Neuronal Cell Protective Effects of Phenolic Compounds Derived from Steam Exploded Nematode-Infected Pine

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Pine wilt disease is one of the most serious forest diseases that kills pine trees. Most of the nematode-infected pines are fumigated or locally incinerated and are thus not appropriately utilized. This study explored methods to utilize abandoned nematode-infected pines. The chemical compositions of healthy and nematode-infected pines were analyzed, and the neuroprotective effects of phenolic compounds extracted after steam explosion treatment were investigated. In terms of chemical composition, the nematode-infected pine chips suffered more damage from the steam explosion treatment than the healthy pine chips. In addition, the total phenolic compound content showed a clear difference depending on the presence of infection and steam explosion conditions. The maximum total phenolic compound content of extracted ethanol was found when the nematode-infected pine chip was subjected to steam explosion with a severity factor (Ro) of 3.82. The steam exploded pine extract of 10 μg/mL inhibited glutamate-induced early apoptotic cell death compared to cells treated with 10 mM glutamate alone. These results suggested that steam-exploded pine can be used as an effective natural material for neuronal cell protection.

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Keywords: Pinus densiflora; Pine wilt disease; Steam explosion; Phenolic compound; Neuronal cell protective effects

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INTRODUCTION

Pinus densiflora occurs widely on approximately 1,507,118 ha of land, representing 23.5% of South Korean forest area (Son *et al.* 2007). The pine wood nematode *Bursaphelenchus xylophilus* causes pine wilt disease in South Korea (Shin 2008). Pine wilt disease, first reported in Busan in 1988, causes serious losses and represents a dire threat to the South Korean pine forests (Yi *et al.* 1989). In 2005, damage occurred in 7,811 ha, and the number of diseased trees removed were reported as 138,441, 200,637, and 862,542 from 2003 to 2005, with yearly disease management costs of 6.9, 7.8, and 9.5 million US dollars, respectively. The average annual cost is 8 million US dollars, which has been increasing annually (Shin 2008). In recent years, the direct and effective strategy to control pine wilt disease has been to discover infected pine trees in real-time and to fell them immediately to prevent the dispersion of the disease to the surrounding healthy trees. Most nematode-infected wood is fumigated, locally incinerated, or not advantageously utilized (Kim and Kim 2008; Kim *et al.* 2011; Han *et al.* 2016). Therefore, this study focused on methods to use parts of the nematode-infected wood that are discarded.

Steam explosion treatment involves the use of steam hydrolysis at high temperature and pressure, followed by a sudden reduction in the pressure for physical treatment of the product to produce low-molecular-weight substances. Owing to the disruption of the cell wall matrix, steam explosion has been employed as an effective pretreatment process for extracting and separating bioactive phytochemicals from plant tissues (Sassner *et al.* 2008; Sui *et al.* 2019). Steam explosion has been applied to pretreat *P. morrisonicola*. Optimal operating conditions were reported for maximum yield of phenolic compounds and related antioxidant activity (Chiang *et al.* 2017). Steam explosion is an effective treatment process, since the high-temperature heat treatment process facilitates the removal of pathogenic microorganisms from the wilted pines and conversion of the constituents into phenolic compounds in one step. The cone, bark, leaves, and pollen of *P. densiflora* are known to contain large amounts of phenolic compounds and bioactive phytochemicals, which have been widely used in traditional medicine or for edible purposes (Choi *et al*. 2001; Kim *et al.* 2012; Jan *et al.* 2016). However, pine chips are rarely used as natural raw materials for phenolic compounds or bioactive phytochemicals. In addition, there has been no research on the extraction of polyphenols from nematode-infected wood.

Neurodegenerative diseases are a great public health concern and socioeconomic problem because of the high mortality rates and healthcare costs associated with treatment and care assistance (Sosa-Ortiz *et al.* 2012; Di Paolo *et al.* 2019). The mechanism of neurodegeneration in Alzheimer's disease remains unclear, and oxidative stress and neuronal apoptosis have been reported to play a pivotal role in the progression of the disease (Barber *et al.* 2006; Daulatzai 2017). Additionally, reactive oxygen species (ROS) accumulate in neurons, leading to lipid peroxidation, protein oxidation, DNA damage, and ultimately, cell death (Satoh and Lipton 2007). The consumption of foods rich in phenolic compounds has been associated with a reduced risk of chronic diseases, including neurodegenerative diseases. In this context, phenolic compounds are particularly relevant because of their ability to decrease reactive oxygen species and oxidative damage, leading to the prevention of neuronal death (Torma *et al.* 2017; Martinez *et al.* 2022; Hadrich *et al.* 2022). Thus, phenolic compounds are potential targets of drug development for neuroprotection (Satoh *et al.* 2006).

