.(Fig 4. C and D)

GC-MS/ MS analysis confirmed that there were substances with antioxidant, anti-inflammatory and anti-cancer effects[33].

In view of the above results, CTE have neuroprotective effect through inhibition of ROS by glutamate-induced oxidative stress in HT22 cells, suppression of phosphorylation of the MAPK pathway of ERK, P38, and reduction of pro apoptotic protein expression of Bax and AIF.

Therefore, in the future, the neuroprotective effects of CTE may be useful for the treatment and prevention of degenerative neurological diseases and cognitive disorders such as Parkinson's disease, and Alzheimer's disease, which are caused by glutamate-induced oxidative stress.

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