

# Antihypertensive activities of extracts from tissue cultures of *Vitis thunbergii* var. *taiwaniana*

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**ABSTRACT.** *Vitis thunbergii* var. *taiwaniana* (VTT), the wild grape, is an endemic plant in Taiwan which has long been used as a folk medicine. Two ethanolic extracts (EE), including shoots (S) and root parts (R), from each plant growth regulator-treated VTT tissue culture (TC) plantlets, including TC1 (treated with IAA), TC2 (treated with IBA), TC3 (as the control), and TC4 (treated with NAA), were used to test the angiotensin converting enzyme (ACE) inhibitory activities. It was found that EE of R and S parts from four VTT-TC plantlets exhibited ACE inhibitory activities and the TC-R had better ones. The EE of whole plantlet exhibited dose-dependently ACE inhibitory activities (expressed as IC<sub>50</sub>), and the orders were TC1 (98.67 µg/mL) > TC3 (99.04 µg/mL) > TC4 (102.46 µg/mL) > TC2 (132.05 µg/mL). The hot water extracts (HWE) of TC1-A and HWE-TC1-B exhibited dose-dependently ACE inhibitory activities and the IC<sub>50</sub> were 29.51 and 32.79 µg/mL, respectively. For short-term antihypertensive activity of EE-TC1-R *in vivo*, the spontaneously hypertensive rats (SHRs) were fed by a single oral administration (20 mg/Kg of SHR), and the changes of systolic blood pressure (SBP) and diastolic blood pressure (DBP) during 24-h were measured. It was found that EE showed antihypertensive activities and the highest lowering effects was reached at 4<sup>th</sup>-h after being orally administered and the reductions of SBP and DBP were 16.9 and 17.7 mmHg, respectively. It can also be noted that the SBP reduction could last over 24-h. For long-term antihypertensive activity of EE-TC1-R *in vivo*, SHRs were fed orally once a day for four weeks (30 mg/Kg of SHR), and the changes of systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured weekly. It was found that the reductions of SBP, but not DBP, were 12.45, 17.13, and 10.05 mmHg, respectively, at the 2<sup>nd</sup>, 3<sup>rd</sup>, and 4<sup>th</sup>-week and showed significantly different ( $P < 0.05$  or  $P < 0.01$ ) compared to the blank. The amplexipin C and (+)-ε-viniferin were identified in the EE-TC1-R. This will be beneficial to develop the extracts of VTT as functional foods for blood pressure regulation.

**Keywords:** Blood pressure; Plantlet of tissue culture (TC); Spontaneously hypertensive rats (SHRs); *Vitis thunbergii* var. *taiwaniana* (VTT).

## INTRODUCTION

A number of risk factors are associated with stroke, including age, gender, elevated cholesterol, smoking, alcohol, excessive weight, race, family history, and hypertension (Mark and Davis, 2000). Although some of these risk factors cannot be modified, one factor that can be controlled and has the greatest impact on the etiology of stroke is high blood pressure (Dunbabin, 1992).

Several classes of pharmacological agents have been used in the treatment of hypertension. One class of anti-hypertensive drugs known as angiotensin I converting enzyme (ACE) inhibitors (i.e. a peptidase inhibitor) are associated with a low rate of adverse side-effects and are the preferred class of anti-hypertensive agents for treating patients with concurrent secondary diseases (Fotherby and Panayiotou, 1999). ACE (EC 3.4.15.1) is a dipeptide-liberating exopeptidase which has been classically associated with the renin-angiotensin system regulating peripheral blood pressure (Mullally et al., 1996). The potent ACE inhibitors were frequently derived from food

