Original Article

Investigation of anti-allergic and anti-inflammatory activities of Prasaprohyai ethanolic extract under forced degradation and accelerated storage conditions

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Abstract

Introduction: Prasaprohyai (PPY) is a Thai traditional medicine remedy in the National List of Essential Medicines. It has long been used to treat fever, cold and asthma. However, PPY ethanolic extract has never been reported on the chemical constituents and biological stability. Therefore, this study was to investigate the stability testing on stress and accelerated conditions of PPY ethanolic extract regarding the anti-allergic and anti-inflammatory activities.

Method: PPY ethanolic extract was tested by force degradation method on stress conditions following the International Conference on Harmonisation (ICH) recommended such as moisture-, acid- and alkaline-hydrolysis, heat and oxidation. PPY ethanolic extract was also stored under accelerated conditions (40°C, 75% RH) for 6 months. The constituents of PPY ethanolic extract of all tests were evaluated by using gas chromatography-mass spectrometry (GC-MS). Anti-allergic activity was investigated by measuring the release of β-hexosaminidase from RBL-2H3 cells. Anti-inflammatory activity was also investigated by determining the production of nitric oxide (NO) from RAW 264.7 cells.

Results: Under stressed conditions, PPY ethanolic extract was unstable regarding anti-allergic and anti-inflammatory activities except for alkaline-condition. The extract showed stability of NO production inhibition activity when compared with normal condition. Under accelerated storage conditions, PPY ethanolic extract was stable on both anti-allergic and anti-inflammatory activities. Its chemical content was also stable when compared with day 0.

Conclusion: Force degradation test is benefit for pre-formulation or selection of dosage form of PPY. It is concluded that the dosage form of PPY should avoid moisture, high temperature, oxidation and acid conditions. For accelerated storage conditions, PPY ethanolic extract is stable at least two years at room temperature without loss of *in vitro* anti-allergic and anti-inflammatory activities when it is stored in a tight container and protected from light. This study supports the preparation of PPY dosage form for treatments of fever, cold and allergy.

Key words: Accelerated storage conditions, Anti-allergic, Anti-inflammatory, Forced degradation, Prasaprohyai remedy

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Introduction

Forced degradation studies or stress testing are important to know how the extracts change in different conditions such as hydrolysis, oxidation and thermolysis. This data can be useful for the manufacturing process, prediction of degradation products, estimation of the stability of products[1, 2]. Accelerated stability testing is a method used to quickly estimate the shelf-life, storage stability, and quality control of products[3, 4]. Accelerated stability is specified by the International Committee for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) to determine the expected shelf-life of the drug[5].

Prasaprohyai (PPY) is a Thai traditional medicine remedy in the National List of Essential Medicines (NLEM). It is well-known for relieving fever, common cold and asthma[6]. This remedy consists of 19 plants according to NLEM: *Kaempferia galanga* L. (Zingiberaceae), which is the main component used 20 parts and the other components in equal proportions of 1 part each: *Amomum testaceum* Ridl. (Zingiberaceae), *Anethum graveolens* L. (Apiaceae), *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav. (Apiaceae), *Angelica sinensis* (Oliv.) Diels (Apiaceae), *Artemisia annua* L. (Asteraceae), *Atractylodes lancea* (Thunb.) DC. (Asteraceae), *Cuminum cyminum* L. (Apiaceae), *Dracaena loureiroi* Gagnep. (Asparagaceae), *Foeniculum vulgare* Mill. (Apiaceae), *Lepidium sativum* L. (Brassicaceae), *Ligusticum sinense* Oliv. (Apiaceae), *Mammea siamensis* (Miq.) T. Anderson (Calophyllaceae), *Mesua ferrea* L. (Calophyllaceae), *Mimusops elengi* L. (Sapotaceae), *Myristica fragrans* Houtt. (Myristicaceae), *Nelumbo nucifera* Gaertn. (Nelumbonaceae), *Nigella sativa* L. (Ranunculaceae), and *Syzygium aromaticum* (L.) Merr. & L. M. Perry (Myrtaceae).