The aim of this study was to apply steam explosion treatment to infected pine chip and evaluate the neuronal cell protective effects of extracted phenolic compounds.

EXPERIMENTAL

Materials

The nematode-infected pine used in this study was collected from the Gajwasan area in Jinju. It was immersed in a solution of ethyl alcohol to lose pathogenicity, dried completely, and sealed on-site. Healthy pine (*Pinus densiflora*) was collected from an experimental forest at Gyeongsang National University, Jinju, South Korea. Nematode-infected pine and healthy pine were chipped to a particle size of approximately 2 × 2 × 0.5 cm.

Steam Explosion Treatment

The nematode-infected and healthy pine were subjected to steam explosion in a customized batch pilot unit equipped with a 1-L reaction vessel that was designed to reach the maximum operating pressure of 30 kg/cm^2 used in previous studies (Jung *et al*. 2022).

The raw materials were steam exploded for different time periods (5 to 7.5 min) and at a range of temperatures (200 to 220 °C). The severity of steam explosion [Eq. (1)] is designated by a single factor, the severity factor R_o, which combines the effects of time (t, min) and temperature (T, °C) (Heitz *et al.* 1987).

$$\text{Severity factor } (R_o) = \log[t \times \exp((T\text{-}100)/14.75)] \quad (1)$$

After the steam explosion treatment, the material was ground and sieved to a maximum particle size of 60 mesh. The steam-exploded material was stored in a desiccator at room temperature until the chemical analysis was carried out.

Chemical Composition Analysis

The carbohydrates (cellulose and hemicellulose) and lignin (acid-insoluble and acid-soluble) of healthy, nematode-infected, and steam-exploded pines were determined according to the NREL (National Renewable Energy Laboratory) procedure (Sluiter *et al.* 2008). The healthy, nematode-infected, and steam-exploded pines were subjected to carbohydrate and lignin determination based on the monomer content measured after a two-step acid hydrolysis procedure to fractionate the fibers. The first step was the use of 72% (w/w) H_2SO_4 at 30 °C for 60 min. In the second step, the reaction mixture was diluted with 4% (w/w) H_2SO_4 and autoclaved at 121 °C for 1 h. The hydrolysis liquid was analyzed for sugar content using high-performance liquid chromatography (Agilent 1100 Series HPLC System; Agilent, USA). The remaining acid-insoluble residue was considered to be acid-insoluble lignin.

Preparation of Extract

Extraction was carried out by soaking 35 g of dried powdered sample in absolute ethanol for 48 h at room temperature (22 to 26 °C). The sample was re-extracted twice. The resulting supernatants were combined, filtered, and evaporated. The extract was dissolved in distilled water, passed through a 0.2 μm filter, and stored at -20 °C until use.

Determination of Total Phenolic Compound

The total phenolic content of the extracts was determined using the Folin-Ciocalteu method (Singleton *et al.* 1999). An aliquot of 100 μL of the appropriate dilution of the extracts was stirred for 1 min with 500 μL of freshly prepared Folin-Ciocalteu reagent and 6 mL of distilled water. After the mixture was shaken, 2 mL of 15% (w/v) sodium carbonate was added and the mixture was shaken again for 30 s. Finally, the solution was diluted 10 mL with distilled water. After 30 min of reaction at ambient temperature, the absorbance at 756 nm was measured using glass cuvettes. Using gallic acid as a standard, the total phenolic content of the extracts was expressed as gallic acid equivalents.

Neuroprotective Effect Assay

Mouse hippocampal HT22 cells (a generous gift from Professor David Schubert at the Salk Institute, San Diego, CA, USA) were maintained in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/ streptomycin at 37 °C in a humidified incubator with 5% CO_2. The cells were plated and incubated overnight prior to treatment with glutamate alone or in combination with different concentrations of the extract for the indicated times. Cell viability was assessed by measuring the MTT reduction capacity of mitochondrial enzymes in viable cells. The MTT solution was added to culture medium at a final concentration of 0.5 mg/mL and left

in the dark for 4 h at 37 °C. Further, all the solutions were removed, and the formazan crystals were solubilized using a DMSO (dimethyl sulfoxide)-ethanol mixture (1:1, v/v). The absorbance was read at 550 nm using an EnSpire® Multimode Plate Reader (Perkin-Elmer, Waltham, MA, USA). The results were expressed as a percentage relative to the untreated control. Apoptotic cell death was quantified by flow cytometry using the FITC Annexin V Apoptosis Detection Kit with propidium iodide (PI) (BioLegend, San Diego, CA) according to the manufacturer's protocol. The cells were harvested at the end of treatment, washed with phosphate-buffered saline (PBS), and resuspended in binding buffer before staining with FITC-conjugated Annexin V and PI solution for 15 min in the dark. The fluorescence intensity of the stained cells was immediately analyzed using a BD FACS Calibur™ flow cytometer (BD Bioscience, Heidelberg, Germany). Data were collected from at least 10,000 cells per group and the results were expressed as the percentage of apoptotic cells.