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proteins (Ariyoshi, 1993; Hsu et al., 2002). However, pomegranate juice (Aviram and Dornfeld, 2001), flavan-3-ols and procyanidins (Actis-Goretta et al., 2003), myricetin galloylglycosides (Lee et al., 2006), small molecules of methanol-soluble,  $\beta$ -elimination products from preparations of alginic acid hydroxamate (Liu et al., 2007), geraniin (Lin et al., 2008), and tannins (Liu et al., 2003) were also reported to have ACE inhibitory activity. Fujita et al. (2000) found that the octapeptides of FFGRCVSP ( $IC_{50}=0.4 \mu M$ ) and ERKIKVYL ( $IC_{50}=1.2 \mu M$ ) were potent ACE inhibitors, but none of them were effective in animal models to reduce the blood pressure of SHR. Sato et al. (2002) pointed out that three dipeptides, including AW ( $IC_{50}=18.8 \mu M$ ), VW ( $IC_{50}=3.3 \mu M$ ), and LW ( $IC_{50}=23.6 \mu M$ ), were potential ACE inhibitory peptides. However, none of them were able to effectively reduce the blood pressure of SHR in animal models. Clearly, ACE inhibitory candidates *in vitro* might not have the antihypertensive effects on SHR *in vivo*.

*Vitis thunbergii* var. *taiwaniana* (VTT), as a wild grape, belonged to the Vitaceae family and *Vitis* genus, is an endemic plant in Taiwan which has long been used as folk medicines for treatments of hepatitis, jaundice, diarrhea, and arthritis (Chiu and Chang, 1995). The Endemic Species Research Institute, Council of Agriculture has cataloged VTT as an original medicinal plant in Taiwan. The active components from *Vitis* genus were reported to be oligostilbenes (Li et al., 1996; Teguo et al., 1998; Huang et al., 2001; Huang et al., 2005; Chen and Wang, 2009) and polyphenols (Dou et al., 2003). The methanolic root extracts of VTT were reported to have anti-methicillin-resistant *Staphylococcus aureus* activities (Peng et al., 2008). In this report, ethanolic extracts (EE) and hot water extracts (HWE) of plant growth regulator-treated VTT tissue culture (TC) plantlets were used to examine the ACE inhibitory activities *in vitro*. The EE of VTT-TC were then to investigate the short-term and long-term antihypertensive activity by oral administrations of TC1-R to SHR *in vivo*. These results showed that it will be beneficial to develop the extracts of VTT as functional foods for blood pressure regulation.

## MATERIALS AND METHODS

### Materials

ACE (I unit, rabbit lung) was purchased from Fluka Chemie GmbH (Switzerland); N-(3-[2-furyl] acryloyl)-Phe-Gly-Gly (FAPGG), and other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

### Plants

Four VTT-TC plantlets, including TC1, treated with IAA (indole-3-acetic acid); TC2, treated with IBA (indole-3-butyric acid); TC3, as the control, without plant growth regulator treatment; and TC4, treated with NAA (naphthaleneacetic acid), which were provided by Dr. Wen, Chi-Luan (Taiwan Seed Improvement and Propagation

Station, Council of Agriculture, Taichung, Taiwan), were used in this study. The VTT plantlet was identified by Dr. Hsu, Tsai-Wen (Endemic Species Research Institute, Nantou, Taiwan). The photographs of whole TC plantlets were shown in Figure 1.

### Preparation of ethanolic extracts and hot water extracts from VTT-TC

After being washed with distilled water thrice to remove the culture medium, the whole TC plantlets with or without being freshly cut into shoot (S) and root (R) portions. For ethanolic extracts (EE), 1,000 mL of absolute ethanol was added into whole TC plantlets or S portions (wet weight), or 500 mL of absolute ethanol was added into R portions (wet weight) and then extracted at room temperature for one week. After being filtered, the residue was extracted with ethanol once. The filtrates were collected and concentrated as EE extracts. For hot water extracts (HWE), the whole plantlets of VTT-TC1 were air-dried at 37°C oven and cut into pieces and placed into tea bags and then were sealed. The VTT tea bags were extracted twice by 100°C hot water in the ratio of 1/10 (w/v) for 30 min. After being filtered, the filtrates were collected and then lyophilized as the HWE-TC1. There were two methods to culture VTT-TC1 (treated with IAA plant regulators) plantlets on the same 90-days for HWE-TC1 preparations (including HWE-TC1-A and HWE-TC1-B). TC1-A represented the cultured method of replacing the old culture medium after 30-days with a new one, and then for another 60 days; TC1-B represented the culture method of directly adding the new culture medium onto the old one after 30-days, and then for another 60 days.

