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NEUROPROTECTIVE EFFECT OF *TERMINALIA CHEBULA* EXTRACTS AND ELLAGIC ACID IN PC12 CELLS

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Abstract

Background: Alzheimer's disease (AD) is one of the common neurodegenerative disorders among elderly. The purpose of this study was to determine the neuroprotective effect and mechanisms of action underlying the *Terminalia chebula* extracts and ellagic acid by using beta-amyloid$_{25-35}$ (Aβ_{25-35})-induced cell toxicity in an undifferentiated pheochromocytoma (PC12) cell line.

Materials and Methods: The *T. chebula* extracts were prepared using the methanol, water, and 95% ethanol. Specifically, the ellagic acid was obtained in our laboratory. Assays including cell toxicity and changes in intracellular reactive oxygen species (ROS) and calcium level were evaluated to examine the neuroprotective effects and mechanisms of the *T. chebula* extracts and ellagic acid.

Results: The methanolic and water extracts of *T. chebula* and ellagic acid exhibited the strongest neuroprotective activity against Aβ_{25-35}-induced PC12 cell damages at 0.5–5.0 μg/ml. The ellagic acid also exhibited partial neuroprotective activity against H_2O_2-induced PC12 cell damages at 0.5–5.0 μg/ml. The methanolic and water extracts of *T. chebula* and ellagic acid protected PC12 cells from Aβ_{25-35}-mediated cell damages and enhanced cell viability thorough two key mechanisms by: (1) inhibiting ROS production and (2) reducing calcium ion influx.

Conclusion: The *T. chebula* represents a promising plant-source as medicine in the application for the treatment of AD. Further investigation focusing on the active component of *T. chebula* extracts e.g., ellagic acid is crucial to verify the neuroprotective efficacy and mechanisms *in vivo*.

Keywords: Neuroprotective effect; *Terminalia chebula*; ellagic acid; beta-amyloid.

Introduction

Alzheimer's disease (AD) is a common and severe neurodegenerative disorder in elderly people, inducing progressive memory loss and cognitive impairments. The neuropathology of AD is very complex and has yet to be completely understood. The extracellular beta-amyloid (Aβ) deposition is a pathogenic mechanism in the AD brain.

Normally Aβ is physiological product. Many studies have found that overproduction of Aβ results in a neurotoxic effect on nerve cells *in vitro* and *in vivo*. The Aβ aggregates into amyloid fibrils, which cause the shrinkage of neurites and denaturing of neurons (Jack et al., 2010; Mayeux and Stern, 2012). Accumulation of Aβ monomers into oligomers and lastly into plaques are both reported to mediate the toxicity of amyloid fibrils or plaques (Galimberti and Scarpini, 2012). The rat pheochromocytoma cell line (PC12 cells) is a common cellular model used in the study of neuroprotective effects (Greene and Tischler, 1976). Therefore, the toxic effect of Aβ in PC12 cells was used as a screening model in the study to identify potential neuroprotective components for AD.

Terminalia chebula Retz. (Combretaceae) has been extensively reported to scavenge free radicals (Chang and Lin, 2012), suppress cough (Ul Haq et al., 2013), treat arthritic disorders (Bag et al., 2013), elevate glucose uptake as PPARα/γ dual agonists (Yang et al., 2013), protect the cells from ischemic damage (Gaire et al., 2013), and inhibit oxidative stress (Na et al., 2004). The radical scavenging from the water, methanol and 95% ethanol extracts of the *T. chebula* has been reported in our laboratory (Chang and Lin, 2012). Besides, the *T. chebula* cold aqueous extract has the potent anti-aging effect *in vitro* (Manosroi et al., 2010). The extract of *T. chebula* seeds protected neuronal cells from the damages in a transient cerebral ischemia (Park et al., 2011).

Phytochemical study has shown that tannic acid, chebulagic acid, chebulinic acid, and corilagin are four major active ingredients of *T. chebula* (Park et al., 2011). Previous study in our laboratory has demonstrated that the methanol extract with the maximum total triterpenoid content and the water extract with the maximum total phenolic and tannin content of the *T. chebula* significantly protected PC12 cell against H_2O_2-induced cell death (Chang and Lin, 2012). In addition, the pure compound namely Tech-ME-A (ellagic acid) from *T. chebula* was purified in our laboratory (Hou et al., 2016). Here, the neuroprotective effects and mechanisms of action underlying the *T. chebula* extracts and ellagic acid were investigated by using $A\beta_{25-35}$-induced cell damages in PC12 cells.

