ASIAN JOURNAL OF PHARMACOGNOSY
Asian J. Pharmacogn 2(4):19-24

eISSN-0128-1119

Panax stipuleanatus H. T. Tsai et K. M. Feng Extracts Induced the Relaxation of Isolated Smooth Muscles and Elevated eNOS phosphorylation

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ABSTRACT

This study aimed to explore the effect of *Panax stipuleanatus* H. Tsai et K.M. Feng on 3 types of isolated smooth muscles: trachea, bladder, and corpus cavernosum. Five extracts of *P. stipuleanatus* including total extract (PST), n-butanol extract (PSBt), n-hexan extract (PSnH), dichloromethane extract (PSDcM), and saponin riched extract were used for smooth muscle relaxation and eNOS phosphorylation tests. The results showed that the total extract (PST) and the n-butanol extract of *P. stipuleanatus* (PSBt) induced the relaxation of trachea, bladder, and corpus cavernosum while PSnH and PSDcM did not show similar effect. Moreover, performing experiment for eNOS phosphorylation in human umbilical vein endothelial cells (HUVEC) we showed that the PST and saponin riched extract elevated the nitric oxide production (NO) as well as the eNOS phosphorylation expression.

Keywords: Panax stipuleanatus, bladder, corpus cavernosum, isolated smooth muscles, trachea, eNOS phosphorylation

INTRODUCTION

Panax stipuleanatus (H. T.Tsai et K. M. Feng) belongs to the genius *Panax* L., naturally growing in high mountain named Hoang Lien Son in Vietnam (Nguyen Van Tap, 2006). A rich content of saponin and ginsenoside is found in stems and leaf of *Panax stipuleanatus.* Ginsenoside in *Panax* L. is well-known to modulate the smooth muscle tone and induce the NO production (Chen, 1995; Tamaoki, 2000). It could be explained by the interation between endothelium and smooth muscle layer on vascular bed through calcium signaling, NO production by eNOS phosphorylation (Kolluru, 2010; Förstermann, 2012). Saponin may exert its beneficial effect on vascular tone by similar pathway as ginsenoside. Therefore, this study was designed to evaluate the effect of different extracts of *Panax stipuleanatus* H. T. Tsai et K. M. Feng on the relaxation of three isolated smooth muscles and the eNOS phosphorylation in endothelial cells.

MATERIALS AND METHODS

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Plant collection: Stem of *Panax stipuleanatus* H.Tsai et K.M.Feng was collected at Hoang Su Phi, Ha Giang province and was classified by National Institute of Medicinal Materials. The herbarium was provided by Assoc. Prof. Dr. Do Thi Ha from Department of Plant Chemistry, National Institute of Medicinal Materials. Five extracts were used as total extract (PST), n-hexan extract (PSnH), dichloromethane extract (PS DCM), n-butanol extract (PSBt) and saponin extract (PS27).

Smooth muscle relaxation test: Wistar rats with body weigh from 150 to 250 g were used for trachea, bladder, and corpus cavernosum isolation. These muscle tissues were cut into rings and incubated with Tyrode buffer containing mM concentration of NaCl: 139.2; KCl: 2.7; $CaCl_2$ 1.8; $MgCl_2$ 0.49; $NaHCO_3$ 11.3; Na_2PO_4 0.4; glucose: 5.5 at 37°C before fixing in two stainless steel stirrup hooks of the Powerlab system. Acetylcholine (0.15 ml; 10^{-5}M) was used as relaxation stimulator. Adding 0.15 ml of 4 concentrations (0.5; 1; 2 and 5 mg/ml) of each *Panax stipuleanatus* extract into the organ chamber. Between each running, the new buffer was replaced. Smooth muscle relaxation signal was recorded using digital acquisition system (AD Instruments, New Zealand).

Cell culture: Human umbilical vein endothelial cells were cultured at 37°C, CO_2 5%, in medium containing 10% of FBS, penicillin (100 units/ml) and streptomycin (100 µg/ml). The number of cell reach 80-90% confluence before using for experiments.

NO production measurement: NO production is indirectly quantified by NO_2^- (nitrit) amount using Griess chemical test (Enzo Life Sciences, Plymouth Meeting, PA). HUVEC was treated with 30 µg/ml herbal extract for 24h, then incubated with nitrite reductase for 1 hour at 37°C to convert nitrate into nitrit. Added 100 µL Griess chemical, then measure optical density at wavelength of 540 nm.