### Determination of the ACE inhibitory activity of different VTT-TC extracts by spectrophotometry

The ACE inhibitory activity was measured according to the method of Holmquist et al. (1979) with some modifications. Twenty  $\mu L$  (20 mU) of commercial ACE (1 U/mL, from rabbit lung) were mixed with 200  $\mu g$  of EE from TC1-R, TC1-L, TC2-R, TC2-L, TC3-R, TC3-L, TC4-R, and TC4-L, or different amounts of whole plantlets of EE (50-250  $\mu g$ ) from TC1, TC2, TC3, and TC4, or different amounts of HWE-TC1-A and HWE-TC1-B (16.39, 32.79, 49.18, and 65.57  $\mu g/mL$ ), and then 1 mL of 0.5 mM FAPGG [dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl] was added. The decreased absorbance at 345 nm ( $\Delta A_{\text{sample}}$ ) was recorded within a 1.5-min span at room temperature and expressed as  $\Delta A_{\text{sample}}/\text{min}$ . The extracted solvent, ethanol or distilled water, respectively, was used in blank experiments and expressed as  $\Delta A_{\text{blank}}/\text{min}$ . The ACE inhibition (%) was calculated as follows:  $[1 - (\Delta A_{\text{sample}}/\text{min} \div \Delta A_{\text{blank}}/\text{min})] \times 100\%$ . Means of triplicates were determined. The 50% inhibition ( $IC_{50}$ ) of ACE activity was calculated as the concentrations of samples that inhibited 50% of ACE activity under these conditions.



**Figure 1.** The photographs of whole plantlet of four typers of tissue culture (TC) of *Vitis thunbergii* var. *taiwaniana* as followed: TC1, IAA (indole-3-acetic acid); TC2, IBA (indole-3-butyric acid); TC3, the control; and TC4, NAA (naphthaleneacetic acid).

### Antihypertensive effects of EE-TC1-R on SHR

The effects of orally-administered EE-TC1-R by feeding tube (2.0 × 80 mm) on the blood pressure of SHR were determined (Lin et al., 2006; Liu et al., 2007; Lin et al., 2008; Liu et al., 2009a, b). All animal experimental procedures followed published guidelines (National Science Council, 1994) and reviewed and approved by the Institutional Animal Care and Use Committee of Taipei Medical University (LAC-95-0076). The male SHRs (8 weeks of age, National Laboratory Animal Center, Taipei) were housed individually in steel cages kept at 24°C with a 12-h light-dark cycle and had free access to a standard mouse/rat chow (Prolab® RMH2500, 5P14 Diet, PMI Nutrition International Brentwood, MO) and water. SHRs were randomly divided into control and sample treatments for blood pressure determinations (six rats per group). For a short-term antihypertensive experiment, 0.5-mL of water-dissolved EE-TC1-R was orally administered to SHR (20 mg/Kg of SHR) once, and tail blood pressure was measured four times at each desired time over 24 h using an indirect blood pressure meter (BP-98A, Softron Co. Ltd. Tokyo, Japan) for systolic blood pressure (SBP) and diastolic blood pressure (DBP) measurements. For long-term antihypertensive effects, the EE-TC1-R was orally administered to SHR once a day for 4-weeks (30

