Original Article

Evaluation of antioxidant and anti-acetylcholinesterase activities and toxicity testing of Prasa-kanphlu formula extracts

Satidmas Chanplang[*], Jaturong Pratuangdejkul[*], Worawan Kitphati[**] Veena Satitpatipan[***], Sopapan Rattanaprapanporn[†], Sumet Chongruchiroj[*]

Abstract

Prasa-kanphlu formula (PKF) is one of gastro-intestinal herbal medicinal product existing in the National List of Essential Medicines (NLEM) and has been used as folklore remedies for a long time. The indications of PKF are carminative and anti-flatulence for the treatment of abdominal discomfort and bloating due to dyspepsia. Nowadays, several PKF products are available in drugstore and hospital uses, but some herbal components in their formulas are different from the original PKF in the NLEM. The PKF used in this study was the modified formula in which *Albizia lebbeck* was replaced by *Albizia procera* and *Raphistemma hooperianum* was excluded when compared with original PKF. The aims of this study were to evaluate antioxidant, anti-acetylcholinesterase (anti-AChE) activities and toxicity testing of this modified PKF. The combined 95% ethanol-aqueous extract of PKF was used throughout the study. Total phenolic content of 204.14 ± 2.17 mg/g gallic acid equivalent (GAE) was found in the PKF extract by Folin-Ciocalteu reagent method. For antioxidant activity, DPPH assay showed satisfied antioxidant activity with the IC_{50} value of 42.06 ± 0.22 µg/ml when compared with 16.50 ± 0.07 µg/ml of ascorbic acid. The FRAP assay showed reliable antioxidant reducing power of PKF extract with the FRAP value of 1.90 ± 0.03 mmol $FeSO_4$/g of extract when compared with 11.56 ± 0.50 mmol $FeSO_4$/g of ascorbic acid. Anti-AChE activity assessment of extract was carried out using the Ellman′s method and the IC_{50} value of 631.15 ± 4.30 µg/ml was observed, while galantamine showed IC_{50} value of 0.64 ± 0.12 µg/ml. Furthermore, toxicity of PKF extract in small organism was determined for the first time using nematode *Caenorhabditis elegan*s and the LD_{50} of 39.19 ± 0.63 mg/ml was reported. In conclusion, the modified PKF extract exhibited both antioxidant and anti-acetylcholine activities that were consistent with its traditional and wisdom use for treatment of dyspepsia and abdominal discomforts as indications mentioned in an original PKF of NLEM.

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Introduction

Dyspepsia is a predominant epigastric pain lasting at least 1 month that can be associated with any other upper gastrointestinal symptoms, such as epigastric fullness, nausea, vomiting, or heartburn.[1] A population-based study in the district of Banpaeo, central region of Thailand, reported a prevalence of 65.98% for dyspepsia and approximately 60-90% of patients with dyspepsia in Thailand eventually end up with a diagnosis of functional dyspepsia (FD) following endoscopic evaluation.[2] Functional or non-ulcer dyspepsia refers to dyspeptic symptom where routine diagnosis investigations, including endoscopy has ruled out organic pathology that is likely to explains the patient`s complaints.[1] FD is a heterogeneous disorder and the various symptoms may be associated with diverse pathophysiological mechanism, such as motility disorders, sensorimotor disorders, visceral hypersensitivity, post-infectious plasticity of the duodenum, *Helicobacter pylori* infection, and other associations.[3] Although FD does not reduce life expectancy but symptoms negatively impact patients quality of life significantly. The management of FD patients is to establish the firm diagnosis of FD and explain the nature and causes of the symptoms, and adjust the behavior of dietary consumption, such as carbonated drinks, use of alcohol, canned food and hot spices that triggered to the dyspeptic symptoms. The medication for eradication of *H. pylori* and medicinal treatment such as proton pump inhibitors (PPIs) or H_2 receptor antagonist, prokinetic agents, antidepressants, and phytotherapy are considered for treatment related to the predominant symptoms.[2,4] To utilize the wisdom of Thai traditional medicines, the National List of Essential Medicine (NLEM) 2019 of Thailand provides the list of several gastro-intestinal herbal medicinal products for alternative treatment of abdominal discomfort, such as Ya-that pra-jop, Prasa-kaprao formula, and Prasa-kanphlu formula.[5]

*Department of Microbiology, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand;
**Department of Physiology, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand;
***Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand;
†Laboratory of biotechnology, Chulabhorn Research Institute, Bangkok, Thailand.