Statistical Analysis

All experiments were performed in at least three biological replicates. The results are presented as average standard deviation (SD). All experiments were performed in triplicates. Data were analyzed using SAS statistical software, and Duncan's multiple range test was used to compare treatment means when p-values were significant ($p < 0.05$).

RESULTS AND DISCUSSION

Effect of Steam Explosion on the Chemical Composition of Healthy Pine and Nematode-Infected Pine

Figure 1 shows a healthy pine chip and a nematode-infected pine chip subjected to steam explosion. The nematode-infected pine chips infected with *B. xylophilus* were used. When the macrostructural morphology of the healthy pine chip and nematode-infected pine were compared, the nematode-infected pine showed a light black color and spot shape as a whole, and the signs of infection were clearly visible. The color of the healthy pine chips and nematode-infected pine chips changed considerably after steam explosion. The color of the healthy pine chip and nematode-infected pine chip gradually darkened. The R_o value for the steam explosion treatment increased, as shown in Fig. 1. These darker products might have been formed from Maillard reactions (Sørensen *et al.* 2008). The color of the steam-exploded uninfected pine chip and infected pine chip was dark brown when R_o was 4.23 due to the relatively high temperature, regardless of infection. In the steam explosion treatment, the nematode-infected pine chip showed a much darker color than that of the healthy pine chip. It is presumed that the nematode-infected pine chips received more damage from the steam explosion treatment than the healthy pine chips.

The main components in the healthy pine chips and nematode-infected pine chips under various steam explosion conditions are shown in Table 1. The percentage of cellulose and hemicellulose (including xylose, mannose, galactose, and arabinose) content in healthy pine chip were 39.1% and 27.1%; however, the cellulose (37.7%) and hemicellulose content (25.3%) were lower in nematode-infected pine chip when compared to those in the healthy pine chip. In addition, the percentage of lignin (including acid soluble lignin and acid insoluble lignin) content of nematode-infected pine chips (33.0%) was also slightly lower than that of healthy pine chips (33.7%). It can be inferred from the results that the differences were due to the hydrolysis of some cellulose and hemicellulose that occurred

during the period of infection by the pinewood nematode, which may have been caused by *B. xylophilus* infection. Previous studies have also reported that nematode-infected pine chips contain lower polysaccharide and lignin contents than healthy pine chips (Huan *et al.* 2020).

Steam Explosion Condition	Healthy Pine Chip	Nematode-infected Pine Chip
Raw		
*R*o 3.64-steam exploded		
*R*o 3.82-steam exploded		
*R*o 4.23-steam exploded		

Fig. 1. Gross examination of healthy pine chip and nematode-infected pine chip after steam explosion treatment. Severity factor (Ro) 3.64, 200 ℃, 5 min; (Ro) 3.82, 200 ℃, 7.5 min; (Ro) 4.23, 220 ℃, 5 min

The chemical composition of healthy pine chips and nematode-infected pine chip was different depending on the steam explosion conditions. The cellulose content in steam exploded healthy pine chips ranged between 39.1% and 37.4% when severity (R_o) range from 3.68 to 4.23. Further, the cellulose content in steam-exploded nematode-infected pin chips ranged between 36.1% and 30.5% when R_o ranged from 3.68 to 4.23. Followin steam explosion, the cellulose content was significantly lower in the nematode-infecte pine chips than in the healthy pine chips. As the steam explosion treatment condition increased in severity, the hemicellulose content decreased from 20.3 to 13.5% in health pine chips and 18.5 to 11.2% in nematode-infected pine chips, and the degree of decreas was similar.