mg/Kg of SHR) and the blood pressure was measured once every week before each oral administration. Before each blood pressure measurement, SHRs were warmed for 10 min in a 39°C thermostated box. The 0.5-mL distilled water was used for a blank experiment and the blood pressure was measured after oral administration of distilled water. Means of triplicates were recorded. The measured blood pressure values were collected and averaged from six rats as termed  $BP_{blank}$ . The measured blood pressure values of each rat after being administered sample orally were collected and averaged as termed the  $BP_{sample}$ . The six values calculated from  $BP_{sample} - BP_{blank}$  are averaged and then indicated as lowering effects in blood pressure changes ( $\Delta BP$ ) in sample at the same treatment time after oral administration (such as 2, 4, 6, and 24-h) for short-term antihypertensive activity. Means  $\pm$  SD of triplicates were measured. Student's *t*-test was used for comparisons between blank and sample treatment at the same time interval when  $P < 0.05$  (\*) or  $P < 0.01$  (\*\*).

### HPLC chromatogram of EE-TC1-R of *Vitis thunbergii* var. *taiwaniana*

The HPLC chromatogram of EE-TC1-R was performed by XTerra MS C18 HPLC column (3.5  $\mu$ m, 2.1 × 150 mm). The mobile phase was mixed in gradients with 95%

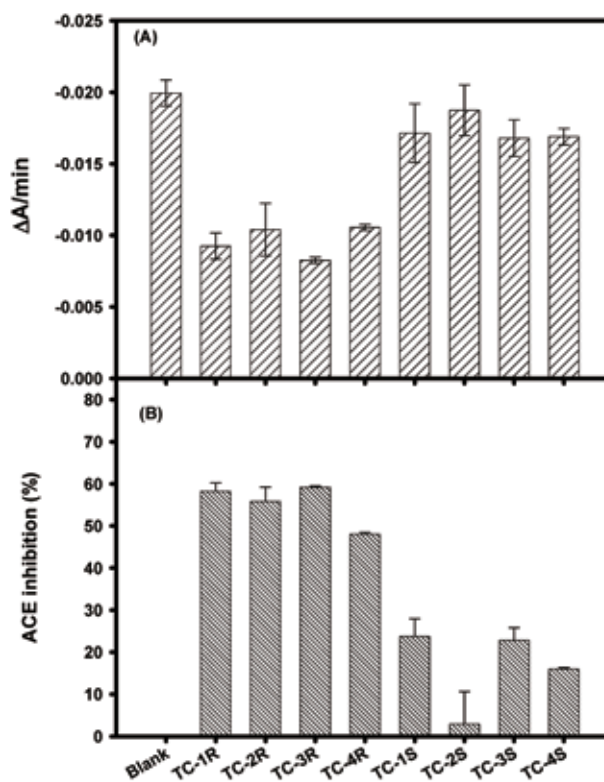
Solvent A and 5% Solvent B (0 min) to 70% Solvent A and 30% Solvent B (50 min). Solvent A: 0.1% TFA, and Solvent B: acetonitrile. The detector was set at UV 280 nm. The identified component, ampelopsin C (35.8 min) and (+)- $\epsilon$ -viniferin (38.6 min), were compared to each isolated pure compound and electrospray ionization mass spectra (ESI-MS) (Chen and Wang, 2009).

## RESULTS AND DISCUSSION

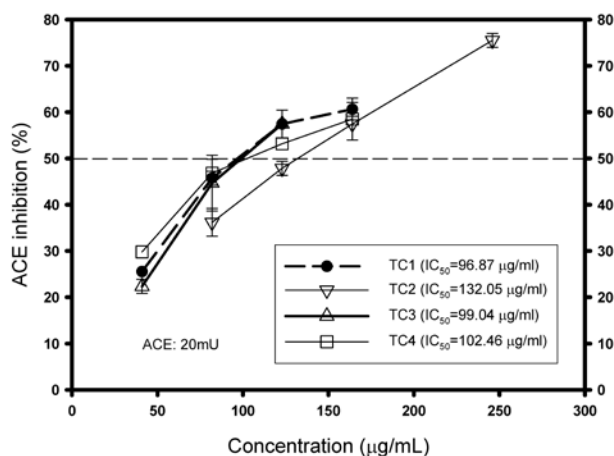
*Vitis thunbergii* var. *taiwaniana* (VTT), as a wild grape, belonged to the Vitaceae family and *Vitis* genus, is an endemic plant in Taiwan which has long been used as folk medicines for treatments of hepatitis, jaundice, diarrhea, and arthritis (Chiu and Chang, 1995). The Endemic Species Research Institute, Council of Agriculture has cataloged VTT as an original medicinal plant in Taiwan. Therefore, the evidence-based biological functions of VTT might be main criteria to develop as functional foods.