Corresponding author: Jaturong Pratuangdejkul, PhD
E-mail: jaturong.pra@mahidol.ac.th

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Prasa-kanphlu formula (PKF) is a gastrointestinal herbal medicinal product existing in the NLEM and used as folklore remedies for a long time. As indications in NLEM, PKF is used as carminative and anti-flatulence agents for treatment of abdominal discomfort, flatulence and bloating due to dyspepsia. The oral administrative dose of PKF (powder, tablet, or capsule) is 1 g for 3 times a day after meals.[5] The original PKF in NLEM composes of 30 types of natural materials which are 28 types of medicinal plants and 2 kinds of minerals (camphor and sulfur). The major component of PKF formula is clove; *Syzygium aromaticum* (50% w/w); other components are shown in Table 1.[5] However, Thai FDA-registered Prasa-kanphlu products available in drug-stores and hospitals contain some herbal components differing from the original PKF in NLEM, because some medicinal plants, such as *Raphistemma hooperianum* and *Albizia procera,* are difficult to find and usually not commercially available. Accordingly, most commercial and hospital formulas of Prasa-kanphlu products were modified by cutting off or replacing these medicinal plants with others. The modified PKF used in this study was the same formula as Prasa-kanphlu product from Siriraj hospital.[6] The changes of herbal components in modified PKF used in this study were 1) replacing *Albizia lebbeck* with *A. procera*, and 2) excluding *R. hooperianum* (Table 1). For this modified PKF, quality control analysis of crude drug components was previously evaluated and reported by Saetan J.[6] Moreover, the authors showed stimulating effect on ileum contraction in guinea pigs when treated with combined 95% ethanol-aqueous PKF extract, indicating the consistent effect with its traditional use as antiflatulence.[6]

In this study, we focused on antioxidant activity of the modified PKF because excess of reactive oxygen species (ROS) might have deleterious effects on normal physiological processes of gastrointestinal (GI) tract. Accordingly, antioxidants that perform by either simply acting as a scavenger or inhibiting oxidative stress pathways in a tissue- and environment-specific manner might be beneficial at certain stages of specific GI symptoms. Moreover, our interest was to study anti-acetylcholinesterase activity of PKF extract to support the previous result showing that PKF extract stimulated ileum contraction in guinea pigs.[6] The inhibitory effect of PKF on acetylcholinesterase activity might promote GI motility that help relieve abdominal discomfort, flatulence, and bloating due to dyspepsia.

Although the major component of PKF, *S. aromaticum* (clove) was reported for high antioxidant and potential anti-cholinesterase activities,[6,7,8] other components in PKF, even presenting in smaller amounts, might have significant effects and should not be omitted without consideration. Thus, this study aimed to evaluate the antioxidant and anti-acetylcholinesterase activities of a combined 95% ethanol-aqueous extract obtained from the modified PKF. Furthermore, the toxicity of PKF extract in a small organism was determined for the first time using *Caenorhabditis elegans,* to provide an idea to use a nematode model for toxicity screening prior to testing in mammalian model. Our data provided the explanation for biological activities related to plausible pharmacological actions of modified PKF to support traditional use as indicated in the original PKF.

Table 1 Percentage of each component in the original and modified Prasa-kanphlu formulas (PKF)