Table 1. Chemical Composition of Healthy Pine Chip and Nematode-infected Pine Chip Before and After Steam Explosion

Steam Explosion Condition	Material	Chemical Composition (%)[a]						
		Cellulose	Hemicellulose				Lignin	
			Xylose	Mannose	Galactose	Arabinose	Acid soluble lignin	Acid insoluble lignin
Raw	Healthy	39.1	15.1	5.9	4.5	1.6	2.0	31.7
	Nematode-infected[b]	37.7	14.5	5.4	4.2	1.2	1.8	31.2
R_o 3.64[d] steam exploded	Healthy	39.1 (37.3)[c]	8.1 (7.7)	6.2 (5.9)	4.9 (4.7)	1.1 (1.0)	2.6 (2.5)	37.2 (35.5)
	Nematode-infected	36.1 (33.5)	7.6 (7.0)	5.6 (5.2)	4.7 (4.4)	0.6 (0.6)	3.0 (2.8)	39.4 (36.5)
R_o 3.82[e] steam exploded	Healthy	38.2 (35.0)	7.6 (7.0)	5.8 (5.3)	3.6 (3.3)	0.0 (0.0)	2.9 (2.7)	40.9 (37.5)
	Nematode-infected	34.6 (29.9)	4.5 (3.9)	4.4 (3.8)	3.2 (2.8)	0.0 (0.0)	3.1 (2.7)	48.7 (42.0)
R_o 4.23[f] steam exploded	Healthy	37.4 (30.8)	4.6 (3.8)	5.7 (4.7)	3.2 (2.6)	0.0 (0.0)	3.1 (2.6)	45.1 (37.1)
	Nematode-infected	30.5 (24.2)	3.9 (3.1)	4.3 (3.4)	3.0 (2.4)	0.0 (0.0)	2.5 (2.0)	53.6 (42.6)

[a]Percentages calculated from values on a dry-weight basis.
[b]Infected with *B. xylophilus*
[c]Data are expressed in parentheses as a percentage based on dry weight of raw material.
[d]R_o 3.64; 200 °C, 5.0 min
[e]R_o 3.82; 200 °C, 7.5 min
[f] R_o 4.23; 220 °C, 5.0 min

As the steam explosion conditions increased in severity (R_o) 3.64 to 4.23, the lignin content increased from 37.2% to 45.1% in healthy pine chip and from 39.4% to 53.6% in nematode-infected pine chips. The degree of increase in nematode-infected pine chips was larger than that in healthy pine chips. This was not a gravitational increase in lignin content, indicating that some cellulose or hemicellulose content of healthy and nematode-infected pine chips was digested during fungal infection, resulting in an increased percentage of lignin content (Ohgren *et al.* 2007).

Ethanol is known to be the most cost-effective solvent for commercial applications (Yu *et al.* 2002), and therefore, ethanol was selected as the extraction solvent in the present study. The extraction yields of healthy pine chips and nematode-infected pine chips under various steam explosion conditions were determined (Fig. 2). The healthy pine chips and nematode-infected pine chips extracted with ethanol gave extraction yields of 8.7 and 9.8%, respectively. Steam explosion resulted in a significant increase in the extraction yield of ethanol solvents. The extraction yield in healthy pine chips increased from 8.7% (untreated) to 10.5 %for the severity (R_o) 4.23 in steam exploded healthy pine chips. Similarly, the extraction yield in nematode-infected pine chip increased from 9.8% (untreated) to 11.4% at the severity (R_o) 3.82 in steam exploded nematode-infected pine chip. The extraction yield was highest for severity (R_o) 3.82 in steam exploded nematode-infected pine chips. These results indicated that the infected tree is suitable for explosion treatment, and the increase in the extract after explosion treatment may be due to the decomposition of high molecular weight components and change in these components from insoluble to soluble components in the solvent (Liu and Wyman 2003).