The screening of ACE inhibitory activities *in vitro* might be reasonable for further antihypertensive activities in animal models. Figure 2 showed the 200  $\mu$ g of EE extracts from R and S portions of four VTT-TC on 20 mU ACE. It was clear that the FAPGG was hydrolyzed by ACE to produce FAP and resulted in the reduction of absorbance at A345 nm (Holmquist et al., 1979). It could be found that the blank (ACE and FAPGG) exhibited the highest reduction of  $\Delta A_{\text{blank}}/\text{min}$  and the R portion had better effects than S portion to inhibit the reductions in  $\Delta A_{\text{sample}}/\text{min}$  (Figure 2A). It was calculated for ACE inhibition under the same 200  $\mu$ g of EE extracts (Figure 2B), the orders of each ACE inhibition of TC-VTT were TC3-R (59.22%)  $\sim$  TC1-R (58.25%)  $\sim$  TC2-R (55.83%)  $>$  TC4-R (48.06%)  $\gg$  TC1-S (23.79%)  $\sim$  TC3-S (22.82%)  $>$  TC4-S (16.02%)  $>$  TC2-S (2.91%). It was preliminarily found that the IAA-treated TC-VTT (TC1) had closely ACE inhibitory activities to the control TC-VTT (TC3) and better than other plant growth regulator-treated VTT-TC. Therefore, the dose effects of EE from the whole plantlets of VTT-TC were investigated and the results were shown in Figure 3. It was clear that the dose-dependent ACE inhibition was found and the  $IC_{50}$  of ACE inhibitory activity was 96.87, 132.05, 99.04, and 102.46  $\mu$ g/mL, respectively, for TC1, TC2, TC3, and TC4. The IAA-treated plantlet (TC1) had higher ACE inhibitory activities compared to the control plantlet (TC3) used in this study. It was the first report that EE of VTT-TC exhibited ACE inhibitory activities.

For contamination prevention during medium replacements for TC, two cultured methods for TC1 were used. TC1-A represented the cultured method of replacing the old culture medium after 30-days with a new one, and then for another 60 days; TC1-B represented the culture method of directly adding the new culture medium onto the old one after 30-days, and then for another 60 days. Both whole TC plantlets were washed, air-dried at 37°C and then cut into pieces and placed and simulated as tea bags. Figure 4 showed the effects of different



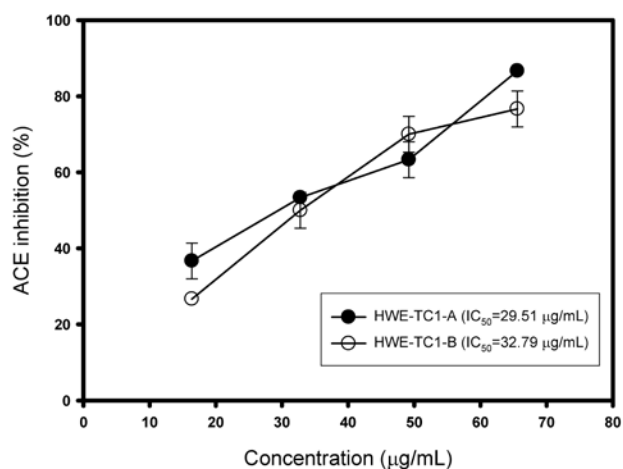
**Figure 2.** Effects of ethanolic extracts (200  $\mu$ g) of two portions (S, shoots; R, roots) of four tissue culture (TC) of *Vitis thunbergii* var. *taiwaniana* on 20 mU ACE inhibition by continuous spectrophotometric methods. (A) The decreased absorbance at 345 nm ( $\Delta A_{\text{sample}} - \Delta A_{\text{blank}}$ ) were recorded during 1.5 min at room temperature and expressed as  $\Delta A/\text{min}$ . (B) The ACE inhibition (%) was calculated according to the equation of  $[1 - (\Delta A_{\text{sample}}/\text{min} \div \Delta A_{\text{blank}}/\text{min})] \times 100\%$ .