% w/w	Components	Part of use	Original (NLEM)	Modified
			30 items; 28 plants & 2 minerals	29 items; 27 plants & 2 minerals
50%	*Syzygium aromaticum*	Buds	✓	✓
4%	*Albizia lebbeck*	Bark	✓	Replaced by *Albizia procera*
3.2%	*Aquilaria crassna*	Agarwood	✓	✓
	Curcuma longa	Rhizome	✓	✓
	Myristica fragrans	Seed	✓	✓
	Raphistemma hooperianum	Root	✓	× Omitted
1.6%	*Acorus calamus*	Rhizome	✓	✓
	Amomum testaceum	Leaf	✓	✓
	Angelica dahurica	Root	✓	✓
	Boesenbergia rotunda	Rhizome	✓	✓
	Cissampelos pareira	Root	✓	✓
	Coriandrum sativum	Seed	✓	✓
	Cuminum cyminum	Fruit	✓	✓
	Kaempferia galanga	Rhizome	✓	✓
	Maerua siamenis	Root	✓	✓
	Nigella sativa	Seed	✓	✓
	Oroxylum indicum	Bark	✓	✓
	Laurus nobilis	Leaf	✓	✓
	Saussurea lappa	Root	✓	✓
	Terminalia nigrovenulosa	Bark	✓	✓
	Vetiveria zizanioides	Root	✓	✓
	Camphor	-	✓	✓
	Sulfur	-	✓	✓
1.2%	*Piper retrofractum*	Flower	✓	✓
	Zingiber officinale	Rhizome	✓	✓
0.8%	*Piper interruptum*	Stem	✓	✓
	Piper sarmentosum	Root	✓	✓
	Plumbago indica	Root	✓	✓
0.4%	*Zingiber montanum*	Rhizome	✓	✓
	Piper nigrum	Seed	✓	✓

Materials and Methods

Plant materials

The modified Prasa-kanphlu formula (PKF) used in this study contained only 27 types of medicinal plants instead of 28 types in the original PKF in the NELM. In modified PKF, *R. hooperianum* was excluded from the formula because it was scarce and unavailable to purchase. Moreover, *A. lebbeck* was replaced by *A. procera* for the same reasons (Table 1).[6] All herbal and mineral materials were purchased from trust-worthy herbal drugstores (Vejchapong, Thai-houchang and Siriraj Hospital). The major component of this formula *S. aromaticum* was selected from many

herbal drugstores by comparing the content of essential oils meeting the requirements of the pharmacopoeia. The identification and quality control of all crude drugs used in this modified PKF were previously performed according to Thai Herbal Pharmacopoeia and all data were completely reported by Saetan J.[6]

Sample extraction

Briefly, the extraction process was carried out by maceration of modified PKF powder in 95% ethanol for 72 hours. The extract was concentrated by filtration and evaporation using rotary evaporator at 60ºC. The residue was further decocted with water for 3 hours, filtered, and concentrated. The ethanolic and aqueous extracts were combined together and dried using spray apparatus and stored at 4 °C until further use.[6] This combined 95% ethanol-aqueous extract of modified PKF was kindly provided by Assoc. Prof. Dr. Noppamas Soonthornchareonnon, Faculty of Pharmacy, Mahidol University.

Determination of total phenolic content (TPC) by Folin-Ciocalteu′s method

Total phenolic contents were determined by Folin-Ciocalteu′s method, modified from Zhang Q, et al.[9] Standard gallic acid was prepared by dissolving in methanol and the solution was diluted to concentrations of 5-50 µg/ ml. The stock solution of sample extract was dissolved in 80% methanol and adjusted to 1 mg/ml final concentration. 25 µl of each sample extract solution and 75 µl of sterile water were added into 96-well microplate. 25 µl of Folin-Ciocalteu reagent was added and the mixture was then shaken and kept in the dark at room temperature for 5 minutes. Finally, 100 µl of sodium carbonate (7.5% w/v) was added. After incubation at room temperature in the dark for 90 minutes, the absorbance of the solution was measured at 750 nm using a microplate reader. The total phenolic content of the sample extracts was expressed as gallic acid equivalent (GAE) in milligram per gram of extract using the standard curve of gallic acid. The assay was carried out in triplicates.

Antioxidant activity assay

The methods used for antioxidant activity assay in this study were divided into 2 methods, radical-scavenging activity (DPPH) assay and ferric reducing antioxidant power (FRAP) assay**.**

a) Radical-scavenging activity (DPPH) assay

The DPPH free radical scavenging activity was determined according to the method described by Prieto, 2012.[10] Briefly, The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) was dissolved in methanol to obtain 0. 4 mM DPPH solution. The sample extract was dissolved in 80% methanol and ascorbic acid as a standard was prepared in methanol. 100 µl DPPH solution was added to 100 µl of different concentrations (0. 98-500 µg) of sample extract solution. The reaction mixture was shaken in a vortex and kept in the dark, by wrapping up with aluminum foil, at 37ºC for 30 minutes. The absorbance of the mixture was measured spectrophotometrically at 517 nm by using microplate reader (TECAN M200, NanoQuant). Each sample was performed in 96-well plates containing a total volume of 200 µl/well, with triplicates. The ability to scavenge DPPH radical was calculated using the following equation:

$$\%\ \text{Absorbance activity} = \frac{[\ \text{Abs control} - (\ \text{Abs sample} - \text{Abs Blank}\ )]}{\text{Abs control}} \text{x100;}$$

where Abs control is the absorbance of control (DPPH and solvent), Abs sample is the absorbance of test sample (DPPH and extract solution) and Abs blank is the absorbance of blank (extract solution, without DPPH).

b) Ferric reducing ability power (FRAP) assay

Reducing power of PKF extract was determined according to Benzie and Strain, 1996,[11] with some modifications. The FRAP reagent was prepared by mixing 300 mM acetate buffer, 10 mM TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) in 40 nM HCl and 20 mM FeCl3•6H2O in 10:1:1 ratio. Then 175 µl of freshly prepared FRAP reagent was mixed with 25 µl of various concentrations of sample and ferrous sulfate as a standard. Subsequently, the mixture was incubated at 37°C for 1 minute. The absorbance was measured at 593 nm using the microplate reader and calculated absorbance at 4 minutes since was measured. Each sample was contained in a 96-well plate at 200 µl/ well with triplicates. The absorbance was calculated using the follow equation:

$$\text{Absorbance} = \text{Abs sample} - \text{Abs blank} - \text{Abs control}$$

The result was expressed as millimole of ferrous sulfate per gram of sample extract.

Acetylcholinesterase (AChE) inhibitory activity by microplate assay

AChE inhibitory activity was determined by 96-well microplate assay based on Ellman′s method, using acetylthiocholine iodide (ATCI) as a substrate.[12,13] Each experiment consisted of 4 wells: control, blank, sample, and blank of sample. Each well contained 25 µl ATCI, 125 µl DTNB (in Buffer III), 50 µl Buffer II, and 25 µl of 10% methanol (in Buffer I), or 25 µl sample (in 10% methanol in Buffer I). Then the mixture was incubated at 25°C for 5 minutes and the absorbance of mixture was measured at 405 nm using the microplate reader. After that, 25 µl of 0.22 U/ml acetylcholinesterase or Buffer II was added into the mixture, followed by incubation at 25°C for 20 minutes. Absorbance was measured at 405 nm. Percentage of inhibition was calculated as follows:

$$\%\text{Inhibition} = \frac{[(\text{Abs control} - \text{Abs blank}) - (\text{Abs sample} - \text{Abs blank of sample})]}{(\text{Abs control} - \text{Abs blank})} \text{x100;}$$

where Abs control is the absorbance of a control

(reagent, methanol and enzyme), Abs blank is the absorbance of a blank of control (reagent and methanol), Abs sample is the absorbance of a sample (reagent, sample and enzyme), and Abs blank of sample is the absorbance of a blank of a sample (reagent, sample and methanol)

Toxicity assessment using *Caenorhabditis elegans* as model [14,15]

a) Nematode strains

The wild-type N2 strain of *C. elegans* was obtained from the Laboratory of Biotechnology, Chulabhorn Research Institute. *C. elegans* cultures were raised on Nematode Growth Medium (NGM) agar which has *Escherichia coli* strain OP50 acquired from the same laboratory as a food source. In 400 ml of NGM, there contains 1.2 g NaCl, 6.8 g agar (1.7% w/v), 1 g peptone (soy) and 390 ml distilled water. After well mixing, the medium was autoclaved and cooled down to 65°C before adding sterile solutions of 1 M $CaCl_2$ 0.4 ml, 1 M $MgSO_4$ 0.4 ml, and 1 M K_2HPO_4 10 ml, pH 6.0. Then 15 ml NGM was poured into 9 cm petri plates and kept in refrigerator until needed.