In previous reports, PPY remedy showed anti-allergic activity by determining the inhibitory effect on β-hexosaminidase release from RBL-2H3 cells and antioxidant activity by using DPPH radical-scavenging assay[7]. PPY ethanolic extract also showed *in vitro* anti-inflammatory effect having inhibitory effect on NO production in RAW 264.7 cells and showed *in vivo* by carrageenan-induced paw edema assays[7, 8]. In addition, this remedy has been reported to possess analgesic[8], anticancer[8], antimalarial[9], antimicrobial[10], antipyretic[8] and cytotoxic[11] activities. However, there is no report about anti-allergy and anti-inflammation activities of PPY ethanolic extract under forced degradation and stability testing under accelerated conditions. Therefore, the present study focused on the anti-allergic and anti-inflammatory activities of PPY ethanolic extract under stress and accelerated conditions using *in vitro* cell-based assays and also focused on the chemical constituents of PPY ethanolic extract under accelerated storage conditions by using GC-MS.

Methods

Chemicals and reagents

4-Nitrophenyl-N-acetyl-β-D-glucosaminide (PNAG), anti-dinitrophenylated bovine serum albumin (DNP-BSA), albumin from bovine serum (BSA), chlorpheniramine, D-(+)-glucose, lipopolysaccharide (LPS), monoclonal anti-dinitrophenyl antibody produced in mouse (Anti-DNP IgE), N-(1-naphthyl) ethylenediamine dihydrochloride, phosphoric acid, prednisolone, sulfanilamide and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Inc. (MO, USA). Calcium chloride dihydrate, citric acid monohydrate, magnesium chloride hexahydrate, potassium chloride, sodium bicarbonate and sodium carbonate were purchased from Merck (Darmstadt, Germany). Fetal bovine serum (FBS), minimum essential medium (MEM), penicillin-streptomycin (P/S), RPMI medium 1640, trypan blue stain and trypsin-EDTA were purchased from Gibco BRL Life Technologies (NY, USA). Phosphate buffered saline (PBS) and piperazine-N,N'-bis (2-ethanesulfonic acid) (PIPES) were purchased from Amresco (OH, USA). Sodium chloride and sodium hydroxide were purchased from Univar (NSW, Australia). Commercial grade ethanol was purchased from Sasol Chemical Pacific LTD (Shenton, Singapore). Analytical

grade reagents (e.g. dimethylsulphoxide, hydrochloric acid, isopropanol) were purchased from RCI Labscan (Bangkok, Thailand). Murine macrophage cell line (RAW 264.7) was obtained from American Type Culture Collection (ATCC® TIB-71™, VA, USA). Rat basophilic leukemia cell line (RBL-2H3) was obtained from American Type Culture Collection (ATCC® CRL-2256™, VA, USA).

Preparation of PPY ethanolic extract

All plant materials were purchased from Australia, China, India, Indonesia and Thailand. The plant species, voucher specimens, plant parts and proportions are shown in Table 1. The voucher specimens were deposited at the herbarium of the Southern Center of Thai Medicinal Plants at the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. All plant materials were cleaned by tap water, sliced thinly, oven-dried at 50°C for 24 h. The dried materials were ground by electric-grinder and the powdered materials kept in an air-tight container at room temperature until used. The powdered materials were mixed according to the proportions of plant combination (Table 1). The powdered PPY (300 g) was extracted by maceration with 95% ethanol (900 ml) at room temperature for 3 days. The extract was filtered through Whatman No.1 filter paper and concentrated to dryness under reduced pressure (45°C) using a rotary film evaporator. The extraction process was repeated again 2 times and the extracts were combined, and dried to constant weight and kept in an air-tight glass container at -20°C until used. The percentage yield of the ethanolic extract of PPY was 18.66%.

Table 1 The plant species, voucher specimens, plant parts and proportions in PPY remedy