**Figure 3.** Effects of different concentrations of ethanolic extracts of whole plantlets of four tissue culture (TC) of *Vitis thunbergii* var. *taiwaniana* on 20 mU ACE inhibition by continuous spectrophotometric methods. The ACE inhibition (%) was calculated according to the equation of  $[1 - (\Delta A_{\text{sample}}/\text{min} \div \Delta A_{\text{blank}}/\text{min})] \times 100\%$ . The 50% inhibition ( $IC_{50}$ ) of ACE activity was calculated as the concentrations of samples that inhibited 50% of ACE activity under these conditions.

concentrations of HWE-TC1-A and HWE-TC1-B on ACE inhibitions. It was found that HWE from whole plantlets of VTT-TC exhibited dose-dependently ACE inhibitory activities and the  $IC_{50}$  of ACE inhibitory activity was 29.51 and 32.79  $\mu\text{g/mL}$ , respectively, for HWE-TC1-A and HWE-TC1-B. It was found that HWE of two cultured methods from whole plantlets of TC1 for ACE inhibition were closely and three-folds better than that of EE (Figure 3).

Some reports had pointed that ACE inhibitory candidates *in vitro* might not have the antihypertensive effects on SHR *in vivo* (Fujita et al., 2000; Sato et al., 2002). Therefore, the EE-TC1-R was orally administered to SHR (20 mg/Kg of SHR) once, and the  $\Delta\text{BP}$  was measured four times at different time intervals within 24 h for short-term effects (Table 1). It was found that EE-TC1-R at dose of 20 mg/Kg of SHR could lower the SBP and DBP of SHR within 24-h. For changes of SBP ( $\Delta\text{SBP}$ ), there were 14.1, 16.3, 9.1, and 10.9 mmHg reductions, respectively, for the 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup>, and 24<sup>th</sup>-h after being orally administered. For changes of DBP ( $\Delta\text{DBP}$ ), there were 11.1, 17.7, 11.9, and 6.7 mmHg reductions, respectively, for the 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup>, and 24<sup>th</sup>-h after being orally administered. It was noted that the SBP reductions could over 24-h by a single oral administration of EE-TC1-R. Figure 5 showed the effects of EE-TC1-R (30 mg/Kg of SHR) on the changes of SBP and DBP of SHR