b) Isolation of adult day-1 nematodes

In order to select only adult day-1 uncontaminated *C. elegans,* stock plates with many gravid adult nematodes and eggs on petri plates were washed with 5 ml S-buffer about 2 times to loosen worms and eggs which were struck in the bacteria before transferred to a 50-ml centrifuge tube. After adding S-buffer to 30 ml, the nematodes solution was centrifuged at 3,000 rpm for 3 minutes. Then the supernatant was carefully aspirated. The worm pellet in 50-ml centrifuge tube was added with distilled water into 15 ml and then 4 ml of 8°C chlorox and 1 ml of 10 N NaOH were added. After shaking for approximately 4 minutes, the tube was again centrifuged at 3,000 rpm for 3 minutes and the supernatant was removed. Total time in this step should not exceed 10 minutes. The white pellet at the bottom of the tube was re-suspended with 30 ml of S-buffer before centrifugation at 3,000 rpm for 3 minutes and removing supernatant. After repeating this step 3 times, 5 ml S-buffer was added into the 50-ml tube to suspend the worm pellet and transferred to an Erlenmeyer flask. The flask was kept in an incubator shaker at 120 rpm, 22°C overnight, to allow embryos to hatch into L1. Then L1s were transferred into NGM agar with *E. coli* OP50 as the food source. The development of *C. elegans* from L1 to L4 larvae takes approximately 48 hours at 22°C. Next, 90 µl of 50 µM 5-Fluoro-2′-deoxyuridine (FUDR) was added into L4s plate and incubated at 22°C overnight. Adult *C. elegans* were then transferred into 50 ml tubes and diluted with S-buffer to approximately 5 ± 2 nematodes per 50 µl.

c) Assay preparation

Experiments were performed in 24-well plates. A total volume of 300 µl/well consisted of 50 µl nematodes stock solution and 250 µl sample extract solution, with 6 replicates per concentrations. After covering with transparent plastics, the 24-well plates were incubated at 22°C for 24 hours in the absence of food.

d) Assay evaluation [15]

After incubation at 22°C for 24 hours, all nematodes in each well were counted and determined as motile, indicating alive, or non-motile, indicating death, through visual inspection under a stereo microscope. If worms exhibited any movement, they were considered motile, while when they were completely straight or curved and no movement during 30 seconds of observation after shaking 10 times, they were considered as non-motile. Drawing horizontal lines at the bottom of each well would facilitate counting nematodes easily.

e) Assay analysis

The percentage of mortality was expressed as the number of dead nematodes in relation to the number of total nematodes. The LD_{50} value of sample (lethal dose of sample to kill 50% of total nematodes) were calculated using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, California, USA). Results were presented as mean ± standard deviation (SD). Negative control was the solvent of sample extracts.

Results

The percentage yield of Prasa-kanphlu formula (PKF) used in this study was 13.55% w/w obtained from the previous study of Saetan J.[6] The total phenolic content (TPC) of PKF extract was 204.14 ± 2.17 mg GAE/ g of extract. Moreover, two different methods of antioxidant activities assays, the DPPH• (2,2-diphenyl-1-picrilhydrazyl) free radical scavenging and the FRAP (Ferric Reducing Antioxidant Power) assays were used to evaluate the antioxidant activities of PKF extract *in vitro*. The results of DPPH assay showed that the IC_{50} of PKF extract was 42.06 ± 0.22 µg/ml while the IC_{50} of eugenol and ascorbic acid were 9.74 ± 1.07 and 16.50 ± 0.07 µg/ml, respectively. All IC_{50} values of DPPH assay were significantly different ($P < 0.05$) when compared with each other. The FRAP value of PKF extract was 1.9 ± 0.03 mmol $FeSO_4$/g, while eugenol and standard ascorbic acid showed 4.17 ± 0.06 and 11.56 ± 0.50 mmol $FeSO_4$/g, respectively. All FRAP values were significantly different ($P < 0.05$) when compared to each other. For cholinesterase inhibitory activity, PKF extract presented lower anti-AChE activity (45.29 ± 0.51% inhibition) than the positive control galantamine (98.62 ± 0.83% inhibition) but higher than eugenol (7.39 ± 5.03% inhibition) when the same concentration of 500 µg/ml was used for testing. All %inhibition values of AChE were significantly different ($P < 0.05$) when compared with each other. Since the PKF extract presented AChE inhibitory activity near 50% at 500 µg/ml, various concentrations of the extract (100–800 µg/ml) were tested to

Table 2 The percentage yield of PKF extraction, total phenolic contents Biological activities of PKF extract as, IC_{50} of DPPH (µg/ml), FRAP value, %Inhibition of AChE at dose of 500 µg/ml and IC_{50} of AChE inhibition (AChEI).

