Quality control, cytotoxicity and inhibitory effect on nitric oxide production of Pathavi Apo Vayo formulary extract

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Abstract

Pathavi Apo Vayo formulary (PAV) is one of Thai traditional medicine that consisted of 21 herbal plant powders and has been used in the treatment of diabetic patients. An uncertainty on efficacy, safety and variation in quality of the products are important factors for herbal medicine usage. Therefore, the aim of this study was to establish quality control of PAV. The effects of PAV on cell viability and nitric oxide production in the different glucose containing media were also investigated. The 21 herbal plants and PAV were extracted using 50% ethanol. The possible chemical fingerprint and chemical markers of these plants were identified by using TLC and HPLC comparing with PAV. Normal glucose medium (NGM) and high glucose medium (HGM) were used to culture RAW264.7 cells in this study. The cytotoxicity and nitric oxide (NO) production were studied by using MTT assay and Griess reaction, respectively. The R_f values of 21 plant extracts on TLC fingerprint were similar with the R_f values of PAV extract. Gallic acid was the major content in PAV and plant extracts using qualitative HPLC. PAV extract showed low cytotoxicity (IC_{50} of 1,139.48 ± 36.22 and 1,134.69 ± 13.55 µg/mL, respectively) and could inhibit NO production (IC_{50} of 128.49 ± 4.68 and 127.57 ± 14.02 µg/mL, respectively) in NGM and HGM without a statistical difference. In conclusion, the chemical composition of plant extracts was remaining content in PAV extract and gallic acid can be used as a chemical marker. This PAV extract had low cytotoxicity and inhibited NO production.

Keywords: Pathavi Apo Vayo formulary, Quality control, Medicinal plants, High glucose, Cytotoxicity

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Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia which is an increasing of blood glucose levels due to absolute or relative insulin production deficiency. Nitric oxide (NO), a critical inflammatory mediator, plays an important role in the inflammation of type II diabetes mellitus (T2DM) [1]. The synthesis of NO is catalyzed by inducible nitric oxides synthase (iNOS), endothelial cells nitric oxide synthase (eNOS) and neuronal nitric oxides synthase (nNOS). iNOS is induced by inflammatory cytokines such as interleukin-1 (IL-1) or tumor necrosis factor-alpha (TNF-α) in macrophages and many other cell types. The increased levels of CD163, CD154 and TNF-α indicate that an inflammatory process occurs in skeletal muscle of T2DM patients. This may contribute to iNOS induction, muscle damage and insulin resistance [2]. The excessive NO increased the inflammatory marker levels in T2DM, which is activated by the PPARγ/eNOS pathway [3]. In addition, condition of insulin resistance increased NO levels in both diabetic and prediabetic patients [4].

In Thailand, diabetes is rising trend in the hospitalization over the last decade. Due to high cost of medical care with long-term treatment, diabetes is considered to be an economic burden and national public health problem [5]. According to the failure of conventional hypoglycemic drugs to satisfactorily maintain normal glucose levels and some serious side effects [6], significant interest in an alternative and complementary medicine especially herbal preparations has been maintained [7]. More than 400 plants are available for the treatment of diabetes. Despite the fact that there are many herbal drugs available for treating diabetes, only a small number of these plants have undergone scientific and medical evaluation to assess their efficacy [8].

Quality control is therefore one of the major problems in the rational use of herbal medicines. With many herbal medicines, the active ingredient is unknow. Genetic and environmental factors may influence the synthesis of plant secondary metabolites. Frequently a marker compound is selected and this is used to determine the quality of the herbal medicine [9]. Thin-layer chromatography (TLC) is the method of choice for identifying a wide variety of substance classes because it is simple, rapid and inexpensive method. High-performance liquid chromatography (HPLC) is a popular method for analysis of herbal medicines because it is easy to use and is not limited by the volatility or stability of the sample compounds. In general, HPLC can be used to analyze almost all the compounds in the herbal medicines. For example, preliminary phytochemical studies on fruit of *Emblica officinalis*, the TLC and high performance thin-layer chromatography was performed and the developed plates were visualized in UV 254 nm, 366 nm [10]. *Emblica officinalis* and *Terminalia chebula* extracts containing phenolic compounds were determined by HPLC technique [11, 12]. Thai traditional herbal formula, Sattagavata, Mathurameha, and Tubpikarn were prescribed to diabetic patients for a long time [13]. TLC was also developed for the quality control of three anti-diabetic Thai herbal formulae extracts, showed the presence of rutin and chlorogenic acid which are