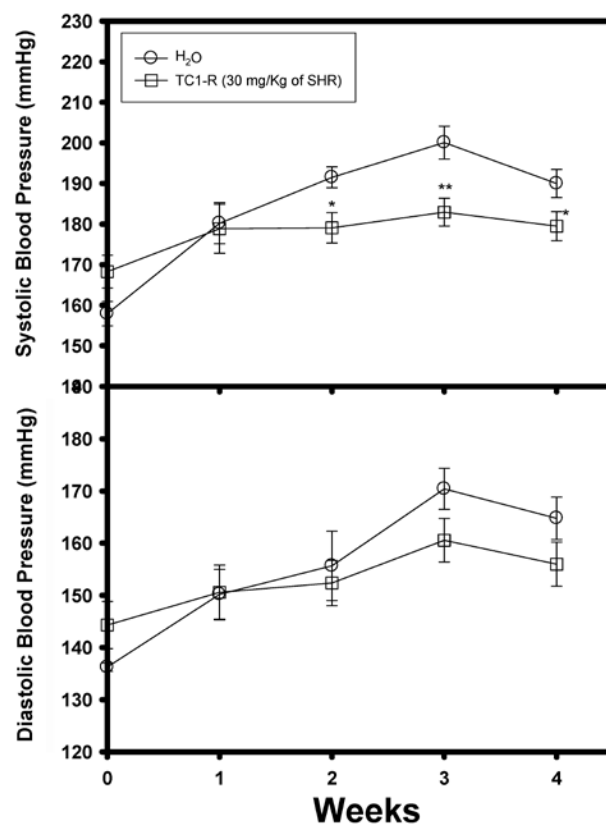


**Figure 4.** Effects of hot-water extracts (HWE) of dried whole plantlets of tissue culture of *Vitis thunbergii* var. *taiwaniana* (TC1, treated with IAA plant regulators) on 20 mU ACE inhibition by continuous spectrophotometric methods. TC1-A represented the cultured method of replacing the old culture medium after 30-days with new culture medium, and then for another 60 days; TC1-B represented the culture method of directly adding the new culture medium onto the old culture medium after 30-days, and then for another 60 days. The dried whole plant was extracted twice by 100°C hot water in the ratio of 1/10 (w/v) for 30 min. After being filtered, the filtrates were collected and then lyophilized as HWE. The ACE inhibition (%) was calculated according to the equation of  $[1 - (\Delta A_{\text{sample}} / \min \div \Delta A_{\text{blank}} / \min)] \times 100\%$ . The 50% inhibition ( $IC_{50}$ ) of ACE activity was calculated as the concentrations of samples that inhibited 50% of ACE activity under these conditions.

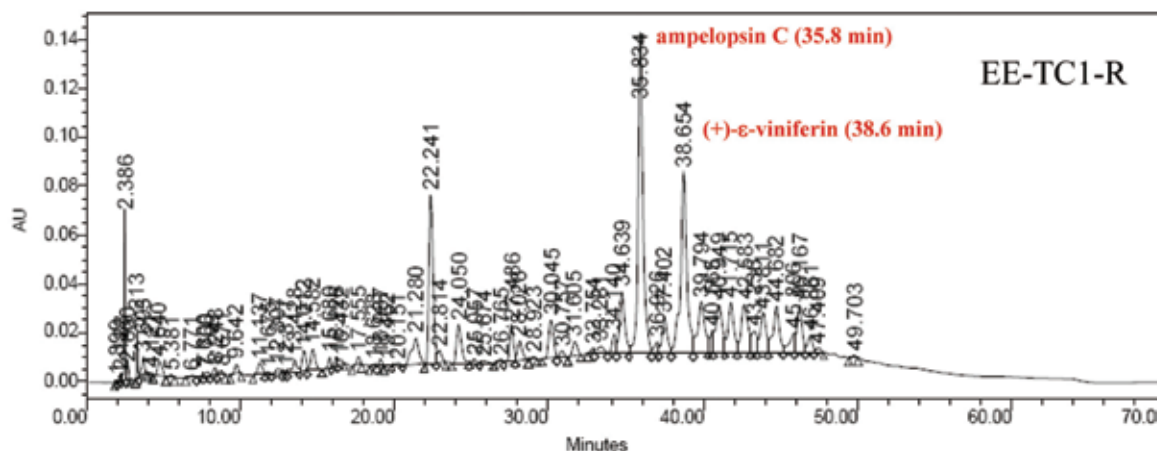
**Table 1.** Effects of ethanolic root extracts of tissue culture of *Vitis thunbergii* var. *taiwaniana* (TC1-R, treated with IAA plant growth regulators) on the changes of blood pressure of spontaneously hypertensive rats (20 mg/Kg of SHR) during 24-h by a single oral administration.