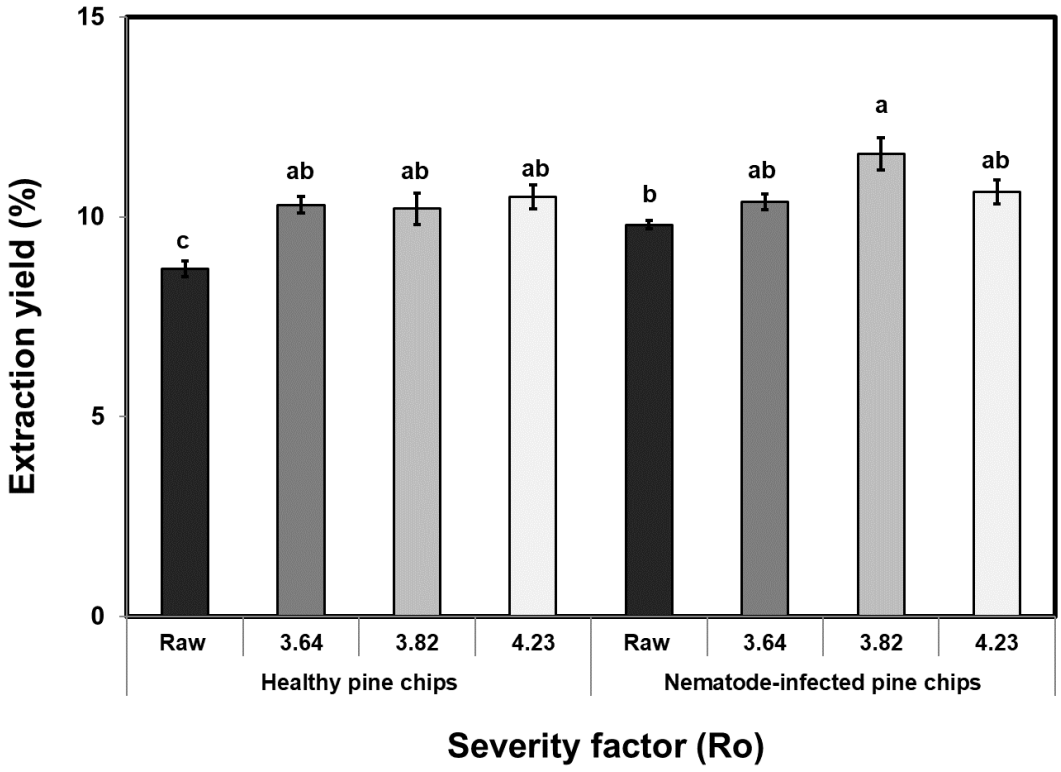


Fig. 2. Effect of infection and steam explosion condition on extraction yield. The data are expressed as the mean ± SD (n = 3). The statistical significance of the results was assessed by Duncan's t-test ($p < 0.05$).

Even if the extraction yield is increased, it may not be an effective component for antioxidant activity; therefore, the total phenolic compound content was measured as an index. Figure 3 shows the influence of steam explosion conditions in the range of severity (Ro) 3.64 to 4.23 on the total phenolic compound content of the healthy pine chips and nematode-infected pine chips. The total phenolic content showed a clear difference depending on the presence of infection and steam explosion conditions. Both healthy pine chips and nematode-infected pine chips showed a tendency of increase in total phenolic compound content when the steam explosion conditions were increased. The maximum total phenolic compound content was found when the nematode-infected pine chip was subjected to steam explosion with a severity (R_o) of 3.82.

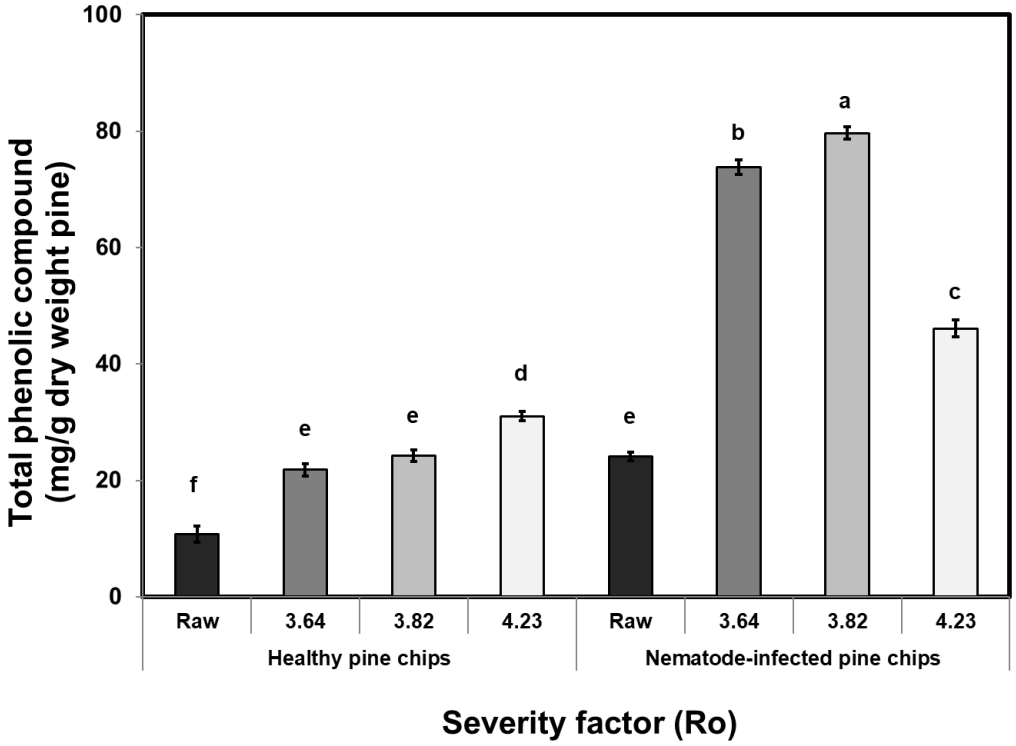


Fig. 3. Effect of infection and steam explosion condition on phenolic compound content. The data are expressed as the mean ± SD (n = 3). The statistical significance of the results was assessed by Duncan's t-test ($p < 0.05$).