Sample	%yield (w/w)	TPC (mg GAE/g)	IC_{50} of DPPH (µg/ml)	FRAP value (mmol $FeSO_4$/g)	%Inhibition of AChE at 500 µg/ml	IC_{50} of AChEI (µg/ml)
Modified PKF extract	13.55*	204.14 ±2.17	42.06 ±0.22[a]	1.9 ±0.03[a]	45.29 ±0.51[a]	631.15 ±4.30
Eugenol	-	9.03 ±0.12	9.74 ±1.07[b]	4.17 ±0.06[b]	7.39 ±5.03[b]	-
Ascorbic acid	-	-	16.50 ±0.07[c]	11.56 ±0.50[c]	-	-
Galan-tamine	-	-	-	-	98.62 ±0.83[c]	0.64 ±0.12

*Percentage yield of PKF extract, as reported by Saetan.[6]
[a,b,c] Significant difference ($P < 0.05$; ANOVA with Tukey *post hoc* test). The value in each group that did not share the same letter were significantly different.

determine the IC_{50} for AChE inhibition. The results showed that IC_{50} for AChE inhibition of PKF extract was 631.15 ± 4.30 µg/ml, while IC_{50} of galantamine was 0.64 ± 0.12 µg/ml**.** All reported values were expressed as mean ± SD of three parallel measurements for each assay. The significant difference ($P < 0.05$) of the values were calculated using one way analysis of variance (ANOVA) with Turkey *post hoc* test (Table 2).

The *in vivo* toxicity testing of PKF extract in a small organism was performed using the nematode *C. elegans* as a model. The morphology of *C. elegans* healthy adult day-1 and death body after treatment with PKF (70 mg/ml) for 24 hours were illustrated in Figure 1. The dead *C. elegans* were observed under white-light stereomicroscope as stretching and non-motile, and undetectable pharyngeal pump. The modified PKF extract induced a high lethality rate (>90%) at 50 mg/ml. The percentage lethality of wild-type *C. elegans* exposed to PKF extract at various concentrations was shown in Figure 2. The calculated LD_{50} for modified PKF extract amounted to 39.19 ± 0.63 mg/ml. The *C. elegans* exposed to the vehicle (deionized water) showed no lethality.

Discussion

The Prasa-kanphlu formula (PKF) extract used in this study was a quality-controlled raw material product kindly provided by Assoc. Prof. Dr. Noppamas Soonthornchareonnon. All quality-controlled data were reported by Saetan J.[6] The PKF used in this study was modified formula, *i.e.*, *R. hooperianum* was omitted and *A. procera* was used instead of *A. lebbeck* in the original PKF in the NLEM. In this modified PKF, *S. aromaticum* (clove) was the same major component (50% w/w) as the original. Therefore, *S. aromaticum* was suggested to be the most pharmacologically active herbal material in this formula. Although the antioxidant by DPPH scavenging and anti-cholinesterase activities of ethanolic and aqueous extracts of *S. aromaticum* (dried flower buds) were reported,[6,7,8] the changes in herbal components in modified PKF might have significant effects on those biological activities and should be examined. Data from the literature on pharmacological activities, like antioxidant and anti-acetylcholinesterase activities of *R. hooperianum* was limited, whereas *A. lebbeck* showed high potency antioxidant activity with DPPH assay. The IC_{50} value of the ethanol and aqueous bark extracts of *A. lebbeck* were 5.86[16] and 1.41[17] µg/ml, respectively. In this modified PKF, *A. lebbeck* was replaced by *A. procera* which belongs to the same family of Mimosoideae.[16] The antioxidant activity of ethanol bark extract of *A. procera* exhibited an IC_{50} of 9.67µg/ml for DPPH assay.[16] This information might support the use of *A. procera* instead of *A. lebbeck* which was more difficult to find or purchase. As recommendation, the administration of whole ground crude PKF (powder, tablet or capsule) was taken orally with therapeutic dose of 1 g, 3 times a day after meals.[5] Hence, the

Figure 1 The *C. elegans* morphology of (***A***) healthy adult day-1 and (***B***) death body after treatment with PKF (70 mg/ml) and incubation at 22°C for 24 hours.