Time after treatment (h)	TC1-R extracts (20 mg/Kg of SHR)	
	$\Delta\text{SBP}$ (mmHg)*	$\Delta\text{DBP}$ (mmHg)*
2	$-14.1 \pm 2.9$	$-11.1 \pm 4.6$
4	$-16.3 \pm 6.8$	$-17.7 \pm 5.7$
6	$-9.1 \pm 5.3$	$-11.9 \pm 4.4$
24	$-10.9 \pm 7.0$	$-6.7 \pm 6.0$

\*The lowering effects in blood pressure changes ( $\Delta\text{BP}$ ) were calculated as  $\text{BP}_{\text{sample}} - \text{BP}_{\text{blank}}$  at the same treatment time after oral administration ( $n=6$ ).



**Figure 5.** Effects of ethanolic extracts of root portions of tissue culture (TC) of *Vitis thunbergii* var. *taiwaniana* (TC1-R, treated with IAA plant regulators) on systolic blood pressure and diastolic blood pressure of spontaneously hypertensive rats (SHR,  $n=6$ ) by oral administration (30 mg/Kg of SHR, dissolved in 0.5-mL distilled water) once a day for four-weeks and 0.5-mL distilled water was used for a control experiment. A difference was considered statistically significant between control and treated group when  $P < 0.05$  (\*) or  $P < 0.01$  (\*\*).



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## 小葉葡萄組織培養苗之抽取物降血壓功效之研究

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小葉葡萄 (*Vitis thunbergii* var. *taiwaniana*) 為台灣特有之變種。本研究以不同植物生長素進行 90 天培養之小葉葡萄組織培養苗為材料 (TC1, IAA; TC2, IBA; TC3, 對照組; TC4, NAA)，發現其莖葉部 (S) 與根部 (R) 之酒精抽取物具有抑制血管收縮素轉化酶抑制活性，但以根部效果較佳。四種組織培養之小葉葡萄苗之酒精抽取物 50% 抑制血管收縮素轉化酶活性 (IC<sub>50</sub>) 分別為 TC1 (98.67 µg/mL) > TC3 (99.04 µg/mL) > TC4 (102.46 µg/mL) > TC2 (132.05 µg/mL)。以 TC1 之熱水抽取物為材料，發現 30 天後倒掉舊培養基，加入新培養基培養 60 天 (HWE-TC1-A)，或是添加新培養基於舊培養基中共同培養 60 天 (HWE-TC1-B)，皆能有效抑制血管收縮素轉化酶活性，IC<sub>50</sub> 分別為 29.51 µg/mL (HWE-TC1-A) 與 32.79 µg/mL (HWE-TC1-B)。先以組織培養苗根部酒精抽取物 (TC1-R) 進行高血壓鼠餵食一次 (20 mg/Kg)，觀察 24 小時血壓變化之試驗。結果顯示，組織培養苗根部醇類抽取物 (TC1-R) 於第四小時達到最低的血壓，其收縮壓與舒張壓分別降低 16.9 毫米汞柱與 17.7 毫米汞柱，24 小時後收縮壓還有 11 毫米汞柱的降幅。以組織培養苗根部酒精抽取物 (TC1-R) 進行高血壓鼠每天餵食一次 (30 mg/Kg)，觀察四週血壓變化。結果顯示，組織培養苗根部酒精抽取物 (TC1-R)，餵食後的收縮壓於第二、三與四週，分別下降 12.45, 17.13 與 10.5 毫米汞柱，並與對照組呈現顯著性差異 ( $P < 0.05$  或  $P < 0.01$ )；但舒張壓並沒有明顯差異。小葉葡萄酒酒精抽取物與熱水抽取物未來可以開發為調節血壓之保健品。

**關鍵詞：**小葉葡萄；組織培養苗；高血壓鼠；血壓。