powerful antioxidants and are beneficial to diabetic conditions [13].

Pathavi Apo Vayo formulary (PAV) is one of Thai traditional medicine that is from Wat Phra Chetuphon's inscriptions. This formulary consists of 21 herbs and has been used for the treatment of diabetes. The screening basic methods could identify the presence of composite plant in PAV. A chromatogram fingerprint of PAV would facilitate the quality control assessments of composite plants in PAV to ensure the reproducibility of ingredients and the consistent efficacy of the product. Accordingly, the aim of this study was to identify possible chemical fingerprint and chemical markers for evaluating the quality of medicinal plants in PAV by using TLC and HPLC. Moreover, the effects of PAV ethanolic extract on the cell viability and nitric oxide production of macrophage were investigated in high glucose medium (HGM) compared with normal glucose medium (NGM) that related to inflammation and hyperglycemic condition in diabetes.

Material and Methods

1. Chemicals

Ethanol (RCI Labscan, Thailand); methanol (RCI Labscan, Thailand); acetone (RCI Labscan, Thailand); CH_2Cl_2 (RCI Labscan, Thailand); D-glucose (Kemaus, Australia); L-glutamine (Himedia, India); *Escherichia coli* LPS (Sigma, USA); DMEM (Gibco, USA); DMSO (Fisher Scientific, UK); FBS (Gibco, Germany) and MTT (Molecular probes, USA) were obtained from local distributors. All other chemicals used were of analytical grade and were obtained commercially.

2. Preparation of Pathavi Apo Vayo formulary and the plant extracts

PAV was the formulary that contained 1 part each of 21 herbal plants. The PAV and each plant ingredients (Table 1) were purchased from Bansamunpaiosot, Bangkok, Thailand. PAV and each plant ingredients of PAV was macerated in 50% ethanol for 7 days. After filtration, the remain debris from extraction was repeated by maceration in 50% ethanol for 7 days. The filtrates from both extractions were combined. The solvent was evaporated and concentrated by using a vacuum evaporator (Buchi, Thailand). The temperature of water bath and coolant were set at 50 ºC and 10 ºC, respectively. The percentage yield for each sample is shown in Table 2.

3. Quality control of raw material and formulary extracts

3.1 Thin layer chromatography fingerprint

For efficacy in clinical practice, the quality control of PAV is continually improving. The ethanolic extracts of 21 plants and PAV were prepared. The experiment was performed on TLC plate, silica gel 60 F254 (Merck, Germany) size 5 x 7 cm. One gram of each plant extracts and PAV were dissolved in 3 mL methanol, then were spotted to the 3 plates. *Cyperus alternifolius* L. (1), *Acorus calamus* L. (2), *Plumbago indica* L. (3), *Ocimum tenuiflorum L.* (4), *Terminalia chebula* Retz. (5), *Zingiber officinale* Roscoe (6), *Coriandrum sativum* L. (7) and PAV (P) were spotted onto the baseline of the plate no. 1 using a capillary tube. *Vitex trifolia* L. (8), *Fagraea fragrans* Roxb. (9), *Cinnamomum porrectum* (Roxb.) Kosterm (10),