Neuronal Cell Protective Effects of Steam Exploded Pine Extract

A definite neuroprotective effect of water extracted steam exploded pine on HT22 cell was demonstrated using the MTT viability test. As a result of measuring the cytotoxicity by varying the concentration of the steam exploded pine extract from 10 μg/mL to 1,000 μg/mL, there was cell viability of over 90% up to a concentration of 200 μg/mL, but at a concentration of 400 μg/mL significant toxicity was noted (Fig. 4a). To compare the glutamate-injury on cell, high concentrations of glutamate-treated and untreated specimens were evaluated at various series of assay. Excessive glutamate triggered oxidative neuronal cytotoxicity and mitochondrial dysfunction due to mechanism that independent to the glutamate receptor (Tang *et al.* 2010). Treatment with different glutamate concentrations, ranging from 1 to 100 mM, induced neuronal cell death in a dose-dependent manner (Fig. 4b). Thus, the glutamate concentrations of 5 and 10 mM resulted in a reduction of approximately 20% of the cells chosen for subsequent

experiments. In the presence of pine extract, glutamate-induced cell toxicity was significantly reduced in a concentration-dependent manner, as determined by 5 mM and 10 mM MTT assays (Figs. 4c and 4d). The 20 μg/mL of pine extract could improve cell viability by more than 50% compared to the control group. These results suggested that steam-exploded pine extract exerts neuroprotective effects against glutamate-induced cytotoxicity in cultured hippocampal neuronal cells.