Material and Methods

Methanol (Echo Chemical Corporation, LTD., Miaoli, Taiwan, R.O.C.) and 95% ethanol (Uni-Onward Corporation, Taipei, Taiwan, R.O.C.) were purchased as ACS grade reagents. Thirty-five percent H_2O_2 was purchased from Riedel-de Haën (Seelze, Germany). $A\beta_{25-35}$ was purchased from Kelowna International Scientific Inc. (Taipei, Taiwan, (R.O.C.). N-acetyl-Asp-Glu-Val-Asp-al (AC-DEVD-CHO) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were originally obtained from Sigma-Aldrich Corporation, Shanghai, China. Curcumin was purchased from Acros Organics (ECHO Chemical Corporation, LTD., Taichung, Taiwan, R.O.C.). PC12 cell line was purchased from ATCC (Manassas, VA, USA).

Extraction procedure

The air-dried fruit of the *T. chebula* was purchased from Xin Long Pharmaceutical Limited Company (Taichung, Taiwan, R.O.C.). The fine powder was ground from the air-dried fruit using a stainless steel blender (Waring Commercial, Torrington, CT, U.S.A.). The dried powder (2 g) was each extracted three-times with methanol (20 ml), deionized water (20 ml) and 95% ethanol (20 ml), respectively. The mixtures were agitated for 15 min at room

temperature in an ultrasonic cleaner (model DC200H, Chemist Scientific Corporation, Taipei, Taiwan, R.O.C.) then filtered as described previously (Chang and Lin, 2012). All dried extracts were brown solids, and were stored at -20°C prior to neuroprotective activity assays.

Preparation of the $A\beta_{25-35}$ stock solution

The $A\beta_{25-35}$ was prepared from the amyloid precursor protein, and it was pre-aggregated prior to use. Synthetic $A\beta_{25-35}$ was aggregated for 60 h at 4°C and then for 8 h at 37°C with mixing every 2 h before use as described previously. The stock solution (1 mM in water) was diluted to the desired concentration immediately before use and it was added to cell culture medium (Chang et al., 2013).

Protective effects

PC12 cell cultures were identical to those in our previous study (Chang and Lin, 2012). Various concentrations of test samples were 0, 0.5, 2.5 and 5.0 μg/ml. The MTT cell viability assay as done in our previous study was used for the protective effect assay (Chang et al., 2013).

Assay for intracellular reactive oxygen species (ROS)

Various concentrations of test samples were 0, 0.5, 2.5 and 5.0 μg/ml. The intracellular ROS was assayed as those in our previous study (Chang et al., 2013). We used a fluorescence spectrophotometer apparatus to measure fluorescence intensity at 485 nm (excitation wavelength) and 520 nm (emission wavelength). The final data were showed in relative fluorescence units.

Assay for intracellular calcium

Various concentrations of test samples were 0, 0.5, 2.5 and 5.0 μg/ml. The intracellular calcium assay was completed as those in our previous study (Chang et al., 2013). Fluorescence intensity was also detected as above.

Statistical analysis

For evaluation of the sample's protective effect, inhibition of the ROS production, and suppression of the calcium ion influx induced by H_2O_2 or $A\beta_{25-35}$ on PC12 cells were performed. All data are expressed as means ± standard deviation (SD) based on data from three independent experiments using Microsoft Excel 2003. One-way ANOVA was used to analyze data for statistical significance, followed by a post hoc Tukey's test using SPSS software (SPSS for Windows, Version 10).