Table 1 Plant component of Pathavi Apo Vayo and Pathavi Apo Vayo formulary

Samples	Botanical name and Formulary	Family	Parts used
	*Cyperus alternifolius* L.	Cyperaceae	Stem
	*Acorus calamus* L.	Acoraceae	Rhizome
	*Plumbago indica* L.	Plumbaginaceae	Root, stem
	*Ocimum tenuiflorum* L.	Lamiaceae	Leaf, stem
	*Terminalia chebula* Retz.	Combretaceae	Fruit
	*Zingiber officinale* Roscoe	Zingiberaceae	Rhizome
	*Coriandrum sativum* L.	Apiaceae	Fruit
	*Vitex trifolia* L.	Lamiaceae	Leaf
	*Fagraea fragrans* Roxb.	Gentianaceae	Leaf
	*Cyperus rotundus* L.	Cyperaceae	Rhizome
	*Holarrhena pubescens* Wall. Ex G. Don	Apocynaceae	Stem bark, wood

Table 1 Plant component of Pathavi Apo Vayo and Pathavi Apo Vayo formulary (continued)

Samples	Botanical name and Formulary	Family	Parts used
	*Aniseia martinicensis (Jacq.)* Choisy. Share.	Convolvulaceae	Climber
	*Kaempferia galanga* L.	Zingiberaceae	Rhizome
	*Allium sativum* L.	Amaryllidaceae	Rhizome
	*Lagenaria siceraria* (Molina) Standl.	Cucurbitaceae	Climber, Leaf
	*Terminalia bellirica* (Gaertn.) Roxb.	Combretaceae	Fruit
	*Phyllanthus emblica* L.	Phyllanthaceae	Fruit
	*Piper retrofractum* Vahl	Piperaceae	Fruit
	*Piper nigrum* L.	Piperaceae	Seed
	*Azadirachta indica* A. Juss.	Meliaceae	Leaf
	Pathavi Apo Vayo	-	-

Table 2 Percentage of yield and chemical marker content of PAV and plant extracts

Botanical name and Formulary	% Yield of extract	Gallic acid (µg/10 mg extract)	Vanillic acid (µg/10 mg extract)	Caffeic acid (µg/10 mg extract)
Cyperus alternifolius L.	5.43	NF	3.84 ± 0.13	3.63 ± 0.04
Acorus calamus L.	8.88	2.33 ± 0.06	1.83 ± 0.40	NF
Plumbago indica L.	14.94	8.38 ± 0.04	3.30 ± 0.01	NF
*Ocimum tenuiflo*rum L.	7.45	4.55 ± 0.45	4.60 ± 1.83	3.89 ± 0.10
*Terminalia chebul*a Retz.	10.30	98.79 ± 1.90	7.22 ± 0.92	NF
Zingiber officinale Roscoe	11.12	4.45 ± 0.40	0.29 ± 0.00	NF
Coriandrum sativum L.	5.40	0.43 ± 0.02	2.98 ± 0.01	4.71 ± 0.11
Vitex trifolia L.	15.60	NF	NF	NF
Fagraea fragrans Roxb.	19.69	4.47 ± 0.45	NF	NF
*Cinnamomum porrectu*m (Roxb.) Kosterm	3.34	NF	6.53 ± 0.47	NF
Cyperus rotundus L.	21.70	4.78 ± 0.15	NF	NF
Holarrhena pubescens Wall. Ex G. Don	10.13	5.99 ± 0.10	2.91 ± 0.04	NF
Aniseia martinicensis (Jacq.) Choisy. Share.	4.50	20.39 ± 5.25	14.37 ± 1.25	NF
Kaempferia galanga L.	9.93	14.15 ± 1.93	2.35 ± 0.21	NF
Allium sativum L.	61.38	NF	2.18 ± 0.01	NF
Lagenaria siceraria (Molina) Standl.	11.75	2.60 ± 0.23	NF	NF
Terminalia bellirica (Gaertn.) Roxb.	8.94	84.78 ± 0.16	NF	NF
Phyllanthus emblica L.	12.94	468.28 ± 0.36	NF	NF
Piper retrofractum Vahl	6.94	4.34 ± 0.37	NF	4.34 ± 0.37
Piper nigrum L.	5.66	NF	NF	NF
Azadirachta indica A. Juss.	7.06	4.02 ± 0.00	NF	NF
Pathavi Apo Vayo	13.87	28.94 ± 0.07	NF	3.73 ± 0.05

Note: NF is not found.