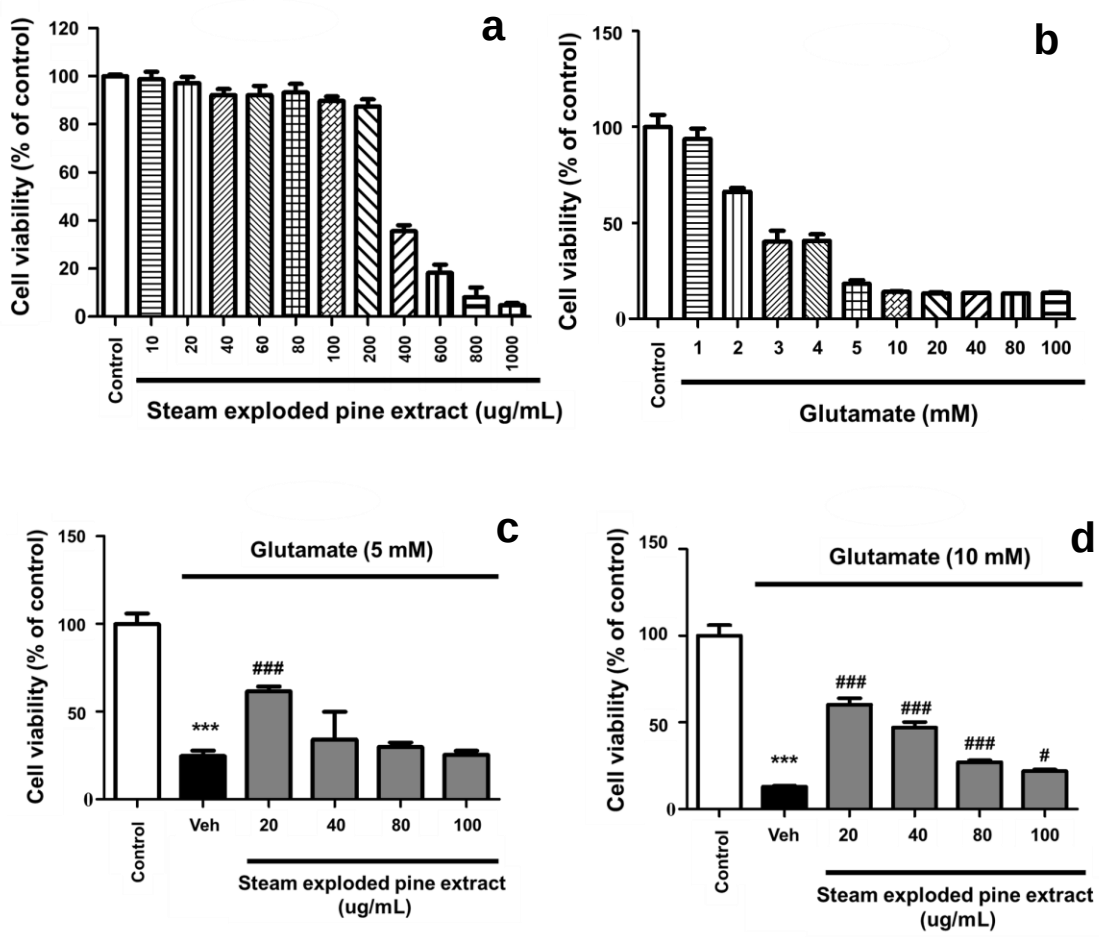
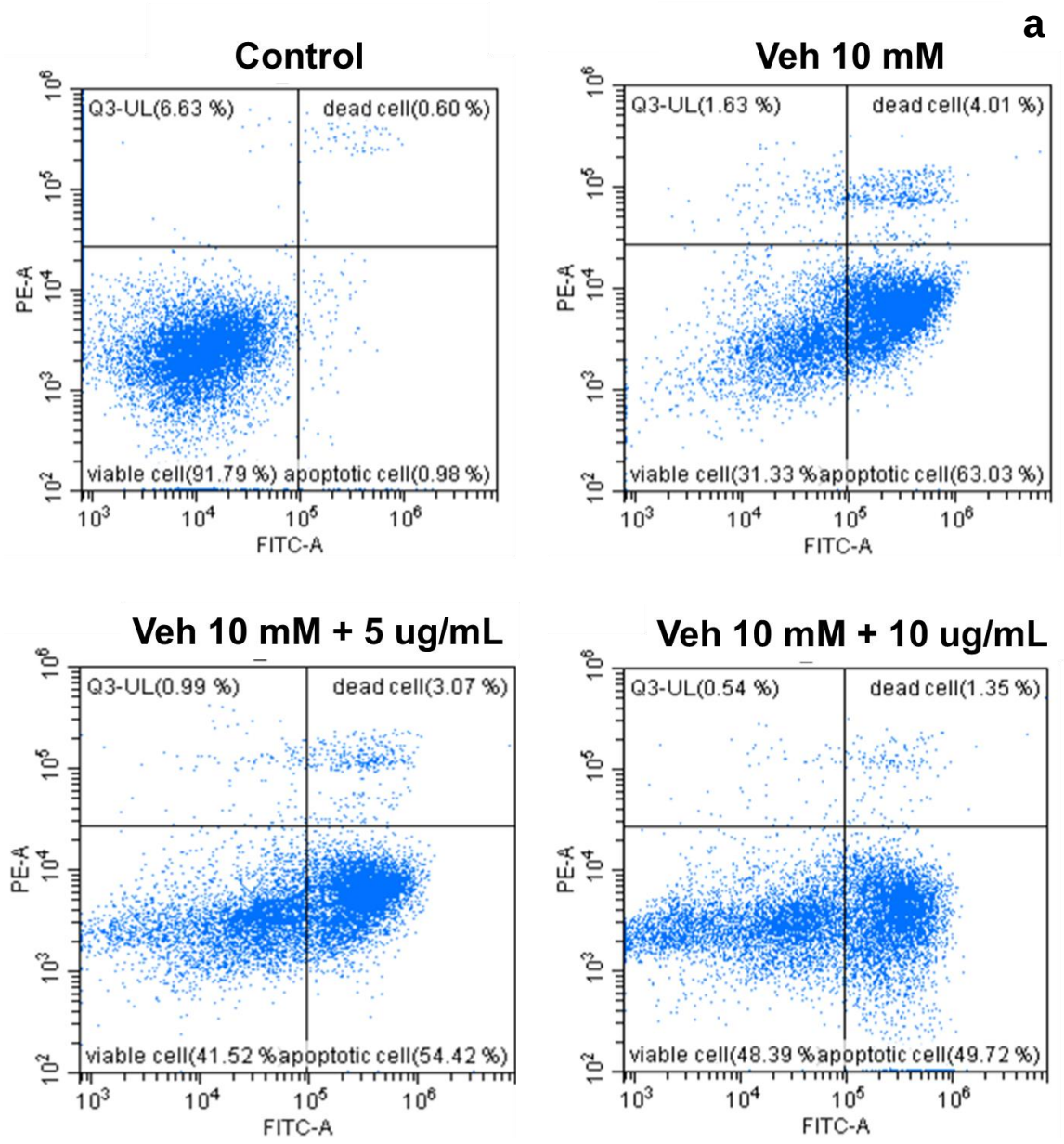