Plant species	Thai name	Source	Part used	Ratio	Voucher specimen number
Amomum testaceum Ridl.	Krawan	Thailand	Fruit	1	SKP 206 01 11 01
Anethum graveolens L.	Thian ta takkataen	India	Fruit	1	SKP 199 01 07 01
Angelica dahurica (Hoffm.) Benth. & Hook. F. ex Franch. & Sav.	Kot so	China	Root	1	SKP 199 01 04 01
Angelica sinensis (Oliv.) Diels	Kot chiang	China	Root	1	SKP 199 01 09 01
Artemisia annua L.	Kot chula lampha	China	All parts	1	SKP 051 01 01 01
Atractylodes lancea (Thunb.) DC.	Kot khamao	China	Rhizome	1	SKP 051 01 12 01
Cuminum cyminum L. (Apiaceae)	Thian khao	India	Fruit	1	SKP 199 03 03 01
Dracaena loureiroi Gagnep.	Chan daeng	Thailand	Stem	1	SKP 065 04 12 01
Foeniculum vulgare Mill.	Thian khao plueak	India	Fruit	1	SKP 199 06 22 01
Kaempferia galanga L.	Proh hom	Thailand	Rhizome	20	SKP 206 11 07 01
Lepidium sativum L.	Thian daeng	India	Seed	1	SKP 057 12 19 01
Ligusticum sinense Oliv.	Kot hua bua	China	Rhizome	1	SKP 199 12 19 01
Mammea siamensis (Miq.) T. Anderson	Saraphi	Thailand	Flower	1	SKP 083 13 19 01
Mesua ferrea L.	Bunnak	Thailand	Flower	1	SKP 083 13 06 01
Mimusops elengi L.	Phikun	Thailand	Flower	1	SKP 171 13 05 01
Myristica fragrans Houtt.	Chan thet	Australia	Stem	1	SKP 121 13 06 01

Table 1 The plant species, voucher specimens, plant parts and proportions in PPY remedy

Plant species	Thai name	Source	Part used	Ratio	Voucher specimen number
Myristica fragrans Houtt.	Mace	Thailand	Aril	1	SKP 121 13 06 01
	Nutmeg	Thailand	Seed	1	SKP 121 13 06 01
Nelumbo nucifera Gaertn.	Bua luang	Thailand	Pollen	1	SKP 125 14 14 01
Nigella sativa L.	Thian dam	India	Seed	1	SKP 160 14 19 01
Syzygium aromaticum (L.) Merr. & L. M. Perry	Kan phlu	Indonesia	Flower	1	SKP 123 19 01 01

Forced degradation studies (Stressed degradation)

Stress degradation of PPY ethanolic extract was determined under moisture hydrolysis, acid hydrolysis, alkaline hydrolysis, thermal degradation and oxidation degradation conditions. These conditions were established by the International Conference on Harmonisation (ICH) guideline[5]. Moisture hydrolysis, 3 drops of DI water was added to 10 mg of PPY ethanolic extract and incubated in a water bath at 80°C for 3 h. For acid and alkaline hydrolysis, 3 drops of 3 N HCl and 3 N NaOH were added in PPY extracts and incubated in a water bath at 80°C for 3 h. Each sample of acid and alkaline condition was neutralized with alkaline and acid respectively before being tested. Thermal degradation of PPY ethanolic extract was carried out by heating at 80°C for 3 h. For oxidation degradation of PPY ethanolic extract, 30% H_2O_2 was added and kept at 80°C for 3 h. All the samples were evaluated for their anti-allergic and anti-inflammatory activities by comparison with normal condition sample.

Stability study (Accelerated condition testing)

PPY ethanolic extract was kept in a capped glass container in environmental chambers-climatic cabinets under accelerated conditions, 40 ± 2°C/75 ± 5% RH for 6 months according to the International Conference on Harmonisation (ICH) guideline[5]. PPY ethanolic extracts were withdrawn on day 0, 15, 30, 45, 60, 90, 120, 150 and 180 and were evaluated for their chemical contents, anti-allergic and anti-inflammatory activities.

Evaluation of chemical stability

All PPY ethanolic extracts under accelerated conditions were analyzed by GC-MS. The sample (10 mg) was adjusted to 4.5 ml with methanol. The solution was filtered before injection. GC-MS was performed with a Thermo Focus GC plus Polaris Q plus Triplus auto injector (Thermo Fisher Scientific, MA, USA). The column used was a Zebron ZB-5ms capillary GC column measuring 30 m × 0.25 mm with a film thickness of 0.25 μm (Phenomenex, CA, USA). The carrier gas used was helium, in splitless mode. The injection volume was 2 μl. The oven temperature was programed initially at 60°C and then 5°C increments/min to 200°C. Then, the temperature was programed to increase to 300°C at a rate of 10°C increments/min ending with a 10 min period, with the injector temperature of 250°C. The total run time was 48 min. The GC-MS was analyzed at a 70 eV electron impact, with an ion source temperature of 200°C. The spectra of the components were compared with the database of spectra of known components stored in the GC-MS library.