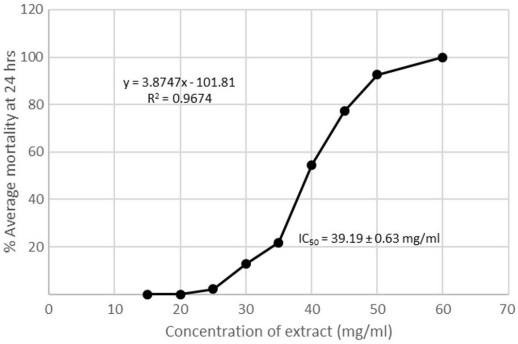

Figure 2 The plot of percentage lethality of wild-type *C. elegans* as a function of various concentrations of combined 95% ethanol-aqueous Prasa-kanphlu extract.

PKF extract used in this study was a combination of ethanolic and aqueous extracts, accommodating both semi-polar and polar substances from a serial extraction technique. The antioxidant and anti-acetylcholinesterase activities of combined ethanol-aqueous PKF extract were examined to appreciate the possible pharmacological effect that was consistent with its traditional use of whole ground modified PKF as indicated in thebNLEM.

As previously reported, the percentage yield of the combined ethanol-aqueous extract of PKF used in this study was 13.55% w/v.[6] The percentage yield of essential oil in PKF was 7.73% v/w and eugenol was found to be the most abundant compound (64.74%) in PKF.[6] The total phenolic content (TPC) of combined 95% ethanol-aqueous PKF extract (204.14 ± 2.17 mg GAE/g of extract) tended to relate with TPCs of ethanolic and aqueous *S. aromaticum* seeds extracts (310 ± 6.87 and 213 ± 9.61 mg GAE/g of extract, respectively).[18] The antioxidant activity by DPPH assay of PKF extract showed IC_{50} value of 42.06 ± 0.22 µg/ml which tended to relate with that of the ethanolic extract of *S. aromaticum* seeds (IC_{50} value of 42 ± 7.4 µg/ml).[18] The results of antioxidant activities of PKF extract, eugenol and ascorbic acid from DPPH assay were consistent with our findings of those from FRAP values. The substance with high radical scavenging activity (low IC50 value by DPPH assay) normally exhibited the high reducing power (high FRAP value). As can be seen, the results of total phenolic content of PKF extract tended to be correlated with the antioxidant activity by DPPH and FRAP assays. The overall results of percentage of yield, total phenolic content along with antioxidant activities by DPPH and FRAP assays of the combined 95% ethanol-aqueous extract of Prasa-kanphlu modified formula exhibited reasonable antioxidant activity when compared with that of eugenol (the most suggested active compound) and ascorbic acid (the positive control). The results of antioxidant activity in PKF extract might be designated by the percentage of essential oil in the PKF extract. This was because of clove oil in *S. aromaticum* was found to be an effective antioxidant when measured by different methods such as FRAP, DPPH and ABTS assays.[19]

Moreover, the previous study of PKF extract at 0.1 mg/ml demonstrated a stimulating effect on ileum contraction of 36.67% when compared with acetylcholine (5 ng/ml) in guinea pigs.[6] The ileum contraction response of PKF extract was gradually increased as the concentration increased. Although the result of main raw material of PKF (*S. aromaticum* and *O. indicum*) showed the reverse effect on isolated ileum, the other medicinal plants in PKF such as *S. lappa* and Z. *officinale* had been reported with spasmodic effects from the literature search.[6] Acetylcholine (ACh) neurotransmitter renders the contraction of GI smooth muscle cells that affects GI movement. As commonly known, AChE is involved in ACh hydrolysis. Thus, the inhibition of AChE may stimulate GI motility and possibly used as concept for potential treatments for conditions associated with inadequate GI motility.[20,21] Accordingly, this study focused on the anti-AChE activity of PKF extract to plausibly explain the stimulating effect on ileum contraction as mentioned earlier and support the traditional use of PKF as a carminative and anti-flatulence agent for many GI disorders. PKF extract showed anti-AChE activity of 45.29 ± 0.51% inhibition at 500 µg/ml and IC_{50} of inhibition was 631.15 ± 4.30 µg/ml. While AChE inhibitory effect of galantamine (positive control) at 500 µg/ml was 98.62 ± 0.83% inhibition. From literature review, the ethanolic and aqueous extracts of *S. aromaticum* seeds showed anti-AChE activities at 500 µg/ml of 52.2 ± 0.51 and 50.6 ± 2.6% inhibition, respectively[18] which were similar to the percentage of AChE inhibition at 500 µg/ml PKF extract in this study.