Results and Discussion

Protective effects

We have reported that the neuroprotective effect of the *T. chebula* extracts may be attributed to their antioxidant properties. The methanol and water extracts exhibit the greater neuroprotective effects against H_2O_2-induced toxicity toward PC12 cells (Chang and Lin, 2012). According to our previous study, the induction of cell damages using 40 μM H_2O_2 for 12 h and 0.01 μM $A\beta_{25-35}$ for 24 h were used in the experiments (Chang et al., 2013). Cell viability decreases to 49.7 ± 7.4% when PC12 cells exposed to H_2O_2 (40 μM) for 12 h. We found that the ellagic acid inhibited H_2O_2-induced toxicity at 0.5–5.0 μg/ml (cell viability = 65.3–71.1%). To further study the neuroprotective effects of the *T. chebula* extracts and ellagic acid, we used $A\beta_{25-35}$-induced PC12 cell death to evaluate the neuroprotective effects by these samples. Table 1 shows the protective effects. Cell viability decreases to 48.6 ± 5.1% when exposed to $A\beta_{25-35}$ (0.01 μM) for 24 h. Using AC-DEVD-CHO and curcumin as two positive controls, the extracts and ellagic acid all concentration-dependently inhibited $A\beta_{25-35}$-induced toxicity at concentrations ranging from 0.5–5.0 (μg/ml) up to 24 h with potency in the following as descending order: methanol extract > water extract > ellagic acid > curcumin > AC-DEVD-CHO > 95% ethanol extract. No significant cytotoxicity of the extracts and ellagic acid could be observed between 0.5–5.0 μg/ml up to 24 h (data not shown). The methanol extract, water extract, ellagic acid and curcumin significantly inhibited $A\beta_{25-35}$-induced toxicity at dose rages of 0.5–5.0 μg/ml. It is notice that ellagic acid could significantly reduce $A\beta_{42}$-induced neurotoxicity as that been reported in SH-SY5Y cell model (Feng et al., 2009).

Ellagic acid exhibited neuroprotective effects against oxidative damage in diabetic rats (Uzar et al., 2012). The 95% ethanol extract partially inhibited $A\beta_{25-35}$-induced toxicity at dose rages of 2.5 and 5.0 μg/ml. AC-DEVD-CHO partially inhibited $A\beta_{25-35}$-induced toxicity at a dose of 2.5 μg/ml, but it clearly inhibited $A\beta_{25-35}$-induced toxicity at a dose of 5.0 μg/ml. The methanolic extract displayed the greatest neuroprotective effect among the extracts and ellagic acid. Based on these results, and our previous findings (Chang and Lin, 2012), we proposed that the potent neuroprotective effect of the methanol-extract of *T. chebula* is largely completed by its antioxidant activities achieved by its total triterpenoid content. These findings demonstrate for the first time that the methanol and water extracts of *T. chebula*, and ellagic acid can significantly ameliorate the H_2O_2 and $A\beta_{25-35}$-induced PC12 cell damages.

Table 1: Protective effects of the samples and positive control on $A\beta_{25-35}$-induced PC12 cell damages.

Sample	Concentration (μg/ml)	Cell viability (%)
Control	0	100.0 ± 3.3
$A\beta_{25-35}$	0.01 μM	48.6 ± 5.1$^{\#}$
Methanol extract	0.5	70.4 ± 6.8
	2.5	114.5 ± 5.5*
	5.0	163.9 ± 10.8**
Water extract	0.5	92.4 ± 8.8
	2.5	106.9 ± 5.7*
	5.0	122.3 ± 3.0**
95% Ethanol extract	0.5	40.9 ± 5.7
	2.5	62.5 ± 6.2

	5.0	71.2 ± 3.5
Ellagic acid	0.5	91.2 ± 7.8
	2.5	98.4 ± 6.1*
	5.0	105.1 ± 8.8*
AC-DEVD-CHO	0.5	45.5 ± 7.9
	2.5	64.0 ± 5.2
	5.0	94.6 ± 8.6
Curcumin	0.5	91.6 ± 3.2
	2.5	93.5 ± 6.1
	5.0	102.0 ± 5.4*

These data are showed as mean ± SD for 3 replicates. $^{\#}P < 0.01$ versus the control group without the addition of the sample and $A\beta_{25\text{-}35}$ solutions, $^{*}P < 0.05$; $^{**}P < 0.01$ versus the 0.01 μM $A\beta_{25\text{-}35}$-treated group without the addition of the sample solution.