Measurement of anti-allergic activity by β-hexosaminidase release

The inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cells were modified by using the method of Tewtrakul et al[12] with slight modifications. RBL-2H3 cells were cultured in minimum essential medium (MEM) supplemented with 15% heat-

inactivated fetal bovine serum (FBS), 10,000 units/ml penicillin and 10,000 μg/ml streptomycin. Cells were maintained in an incubator at 37°C in a humidified atmosphere of 5% CO_2/95% air. RBL-2H3 cells (2×10^5 cells/well) were seeded in 24-well plates and allowed to adhere for 2 h. Cells were sensitized with 0.45 μg/ml monoclonal anti-dinitrophenyl antibody (anti-DNP IgE) and incubated for 24 h. Cells were then washed with Siraganian buffer [Siraganian buffer; containing 119 mM NaCl, 5 mM KCl, 5.6 mM D-(+)-glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 0.1% bovine serum albumin (BSA) and 40 mM NaOH, pH 7.2], and then incubated in 160 μl of Siraganian buffer for 10 min. Various concentrations of test samples (20 μl) were then added to each well and incubated for 10 min, followed by addition of 20 μl of anti-dinitrophenylated bovine serum albumin (DNP-BSA, final concentration is 10 μg/ml) for 20 min. After that, aliquots (50 μl) of supernatant were transferred into 96-well plates and mixed with 50 μl of substrate solution (1 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide in 0.1 M citrate buffer, pH 4.5) for 2 h. The reaction was stopped by adding 200 μl of a stopping solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance at 405 nm was measured using a microplate reader. The test sample was dissolved in DMSO and the solution was added to Siraganian buffer (final DMSO concentration was 0.1%).

The percentage of inhibitory effect on β-hexosaminidase release by the test samples was calculated by the following equation, and the IC_{50} values were calculated from the Prism program.

$$\text{Inhibition (\%)} = [1 - (T - B - N)/(C - B - N)] \times 100$$

Where Control (C): DNP-BSA (+), test sample (-); Test (T): DNP-BSA (+), test sample (+); Blank (B): DNP-BSA (-), test sample (+); Normal (N): DNP-BSA (-), test sample (-)

Measurement of anti-inflammatory activity by LPS-induced NO production

The inhibitory effect on NO production from RAW 264.7 cells was determined using the method of Tewtrakul and Itharat[13] with slight modifications. RAW 264.7 cells were cultured in RPMI medium 1640 supplemented with 10% heat-inactivated FBS, 10,000 units/ml penicillin and 10,000 μg/ml streptomycin. Cells were maintained in an incubator at 37°C in a humidified atmosphere of 5% CO_2/95% air. RAW 264.7 cells (1×10^5 cells/well) were seeded in 96-well plates and allowed to adhere for 24 h. The medium was replaced with fresh medium (100 μl/well) containing 10 ng/ml LPS and treated with various concentrations of test samples (100 μl/well) for 24 h. After LPS stimulation for 24 h, aliquots (100 μl) of supernatant were transferred into 96-well plates and mixed with Griess reagent (100 μl) (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid). After taking of the supernatant, cell viability was determined using the MTT assay. Briefly, MTT solution (10 μl, 5 mg/ml in PBS) was added to each well and incubated for 2 h. The medium was then removed and isopropanol containing 0.04 M HCl was added to each well to dissolve the formazan production in the cells. The absorbance at 570 nm was measured using a microplate reader. The test sample was considered cytotoxic to cells when viable cell in sample group was less than 70% of the control group. The test sample was dissolved in DMSO and added to RPMI medium 1640 (final DMSO concentration was 0.2%).

The percentage of inhibitory effect on NO production by the test samples was calculated by the following equation, and the IC_{50} values were calculated using the Prism program.

$$\text{Inhibition (\%)} = [(\text{OD control} - \text{OD sample}) / \text{OD control}] \times 100$$

Statistical analysis

The results are reported as mean ± standard error of the mean (SEM) of three independent experiments. The IC_{50} values were calculated using the Prism program. Data were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. *P*-value less than 0.05 indicate statistical significance.