Cyperus rotundus L. (11), *Holarrhena pubescens* Wall. Ex G. Don (12), *Aniseia martinicensis* (Jacq.) Choisy. Share. (13), *Kaempferia galanga* L. (14) and PAV (P) were spotted onto the plate no. 2. *Allium sativum* L. (15), *Lagenaria siceraria* (Molina) Standl. (16), *Terminalia bellirica* (Gaertn.) Roxb. (17), *Phyllanthus emblica* L. (18), *Piper retrofractum* Vahl (19), *Piper nigrum* L. (20), *Azadirachta indica* A. Juss. (21) and PAV (P) were spotted onto the plate no. 3. The plates were developed by TLC tank and a mobile phase was acetone : CH_2Cl_2 (1:9). The chromatography was detected under the ultra violet (UV) light chamber at 254 and 365 nm. A vanillin-sulfuric acid reagent was sprayed on a chromatographic plate and then photographed. The retention factor (R_f) values were calculated as described below:

R_f = Distance traveled by solute / Distance traveled by solvent front

3.2 High performance liquid chromatography (HPLC)

Reversed-phase C18 column (4.6 × 150 mm, 5 µm) (SORBAX, Agilent, USA) was used in HPLC system (Alltech, USA). Mobile phase system was 1% acetic acid: ACN in ratio of 97:7 with flow rate 1.0 mL/min and detected at UV 277 nm. The PAV (10 mg) was dissolved in acetonitrile (1 mL) and then filtrated using 0.45 µm syringe filter. Standard gallic acid, vanillic acid and caffeic acid were diluted to various concentration for calibration curve. Extracts or gallic acid, vanillic acid and caffeic acid (20 µL) was injected. The running time was 35 min. The areas under the curve of each extract were compared with calibration curve of gallic acid, vanillic acid and caffeic acid.

4. Determination of cytotoxicity

Macrophages (RAW264.7 cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with glucose medium (2 mM L-glutamine, 5.5 mM D-glucose for NGM and 2 mM L-glutamine, 15 mM D–glucose for HGM), 1% antibiotic-antimycotic solution and 10% fetal bovine serum that modified method of Torres- Castro et al. [14] and incubated at 37 ºC, 5% CO_2 for 1 week. Cells (2×10^5 cells/well) were seeded into 96-well plate and incubated at 37 ºC, 5% CO_2 for 24 h. The PAV was dissolved in dimethyl sulfoxide (DMSO) for 100 mg/mL and sterile by using 0.45 µm syringe filter as a stock solution. PAV was diluted with culture media to various concentration and then added (100 µL) into the cells. After incubation for 24 h, the cell viability was analyzed by using MTT assay [15]. Fifty microliter of 1 mM MTT was added to each well and then incubated at 37 ºC for 3 h. The formazan was dissolved in DMSO and then was measured at 570 nm using a microplate reader (Infinite M200 Pro, TECAN, Austria). The cell viability was calculated as described below and then expressed a cytotoxicity as 50% inhibitory concentration (IC_{50}).

Cell viability (%) = 100 × (absorbance of treatment / absorbance of control)

5. Determination of nitric oxide production

RAW264.7 (2×10^5 cells/well) were cultured into 96-well plate by using NGM and HGM, and then incubated at 37°C, 5% CO_2 for 24 h. Extracts were diluted with glucose culture media in the absence or presence of 10 µg/mL lipopolysaccharides (LPS) and then added (100 µL)

into the cells. After incubation for 24 h, 50 µL of culture media was pipetted to mix with 50 µL of Griess reagent (1% Sulfanilamide dissolved in 5% phosphoric acid:0.01% N-1-napthylethylenediamine dihydrochloride in a ratio of 1:1). The absorbance was measured at 540 nm. The results were compared between with or without LPS induction and then calculated for nitric oxide inhibition. The results were expressed as IC_{50}.