Assay for intracellular ROS

This assay demonstrates that H_2O_2 and $A\beta_{25\text{-}35}$ induced the production of ROS. After treatment with the samples, the H_2O_2 and $A\beta_{25\text{-}35}$-induced ROS were reduced. The various decreases in PC12 cell H_2O_2-induced ROS productions for the samples, AC-DEVD-CHO and curcumin, in decreasing order of activity, were 95% ethanol extract > AC-DEVD-CHO > methanol extract > water extract > ellagic acid > curcumin. The various reductions in PC12 cell ROS-production activities induced by $A\beta_{25\text{-}35}$ for the samples, AC-DEVD-CHO and curcumin were in decreasing order of ellagic acid > curcumin > AC-DEVD-CHO > water extract > 95% ethanol extract > methanol extract. However, these results were not dose-dependent responses except for 95% ethanol extract and AC-DEVD-CHO under the 40 μM H_2O_2 condition and AC-DEVD-CHO under the 0.01 μM $A\beta_{25\text{-}35}$ condition (Table 2). The 95% ethanol extract's high activity in reduction in H_2O_2-induced ROS production has a low dependence on its neuroprotective effect against H_2O_2 toxicity to PC12 cells (Chang and Lin, 2012). The ellagic acid's high activity in reduction in $A\beta_{25\text{-}35}$-induced ROS production is a high dependence on its neuroprotective effect against $A\beta_{25\text{-}35}$ toxicity to PC12 cells, but it expresses the lowest decrease in H_2O_2-induced ROS production. In addition, H_2O_2-induced ROS production was enhanced by 43.2% after the addition of 2.5 μg/ml ellagic acid to the culture medium. The H_2O_2-induced ROS production was enhanced by 121.1% and 45.2% after the addition of 2.5 and 5.0 μg/ml curcumin to the culture medium. Methanol extract exhibits the good neuroprotective effect against $A\beta_{25\text{-}35}$ toxicity to PC12 cells, but it expresses the lowest decrease in $A\beta_{25\text{-}35}$-induced ROS production.

Table 2: Protective effects of the samples and positive control on the intracellular ROS production induced by H_2O_2 or $A\beta_{25-35}$ in PC12 cells.

Sample	Concentration (μg/ml)	DCF fluorescence intensity (% of control) by H_2O_2	DCF fluorescence intensity (% of control) by $A\beta_{25-35}$
Control	0	100.0 ± 4.2	100.0 ± 5.5
H_2O_2	40 μM	192.5 ± 4.4$^{\#}$	
$A\beta_{25-35}$	0.01 μM		199.8 ± 6.3$^{\#}$
Methanol extract	0.5	96.1 ± 11.0**	146.2 ± 8.5*
	2.5	127.4 ± 10.7*	128.8 ± 8.2*
	5.0	92.4 ± 12.1**	135.3 ± 12.3*
Water extract	0.5	98.7 ± 3.9**	114.2 ± 11.6*
	2.5	120.4 ± 5.1*	98.0 ± 7.2**
	5.0	140.2 ± 5.3*	120.8 ± 9.6*
95% Ethanol extract	0.5	87.9 ± 3.4**	97.2 ± 11.4**
	2.5	85.7 ± 4.2**	141.1 ± 4.3*
	5.0	81.6 ± 4.7**	134.5 ± 9.2*
Ellagic acid	0.5	157.7 ± 4.9	35.8 ± 1.5**
	2.5	235.7 ± 6.2	52.4 ± 2.1**
	5.0	196.9 ± 3.4	48.2 ± 3.3**
AC-DEVD-CHO	0.5	107.8 ± 11.0**	109.0 ± 5.4*
	2.5	92.0 ± 12.4**	104.7 ± 10.2*
	5.0	62.9 ± 5.6**	62.9 ± 5.6**
Curcumin	0.5	110.7 ± 5.2**	43.2 ± 2.3**
	2.5	313.6 ± 11.9**	46.8 ± 2.1**
	5.0	237.7 ± 10.9	110.9 ± 5.9*

These data are expressed as mean ± SD for 3 replicates. $^{\#}P < 0.001$ versus the control group without the addition of the sample and H_2O_2 or $A\beta_{25-35}$ solutions, $*P < 0.01$; $**P < 0.001$ versus the 40 μM H_2O_2 or 0.01 μM $A\beta_{25-35}$-treated groups without the addition of the sample solution.

Assay for intracellular calcium

The intracellular calcium by 40 μM H_2O_2 and 0.01 μM $A\beta_{25-35}$ was significantly improved by 132.2 % and 115.2 %, respectively. Table 3 shows the effects on intracellular calcium, of preincubation or coincubation with samples, and 40 μM H_2O_2 or 0.01 μM $A\beta_{25-35}$. The different decreases in PC12 cell calcium-production activities in response to H_2O_2 for the samples, AC-DEVD-CHO and curcumin in decreasing order of activity were curcumin > 95% ethanol extract > water extract > ellagic acid > methanol extract > AC-DEVD-CHO. The different decreases in PC12 cell calcium-production activities, induced by $A\beta_{25-35}$, for the samples, AC-DEVD-CHO and curcumin in decreasing order of