Prasa-kanphlu was published as gastrointestinal herbal medicinal product in the NLEM, therefore its therapeutic dose (1 g orally, 3 times a day after meal)[5] was assumed to be safe enough for therapeutic use. However, the World Health Organization (WHO) Expert Committee on Food Additives has established the acceptable daily human intake of clove oil at 2.5 mg/kg bodyweight for humans,[22] the long-term use of PKF product should be taken with caution. Since the data of acute and chronic toxicities of PKF in mammalian model is not available yet, another option is to utilize a small model organism, such as the nematode *C. elegans*, which can be handled using *in vitro* techniques for screening of PKF toxicity. Toxicity assay in *C. elegans* can provide a bridge between *in vitro* and mammalian *in vivo* testing because *C. elegans* is a whole animal with intact and metabolically active physiological systems like digestion, reproduction, endocrine, sensory, and neuromuscular structures.[23] Although mammalian models still continue to be used for prediction of toxicity in human, a good correlation of endpoints in *C. elegans* to rat LD_{50}s were demonstrated.[23] Thus, a purpose of toxicity testing of PKF extract on *C. elegans* was to grasp an idea for using a small organism nematode for preliminary toxicity screening of natural products. For this reason, we investigated whether *C. elegans* model would be applied to determine toxicity of PKF extract prior to further *in vivo* tests. We determined lethality of the wild-type strain *C. elegans* as a model for toxicity after treatment with various concentration of PKF extract (25-60 mg/ml). The LD_{50} value could be calculated and amount to 39.19 ± 0.63 mg/ml for PKF extract. The concentration-dependent could be established between the lethal effect and PKF concentration. From literature search, the toxicity of clove oil was tested in two aquarium fish species, *Danio rerio* and *Poecilia reticulate*. At 96 hours, clove oil presented the median lethal dose (LD_{50}) of 18.2 ± 5.52 mg/ml in *D. rerio* and 21.7 ± 0.8 mg/ml in *P. reticulate*.[22] The

use of high concentration of PKF for testing led to an experimental problem for toxicity testing by *C. elegans* due to the limitation of aqueous solubility. However, the lethality of PKF extract for the adult day-1 *C. elegans* could not be used to assume whether observed LD_{50} would or would not correlate with the toxicity in animal models like rodents. Further investigation is needed to elucidate whether the potency for lethality induced by this PKF extract is similar or different in rodents *vs C. elegans*. To this stage, our studies with *C. elegans* suggest that this model organism is suitable to study potential toxicity of PKF extract with the advantages of a short-living, self-fertilizing multicellular organism, and easy manipulation in the laboratory.

Conclusion

The combined 95% ethanol-aqueous extract of modified Prasa-kanphlu formula (PKF) used in this study possessed well antioxidant and anti-acetylcholinesterase activities. Although some herbal components were changed from the original PKF in NLEM, but the existence of related biological activities could explain the possible pharmacological action of this modified PKF. The antioxidant activity of PKF had beneficial effect for reducing the excess of ROS in certain stages of specific GI symptoms leading to normal physiological processes of GI tract. Moreover, the anti-acetylcholinesterase activity of PKF extract could promote GI motility due to the inhibitory effect on acetylcholine hydrolysis pathway. The toxicity of PKF was tested for the first time on nematode *C. elegans* representing the plausible use as preliminary screen prior to test in mammalian model. From our study, we summarized that the biological activities of this modified PKF were consistent to support the wisdom use for treatment of abdominal discomfort, flatulence and bloating due to dyspepsia as indications in an original PKF of NLEM.

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Conflict of Interest

All authors have no actual or potential conflict of interest capable of influencing any scientific judgment.

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