6. Statistical analysis

The experimental results were performed in triplicate and represented in mean ± S.D. Data was analysed by using SPSS version 16.0. Independent t-test was used in the between group comparison of normal glucose medium (NGM) and high glucose medium (HGM). Comparison of differences within groups (Pathavi Apo Vayo formulary concentration) was performed by using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc test. Differences were considered statistically significant at $p<0.05$.

Results and Discussion

1. Quality control of raw material and formulary extracts

PAV formulary was consisted of 21 plants as shown in Table 1. The combination of different parts of plants might affect to the quantity of phytochemical content and the biological activities due to the distinctive chemical profiles [16]. The changing of various physical, chemical, and geographical aspects may contribute to the quality of herbal materials [17]. Therefore, the major chemical composition of raw materials of PAV was determined for quality control.

TLC and HPLC were used to identify possible chemical markers for evaluating the quality of the crude drug such as Pogostemoni herba which is a component of Kampo medicines [18]. Therefore, TLC chromatogram was used to separate and identify marker compounds of each plant remaining content in the PAV extract in this study. All plant extracts under UV light at 254 nm were shown as a dark spot as well as PAV extract (Figure 1A). The detection TLC spot with UV light at 365 nm showed the same position in 12 plant extracts (Figure 1B). Chromatographic fingerprints of PAV and 21 plant extracts obtained in visualization with vanillin-sulfuric reagent were investigated in Figure 1C. Each band of all plant extracts (except *A. sativum* extract) was found in the similar position of the bands from PAV extract. PAV had different compounds because of equal ratio combination of 21 plant powders depending on types of phytochemical content in each composite plant. These shown bands on the TLC plate were probably the main compounds of the plant extracts. High intensity and clearly bands might be the same compounds that found in many composite plants in PAV formulary and might serve as a potential herbal activity. The R_f values of 21 plant extracts were similar with some R_f values of PAV extract after determination by using TLC chromatogram (Table 3). Under UV light at 254 nm, 10 spots of PAV were obtained (R_f of 0.17, 0.22, 0.28, 0.44, 0.52, 0.58, 0.65, 0.72, 0.78 and 0.79) while 3 spots of PAV were obtained under UV light at 365 nm (R_f of 0.62, 0.67 and 0.80). Under visualization with vanillin-sulfuric reagent, 6 spots of PAV were obtained (R_f of 0.27, 0.35, 0.59, 0.70, 0.77 and 0.86). Therefore, these results confirmed that 21 plant extracts were remaining content in PAV extract.

Moreover, to determine standard compounds contained in 21 plant extracts and PAV extract, HPLC was used. The method validation of quantitative HLPC analysis was performed. The r-square data of linearity of gallic acid, vanillic acid and caffeic acid was 0.9995, 0.9998 and 1.0000 with the concentration range of 6.66-80.00, 7.73-80.80 and 6.73-80.80 ppm, respectively. The accuracy in the % recovery of these standard compounds was 98.45%, 97.73% and 99.63%, respectively. The precision in % relative standard deviation (%RSD) of these standard compounds was 1.70%, 1.27% and 0.50%, respectively. Gallic acid, caffeic acid and vanillic acid are the examples of common phenolic compound found in plants [19, 20]. Gallic acid is rich in many herbal plants and has progressively demonstrated robust antioxidative and anti-inflammatory, this compound can be a potential anti-hypoglycemic agent for DM [21]. Caffeic acid is a potent agent against diabetes that acts as an effective antioxidant in reducing serum glucose, lipid profile [22] and anti-inflammation [23]. Vanillic acid reduced diabetes and diabetic nephropathy in rats, these effects might be attributed to its powerful free radical scavenging property and down-regulation of inflammatory cytokines [20]. Therefore, gallic acid, caffeic acid and vanillic acid were used as a standard compound for analysis of PAV and plant extracts by using qualitative HPLC.