activity were ellagic acid > curcumin > water extract > methanol extract > 95% ethanol extract > AC-DEVD-CHO. The 95% ethanol extract's high activity in reduction in H_2O_2-induced calcium production has a low dependence on its neuroprotective effect against H_2O_2 toxicity to PC12 cells (Chang and Lin, 2012). It expresses the lowest decrease in $A\beta_{25-35}$-induced calcium production, but it is a medium dependence on its neuroprotective effect against $A\beta_{25-35}$ toxicity to PC12 cells. The ellagic acid's high activity in reduction in $A\beta_{25-35}$-induced calcium production has a high dependence on its neuroprotective effect against $A\beta_{25-35}$ toxicity to PC12 cells. Methanol extract exhibits the good neuroprotective effect against H_2O_2-induced toxicity to PC12 cells (Chang and Lin, 2012), but it expresses the lowest decrease in H_2O_2-induced calcium production. Table 3 shows that water extract can regulate calcium production, and it is able to decrease H_2O_2 or $A\beta_{25-35}$ toxicity.

Table 3: Protective effects of the samples and positive control on the intracellular calcium elevation as induced by H_2O_2 or $A\beta_{25-35}$ in PC12 cells.

Sample	Concentration (μg/ml)	Intracellular calcium (% of control) by H_2O_2	Intracellular calcium (% of control) by $A\beta_{25-35}$
Control	0	100.0 ± 5.1	100.0 ± 6.3
H_2O_2	40 μM	232.2 ± 8.8$^{\#}$	
$A\beta_{25-35}$	0.01 μM		215.2 ± 7.8$^{\#}$
Methanol extract	0.5	188.7 ± 11.7	113.8 ± 11.6*
	2.5	140.6 ± 3.5*	104.4 ± 5.3**
	5.0	124.4 ± 4.7*	107.9 ± 4.3**
Water extract	0.5	101.4 ± 10.3**	90.5 ± 6.8**
	2.5	96.1 ± 8.7**	117.1 ± 2.3*
	5.0	137.8 ± 11.7*	100.5 ± 6.5**
95% Ethanol extract	0.5	86.9 ± 7.4**	121.6 ± 3.9*
	2.5	103.2 ± 9.7**	115.2 ± 3.5*
	5.0	97.9 ± 5.3**	122.7 ± 5.8*
Ellagic acid	0.5	125.4 ± 9.7*	111.4 ± 5.6*
	2.5	142.6 ± 3.4*	64.0 ± 7.4**
	5.0	147.2 ± 5.2*	56.5 ± 1.4**
AC-DEVD-CHO	0.5	146.1 ± 12.8*	109.0 ± 7.4**
	2.5	147.1 ± 4.2*	144.0 ± 6.1*
	5.0	151.9 ± 8.6*	122.3 ± 10.3*
Curcumin	0.5	82.6 ± 9.2**	82.5 ± 5.6**
	2.5	54.9 ± 7.5**	87.5 ± 9.8**
	5.0	52.5 ± 4.9**	140.6 ± 3.2*

These data are expressed as mean ± SD for 3 replicates. $^{\#}P < 0.001$ versus the control group without the addition of the sample and H_2O_2 or $A\beta_{25-35}$ solutions, $*P < 0.01$; $**P < 0.001$ versus the 40 μM H_2O_2 or 0.01 μM $A\beta_{25-35}$-treated

groups without the addition of the sample solution.

Conclusion

We have demonstrated the ability of the *T. chebula* extracts and ellagic acid to prevent H_2O_2 or $A\beta_{25-35}$-induced PC12 cell damages. Our results reveal that the methanolic and water extracts exhibited favorable neuroprotective activities against H_2O_2 and $A\beta_{25-35}$-induced toxicity towards PC12 cells, and are potential candidates for treating H_2O_2 and $A\beta_{25-35}$-induced neurodegenerative diseases. We also show that ellagic acid ameliorated H_2O_2 and $A\beta_{25-35}$-induced PC12 cell damages. Induction of ROS in PC12 cells by $A\beta_{25-35}$ maybe associated with oxidative stress-mediated cytotoxicity. Ellagic acid has been shown to suppress $A\beta_{25-35}$-induced ROS production in PC12 cells. Further, we have demonstrated that H_2O_2 and $A\beta_{25-35}$ decreased the intracellular calcium production in PC12 cells was modulated by exposure to ellagic acid.

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