Results

Forced degradation studies (Stressed degradation)

PPY ethanolic extract was exposed to stress conditions of moisture hydrolysis, acid hydrolysis, alkaline hydrolysis, thermal degradation and oxidation degradation and also heating at 80°C for 3 hours. Thereafter, these extracts were evaluated for anti-allergic effect on β-hexosaminidase from RBL-2H3 cells and anti-inflammatory effect on NO production from RAW 264.7 cells and compared with normal conditions. PPY ethanolic extract (normal condition) showed potent anti-allergic effect (IC_{50} = 11.71 ± 0.94 μg/ml) which is better than a positive control (chlorpheniramine, IC_{50} = 17.98 ± 0.78 μg/ml). In addition, all PPY ethanolic extracts under stress conditions have significantly less anti-allergic effect on β-hexosaminidase release than under normal condition ($p < 0.05$) (Table 2 and Figure 1). These results indicate that PPY ethanolic extract under stress conditions such as moisture hydrolysis, acid hydrolysis, alkaline hydrolysis, thermal degradation and oxidation degradation was unstable in anti-allergic activity by β-hexosaminidase release from RBL-2H3 cells. PPY ethanolic extract possessed moderate anti-inflammatory effect (IC_{50} = 49.95 ± 1.89 μg/ml) but it exhibited lower anti-inflammatory effect than a positive control (prednisolone, IC_{50} = 0.59 ± 0.32 μg/ml). Moreover, PPY ethanolic extract after exposure to moisture, acid, thermal and oxidation conditions also showed significantly less anti-inflammatory effect on NO production than normal control ($p < 0.05$) (Table 2 and Figure 1), except for alkaline hydrolysis condition, which PPY extract showed no significant difference compared with normal condition. Thus, PPY ethanolic extract was stable regarding anti-inflammatory under alkaline conditions.

Table 2 The inhibitory effect on β-hexosaminidase release from RBL-2H3 cells and nitric oxide production from RAW 264.7 cells by PPY ethanolic extract

Conditions	%Inhibition at various concentrations of PPY ethanolic extract (mean ± SEM, μg/ml)						IC_{50} ± SEM (μg/ml)
	0.1	1	10	30	50	100	
β-Hexosaminidase assay							
Normal condition	-	-12.86 ± 5.68	42.16 ± 4.22	-	63.53 ± 1.17	81.74 ± 2.37	11.71 ± 0.94
Moisture hydrolysis	-	-27.97 ± 2.68	5.53 ± 2.89	-	34.41 ± 4.30	67.19 ± 4.03	81.45 ± 5.01*
Acid hydrolysis	-	-9.01 ± 3.65	11.04 ± 2.41	-	37.26 ± 3.79	67.45 ± 1.07	75.75 ± 3.65*
Alkaline hydrolysis	-	-18.59 ± 7.33	3.65 ± 4.99	-	30.77 ± 1.28	51.00 ± 0.71	98.26 ± 1.34*
Thermal degradation	-	-12.40 ± 15.69	16.53 ± 1.76	-	45.25 ± 3.37	62.64 ± 2.10	61.59 ± 6.50*
Oxidation degradation	-	-21.84 ± 4.82	0.07 ± 2.29	-	31.45 ± 2.26	59.11 ± 2.85	86.25 ± 4.56*
Chlorpheniramine[b]	-	-38.89 ± 8.01	17.82 ± 1.01	-	68.35 ± 1.50	91.33 ± 1.22	17.98 ± 0.78
Nitric oxide assay							
Normal condition	-	-7.92 ± 6.22	5.08 ± 2.11	31.96 ± 1.98	50.21 ± 1.52	89.61 ± 5.20	49.95 ± 1.89
Moisture hydrolysis	-	-9.21 ± 1.03	-4.23 ± 1.74	9.97 ± 3.76	24.70 ± 4.59	54.30 ± 3.63	91.38 ± 7.45*
Acid hydrolysis	-	-7.20 ± 2.04	1.16 ± 0.73	16.68 ± 2.34	30.57 ± 4.16	60.56 ± 3.24	80.88 ± 6.95*
Alkaline hydrolysis	-	-7.75 ± 1.43	4.70 ± 1.76	31.63 ± 0.51	59.10 ± 3.79	91.19 ± 2.05	43.49 ± 2.19
Thermal degradation	-	-12.46 ± 4.70	-8.41 ± 3.69	-3.57 ± 2.80	3.42 ± 1.72	18.01 ± 1.86	> 100
Oxidation degradation	-	-15.73 ± 2.91	-1.48 ± 3.73	13.42 ± 3.69	37.04 ± 4.09	90.66 ± 3.55[a]	Toxic
Prednisolone[c]	39.71 ± 5.79	62.78 ± 8.89	70.42 ± 9.10	79.95 ± 8.32	81.47 ± 6.19	-	0.59 ± 0.32

(-) not tested; [a] Cytotoxicity was observed; [b] Positive control for β-hexosaminidase assay; [c] Positive control for nitric oxide assay;