Interestingly, gallic acid was found higher than vanillic acid and caffeic acid in PAV and plant extracts (Table 2). PAV contained gallic acid and caffeic acid (28.94 ± 0.07 and 3.73 ± 0.05 µg/10 mg extract, respectively). The highest gallic acid content was found in *P. emblica* L. (468.28 ± 0.36 µg/10 mg extract) following with *T. chebula* and *T. bellirica* (Gaertn.) Roxb. (98.79 ± 1.90 and 84.78 ± 0.16 µg/10 mg extract, respectively) whereas

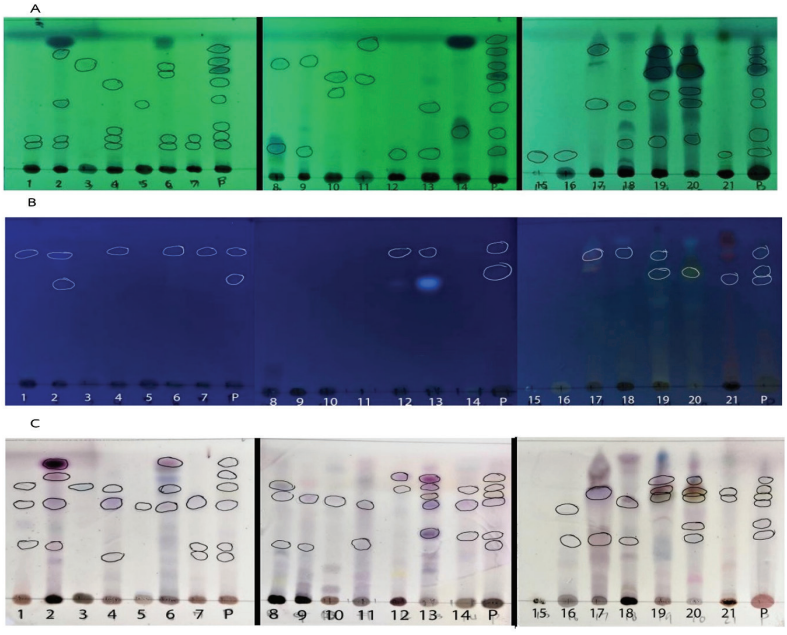

Figure 1 TLC fingerprints of plant and PAV extracts. A) 254 nm, B) 365 nm and C) vanillin-sulfuric acid reagent. Lane 1: *C. alternifolius* L., 2: *A. calamus* L., 3: *P. indica* L., 4: *O. tenuiflorum* L., 5: *T. chebula* Retz., 6: *Z. officinale* Roscoe, 7: *C. sativum* L., 8: *V. trifolia* L., 9: *F. fragrans* Roxb., 10: *C. porrectum* (Roxb.) Kosterm, 11: *C. rotundus* L., 12: *H. pubescens* Wall. Ex G. Don, 13: *A. martinicensis* (Jacq.) Choisy. Share., 14: *K. galanga* L., 15: *A. sativum* L., 16: *L. siceraria* (Molina) Standl., 17: *T. bellirica* (Gaertn.) Roxb., 18: *P. emblica* L., 19: *P. retrofractum* Vahl, 20: *P. nigrum* L., 21: *A. indica* A. Juss. and P: PAV.