Fig. 4. Protective effect of steam-exploded pine extract against glutamate-induced toxicity in HT22 cells. Toxicity of steam exploded pine extract (a), glutamate (b), steam exploded pine extract with 5 mM glutamate (c) and steam exploded pine extract with 10 mM glutamate in HT22 cells was determined by MTT assay after treatment at different concentrations for 24 h. All data are shown as the mean ± SEM of at least three independent experiments. #P < 0.01, ###P < 0.001 vs. control; ***P < 0.001 vs. glutamate alone.

Glutamate induces neuronal cell death *via* necrosis and apoptosis. Nuclear translocation of AIF (Apoptosis-inducing factor) is one of the major downstream mechanisms underlying glutamate-induced neuronal cell death, mediated by ROS formation. Elevated intracellular ROS levels can induce the release of mitochondrial AIF into the nucleus, thereby triggering apoptosis in a caspase-independent manner (Tobaben *et al.* 2011). To observe the protective effects of pine extract on glutamate-induced apoptosis, annexin V/propidium iodide double staining was performed and analyzed using flow cytometry (Fig. 5a). The apoptosis in the glutamate-treated sample increased (68.6%) when compared with the case of the control group (2.44%); however, when the glutamate-treated cells were treated with steam-exploded pine extract, cell death was inhibited by

steam-exploded pine extract in a dose-dependent manner (Fig. 5b). In addition, 10 µg/mL pine extract inhibited glutamate-induced early apoptotic cell death at 54.2% compared to 68.6% of cells treated with 10 mM glutamate alone. These results suggest that steam-exploded pine extracts decreased glutamate-induced HT22 apoptosis.



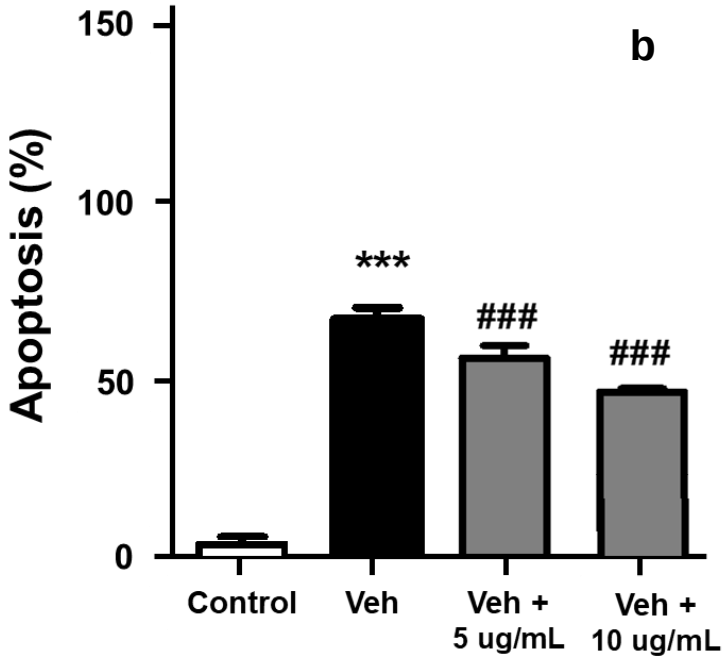


Fig. 5. The steam-exploded pine extract prevented glutamate-induced apoptosis in the HT22 cells. (a) The HT22 cells were exposed to 10 mM of glutamate in the presence of 5 and 10 μg/mL of steam-exploded pine extract for 24 h and double stained with annexin V/propidium iodide to evaluate the number of apoptotic cells. (b) Bars denote the percentage of apoptosis. ###P < 0.001 vs. control; ***P < 0.001 vs. glutamate alone.

CONCLUSIONS

In conclusion, given the data presented here, this study will make a significant contribution to the novel utilization of nematode-infected pines.

1. The neuronal cell protective effects of phenolic compounds were extracted from nematode-infected pine using an environmentally friendly method, *i.e.*, steam explosion treatment followed by ethanol extraction.
2. Ethanol extracts obtained from nematode-infected pine showed higher total phenolic compound content than healthy pine when subjected to steam explosion treatment.
3. The extract derived from steam-exploded infected pine prevented glutamate-induced HT22 apoptosis.

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