Expression of eNOS phosphorylation analysis: HUVEC was lysed by buffer containing NaCl 120 mM, Tris 40 mM (pH 8), NP40 0.1% on ice for 30 minutes, then centrifuged at 13000 rpm for 20 minutes. Protein concentration was measured by Bradford method (Bradford, 1976). Protein was incubated at 95°C in 5 minutes and performed electrophoresis in SDS-polyacrylamide 10%. Phosphorylated eNOS and beta-actin antibodies were used to detect the protein expression (Thermofisher Scientific, Waltham, Massachusetts, USA).

Statistical analysis: Data were analysed using ANOVA and Tukey's posthoc tests for more than 3 group comparisons. The data were expressed as mean ± SE and $P \leq 0.05$ was considered as significant difference.

Ethic approval: This study followed the ethic guideline on Ministry of Health and approved by the ethic committee of School of Medicine and Pharmacy, code IRB-VN01016.

RESULTS

Smooth muscle relaxation induced by herbal extracts: The effect of 4 extracts of *Panax stipuleanatus* including PST, PSnH, PS DCM and PSBt on muscle relaxation was tested. The results ARE showed in Table 1. PST and PSBt clearly induced the relaxation in trachea and bladder muscles in dose dependent manner. The effect started with 1 mg/ml and strong response was observed from 2 mg/ml. Other extracts (PSnH and PS DCM) were almost showed no clear effect at all concentrations.

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Table 1. Effect of *Panax stipuleanatus* extracts on smooth musle relaxation

Extracts	Concentration	Trachea	Bladder	Corpus cavernosum
PST	0.5 mg/ml	±	-	-
	1 mg/ml	+	+	-
	2mg/ml	++	+	+
	5mg/ml	++	++	+
PSBt	0.5 mg/ml	-	-	-
	1 mg/ml	-	+	-
	2mg/ml	+	++	+
	5mg/ml	++	++	+
PSnH	0.5 mg/ml	-	±	±
	1 mg/ml	-	±	±
	2mg/ml	+	±	±
	5mg/ml	+	±	±
PS DCM	0.5 mg/ml	±	±	±
	1 mg/ml	±	±	±
	2mg/ml	±	±	±
	5mg/ml	±	±	±

(-) no relaxation; (±) not identified; (+) weak relaxation; (++) strong relaxation

NO production: The relaxation of smooth muscle could be explained by NO release from endothelium. Therefore, in this study NO production was recorded in HUVEC after incubation with 30 µg/ml of the extracts from *Panax stipuleanatus* for 2 hours. The result was expressed in Figure 1.

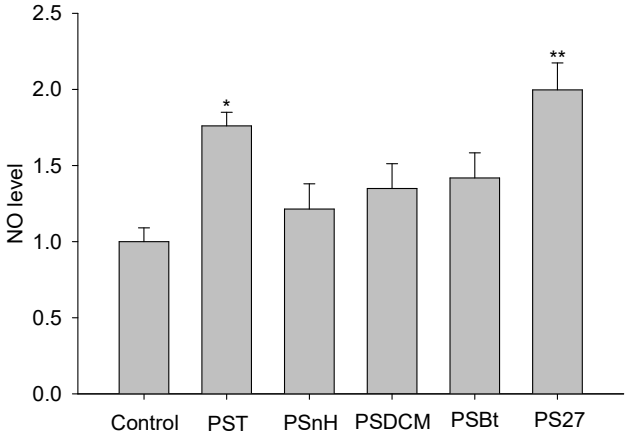

Figure 1. The effect of *Panax stipuleanatus* extracts (30 µg/ml) on NO production in HUVEC. The data were expressed as mean±SE; n=5; *: p<0.01 and **: p<0.001 compared with control group

HUVEC incubated with PST and PS27 extracts elevated NO production 1.76±0.07 an2.00±0.05 times higher than control group ($p < 0.05$). While PSnH, PS_DCM and PSBt did not show any effect on NO production increase in HUVEC ($p>0.05$). Whether this NO production

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increase related to eNOS phosphorylation or not, the protein expression of eNOS phosphorylation was analysed in Figure 2.