Table 3 R_f values of PAV and 21 plant extracts on TLC

	R_f values																					
	P	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
UV 254	0.17	0.17	0.16		0.17		0.16	0.16	0.17	0.17			0.17	0.17		0.17	0.17					0.17
	0.22	0.22	0.22		0.22		0.22	0.22														
	0.28				0.27										0.28				0.28	0.28	0.28	
	0.44		0.44			0.44								0.44				0.44	0.44		0.44	
	0.52																			0.52	0.52	
	0.58			0.58	0.58																	
	0.65						0.65				0.65	0.65								0.65	0.65	
	0.72						0.71		0.71	0.71											0.71	
	0.78		0.77																			
	0.79											0.80						0.78		0.78		
UV 365	0.62																					0.62
	0.67		0.67																	0.67	0.67	
	0.80	0.79	0.79		0.80		0.80	0.80					0.79	0.79				0.80	0.80	0.80		
Vanillin-sulfuric acid	0.27				0.27			0.27	0.27	0.27							0.27	0.27	0.27		0.27	
	0.35	0.35	0.35					0.35				0.35		0.35	0.35						0.35	
	0.59	0.59	0.59		0.59	0.59	0.59	0.59						0.59	0.59		0.59					
	0.70	0.70		0.70	0.70		0.70		0.70	0.70	0.70	0.70		0.70					0.70	0.70	0.70	0.70
	0.77		0.77						0.77				0.77	0.77	0.77			0.77		0.77	0.77	0.77
	0.86						0.86						0.86	0.86						0.86	0.86	

Note: P: PAV, 1: *C. alternifolius* L., 2: *A. calamus* L., 3: *P. indica* L., 4: *O. tenuiflorum* L., 5: *T. chebula* Retz., 6: *Z. officinale* Roscoe, 7: *C. sativum* L., 8: *V. trifolia* L., 9: *F. fragrans* Roxb., 10: *C. porrectum* (Roxb.) Kosterm, 11: *C. rotundus* L., 12: *H. pubescens* Wall. Ex G. Don, 13: *A. martinicensis* (Jacq.) Choisy. Share., 14: *K. galanga* L., 15: *A. sativum* L., 16: *L. siceraria* (Molina) Standl., 17: *T. bellirica* (Gaertn.) Roxb., 18: *P. emblica* L., 19: *P. retrofractum* Vahl, 20: *P. nigrum* L. and 21: *A. indica* A. Juss.

C. sativum had the lowest gallic acid content (0.43 ± 0.02 µg/10 mg extract) among the 16 extracts. The *P. emblica* L. was found to be an interesting source of phenolic compounds, especially gallic acid [11, 24]. The previous research reported that gallic acid was one of the major important compounds of *T. chebula* [12, 25] and *T. bellirica* [26] extracts. Therefore, gallic acid was a major compound in the composite plants of PAV formulary that might serve as a potential herbal activity of PAV formulary. Moreover, caffeic acid was found in four plants and PAV extracts. Gallic acid and caffeic acid, therefore, can be used as the chemical markers of PAV formulary.

2. Cytotoxic activity and nitric oxide production of PAV extract

The cell viability of RAW264.7 cells was analyzed to obtain a safe concentration of PAV on NGM and HGM. PAV showed low cytotoxicity at the concentration that was lower than 800 µg/mL and there was no statistical difference in cells viability between NGM and HGM when treated with PAV. However, the cytotoxicity of PAV extract was increased when treated with the higher concentration in both NGM and HGM (IC_{50} of 1,139.48 ± 36.22 and 1,134.69 ± 13.55 µg/mL, respectively) (Table 4).

Table 4 Cytotoxicity of PAV on RAW264.7 cells in NGM and HGM

PAV concentration (µg/mL)	Cell viability (%)	
	NGM	HGM
0	100.00 ± 0.00	100.00 ± 0.00
100	98.45 ± 3.16	94.29 ± 3.25*
200	106.78 ± 2.11*	98.78 ± 5.03
400	104.68 ± 3.43	101.04 ± 2.89
800	77.57 ± 3.88***	81.05 ± 2.53***
1,600	14.57 ± 1.87***	13.20 ± 0.64***
IC_{50} (µg/mL)	**1,139.48 ± 36.22**	**1,134.69 ± 13.55**

Data are presented as mean ± S.D., *$p < 0.05$, ***$p < 0.001$, significant difference from control (0 µg/mL) using one-way ANOVA followed by LSD post hoc test, n=3 for each group.