* Significant difference at the level $p < 0.05$ compared with normal condition, n = 3

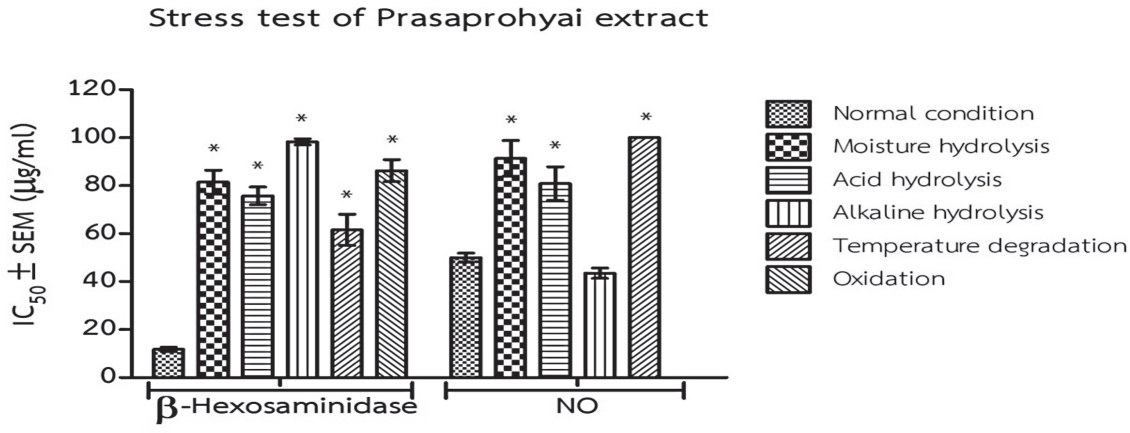

Figure 1 Effects of PPY ethanolic extracts under forced degradation studies on β-hexosaminidase release from RBL-2H3 cells and NO production from RAW 264.7 cells. Data were analyzed using one-way ANOVA and Dunnett's test. Results are presented as the IC_{50} ± SEM values (µg/ml) (n = 3).

*Significant difference ($p < 0.05$) compared with normal condition.

Stability study (Accelerated conditions testing)

PPY ethanolic extract was stored under accelerated conditions at 40°C, 75% RH for 6 months. These extracts were withdrawn on various days (day 0, 15, 30, 45, 60, 90, 120, 150 and 180 and evaluated for their chemical contents by using GC-MS, anti-allergic and anti-inflammatory activities by using *in vitro* cell-based assays and compared with day 0 as shown in Table 3 and Figure 2. The results show that PPY ethanolic extract samples which were kept under accelerated storage conditions were not significantly different on anti-allergic and anti-inflammatory activities when compared with day 0.

Table 3 The IC_{50} values of PPY ethanolic extract on β-hexosaminidase release and NO production after various storage periods under accelerated conditions (n = 3)

Storage period	IC_{50} ± SEM (µg/ml)	
	β-Hexosaminidase release	NO production
Day 0	11.71 ± 0.94	49.95 ± 1.89
Day 15	17.23 ± 1.26	50.97 ± 5.46
Day 30	11.44 ± 1.89	49.46 ± 4.62
Day 45	12.09 ± 2.51	47.76 ± 4.15
Day 60	14.98 ± 1.30	47.32 ± 2.68
Day 90	13.70 ± 1.17	45.35 ± 4.32
Day 120	22.09 ± 6.67	44.17 ± 3.44
Day 150	14.33 ± 2.94	48.01 ± 6.03
Day 180	14.35 ± 1.94	46.60 ± 8.10