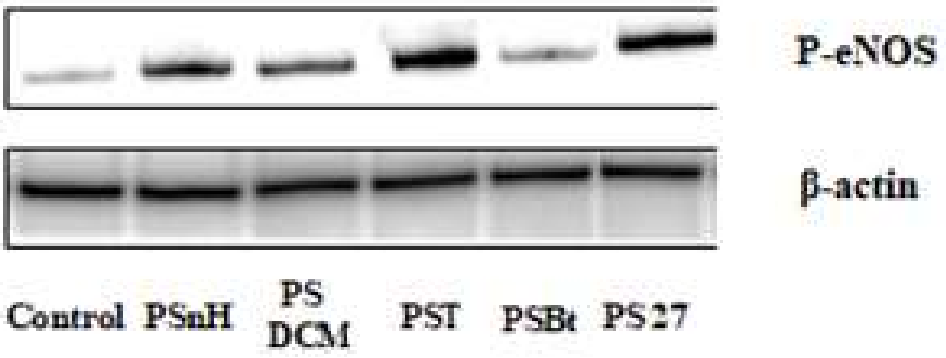

Figure 2. The effect of *Panax stipuleanatus* extracts on protein expression of eNOS phosphorylation in HUVEC after 2 hours incubation with 30 µg/ml plant extracts.

Western Blotting analysis of eNOS phosphorylation (Figure 2) was in line with NO production result. The PST and PS27 extracts dominantly expressed the phosphorylated eNOS in comparison with control group.

DISCUSSION

Panax L. is a valuable medicinal plant with various species for example *Panax ginseng, Panax notoginseng*, and *Panax vietnamensis.* Chemical analysis of these plants showed plenty of saponin compound with dammarane and oleanane structures (Yang et al., 2014) which are known to induce smooth muscle cell and increase NO production (Chen et al., 1995). In this study, total extract (PST) and n-butanol extract (PS Bt) elevated acetylcholine-induced smooth muscle relaxation while other extracts did not. Jang et al. (2012) found that saponin isolated from *P. ginseng* stimulated bladder and urinary smooth muscles both *in vitro* and *in vivo*. N-butanol extract in our study was expected to contain more saponin than other extracts including total extract.

However, the smooth muscle relaxation induced by these two groups was similar. Tran Cong Luan et al. (2009) showed that extract of *Panax* L. by 70% ethanol obtained saponine, triterpene, reduced saccharide and lipid. The effect of total extract on smooth muscle relaxation could be explained by saponin and non-saponin compounds. The effect appeared from extract concentration of 1 mg/ml by dose dependent manner. In line with our study, Choi et al. (1998) observed rabbit smooth muscle relaxation induced by 1 mg/ml of *P. ginseng* extract and reached the maximum effect at 40 mg/ml by dose dependence. Smooth muscle induction could be linked to endothelial nitric oxide signaling (Tamaoki et al., 2000). Kim et al. (2007) showed that 500 µg/ml of *P. ginseng* extract elevated NO production in HUVEC and activated eNOS phosphorylation time dependently ($p<0.01$).

In our study, similar results were reported with butanol (PS Bt) and total (PST) extracts with lower concentration range of 30 µg/ml. This could be due to the variable in bioactive compounds between *P. ginseng* and our *P. stipuleanatus* as well as difference in protocol, method and experiment system of these studies. Ahn et al. (2013) proposed that the extract containing ginsenoside protopanaxatriol of *P. ginseng* release higher NO amount than the total extract of this medicinal plant. In our study, both total and saponin riched extracts named PS27 expressed clearly effect on NO production as well as eNOS phosphorylation. However, n-butanol extract was

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considered containing high saponin concentration showed no significant effect. In the future, we should identify the pure compound(s) of this butanol extract.

CONCLUSION

Total extract (PST) and n-Butanol extract (PS Bt) of *P. stipuleanatus* induced the relaxation of trachea, bladder and corpus cavernosum smooth muscles stimulated by acetylcholine from concentration of 1 mg/ml by dose dependent manner. The total extract (PST) and saponin extract (PS 27) with concentration of 30 µg/ml increased NO release and elevated eNOS phosphorylation in HUVEC.

ACKNOWLEDGEMENT

We acknowledhe financial support from National Project *KHCN-TB.07C/13-18*, North West development program. We thanks to Dr. Do Thi Ha for providing the extract of *Panax stipuleanatus.*

DECLARATION OF CONFLICT OF INTEREST

None of conflict of interest

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Asian J. Pharmacogn 2(4):19-24

eISSN-0128-1119

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