Moreover, as shown in Figure 2, the PAV extract could inhibit LPS-induced NO production in RAW264.7 cells in dose-dependent manner. Especially, the PAV (> 400 µg/mL) could completely inhibit NO production in both NGM and HGM conditions. The IC_{50} values for NGM and HGM were 128.49 ± 4.68 and 127.57 ± 14.02 µg/mL, respectively. PAV extract might have anti-inflammatory activity that led to reduce NO production from LPS-induced RAW264.7 cells in both NGM and HGM conditions. In this study, there was no significant difference in inhibitory activity of PAV on NO production between

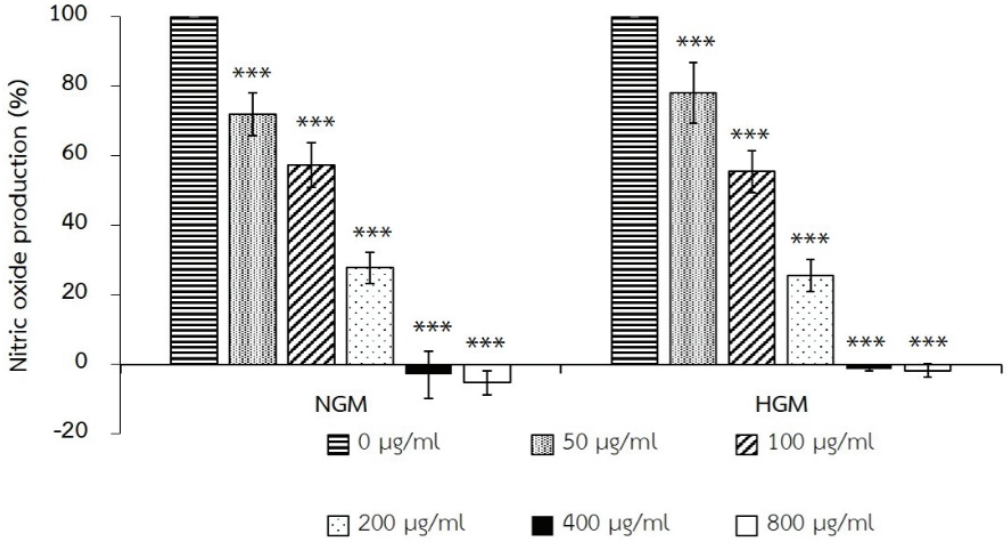

Figure 2 The effect of PAV extract on NO production of LPS-induced RAW264.7 cells in NGM and HGM. Data are presented as mean + S.D., *** $p < 0.001$, significantly different from control (0 µg/mL) using the one-way ANOVA followed by LSD post hoc test, n=3 for each group.

NGM and HGM conditions. This result suggested that the PAV might have non-specific inhibitory effect on NO production and did not depend on the concentration of glucose in the culture media in this study. However, this inhibitory effect of PAV extract on NO production might be the results of anti-oxidative and anti-inflammatory properties from those composite plants. The extract of *P. emblica* L. [11], *T. chebula* fruits [25, 27] and *T. bellirica* [28] have been known to exhibit antioxidant, anti-inflammation and inhibit nitric oxide production. The component plants of PAV, for example, *T. chebula*, *P. emblica*, *Z. officinal*, C. rotundus, *K. galanga*, *A. sativum*, *P. emblica, L. siceraria* and *A. indica* have been independently studied in various reports both *in vitro* and *in vivo* models for their potential hypoglycemic action [29-38]. Gallic acid that found in fruit of *T. bellerica* is the active principle responsible for reduction plasma glucose level after inducing to diabetic male Wistar rats [39]. The role of gallic acid in *P. emblica* facilitated their glucose homeostasis and mediated antidiabetic potential [40].

Conclusion

Gallic acid and caffeic acid were found to contain in PAV extract and many plant extracts in this study. The 21 composite plants were remaining content in PAV extract. The PAV extract showed low cytotoxicity and could inhibit NO production on RAW264.7 cells in both NGM and HGM. The results from this study may be further used for quality control of the medicinal plants, safety, and the other activities of PAV formulary for application on diabetic treatment. Anti-inflammatory activity of PAV should be further studied on gene and protein expressions by using real-time PCR and proteomics, respectively.

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