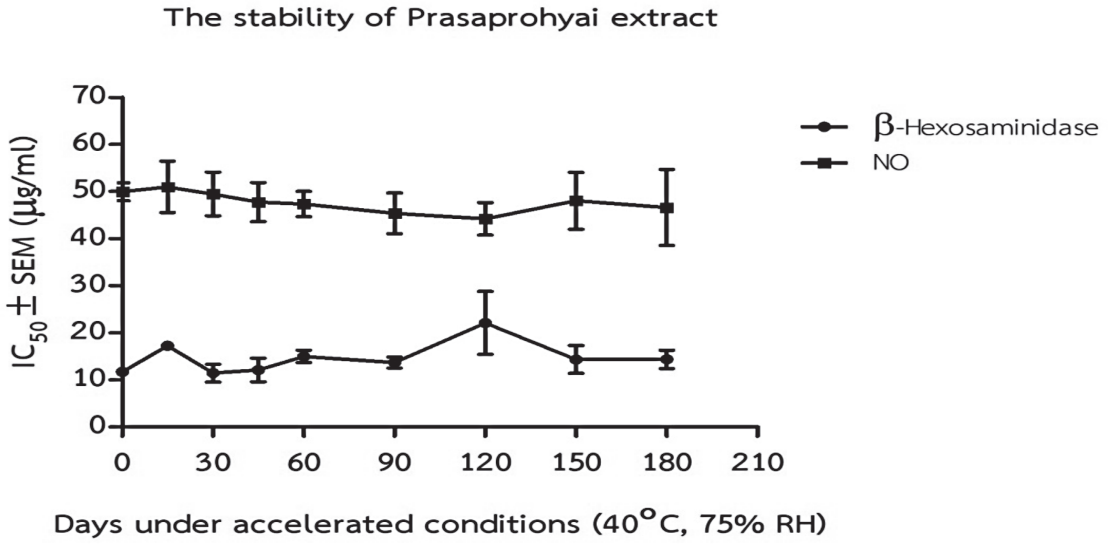

Figure 2 The stability of PPY ethanolic extract on β-hexosaminidase release and NO production under accelerated condition at 40°C and 75% RH for 6 months at the various storage times. Data were analyzed by using one-way ANOVA and Dunnett's test. Results are presented as the IC_{50} ± SEM values (µg/ml) (n = 3).

Chemical composition of PPY ethanolic extract on accelerated condition

From GC-MS analysis, results lead to the identification of a number of compounds from PPY ethanolic extract. The identified compounds, retention times (RT) and percent compositions (%peak area) of PPY ethanolic extract are presented in Table 4. The main compounds identified in PPY ethanolic extract were ethyl p-methoxycinnamate (40.19%), followed by oleic acid ethyl ester (12.23%); 9, 12-octadecadienoic acid, ethyl ester (9.02%); octadecanoic acid (7.22%); austrobailignan-6 (5.27%) and ethyl myristate (3.16%), respectively. GC-MS chromatograms of the chemical stability of PPY ethanolic extract under accelerated storage conditions from day 15 to day 180 in comparison with day 0 are shown in Figure 3. The results show that the chemical compounds of all PPY ethanolic extracts did not change after keeping under accelerated conditions when compared with day 0.

Table 4 Compounds identified in PPY ethanolic extract by GC-MS analysis (n = 2)

Peak	Compounds	Retention time (RT)	%Peak area ± SEM								
			Day 0	Day 15	Day 30	Day 45	Day 60	Day 90	Day 120	Day 150	Day 180
1	Eugenol	15.44	2.28 ± 0.01	2.21 ± 0.02	2.18 ± 0.02	2.14 ± 0.17	2.22 ± 0.01	2.24 ± 0.01	2.29 ± 0.03	2.29 ± 0.00	1.78 ± 0.02
2	Ethyl cinnamate	18.38	2.87 ± 0.04	2.82 ± 0.01	2.77 ± 0.01	2.66 ± 0.17	2.75 ± 0.01	2.76 ± 0.02	2.80 ± 0.04	2.85 ± 0.09	2.44 ± 0.03
3	Pentadecane	19.23	1.33 ± 0.00	1.32 ± 0.01	1.25 ± 0.00	1.20 ± 0.07	1.26 ± 0.00	1.25 ± 0.01	1.29 ± 0.01	1.31 ± 0.08	0.79 ± 0.07
4	Eugenol acetate	19.50	0.21 ± 0.01	0.22 ± 0.00	0.21 ± 0.01	0.20 ± 0.01	0.21 ± 0.00	0.19 ± 0.01	0.18 ± 0.00	0.96 ± 0.78	0.16 ± 0.00
5	Elemicin	20.27	0.18 ± 0.00	0.19 ± 0.00	0.18 ± 0.00	0.17 ± 0.02	0.18 ± 0.00	0.18 ± 0.00	0.19 ± 0.00	0.19 ± 0.00	0.18 ± 0.00
6	Methoxyeugenol	21.42	0.15 ± 0.02	0.13 ± 0.00	0.13 ± 0.00	0.12 ± 0.01	0.13 ± 0.00	0.13 ± 0.00	0.13 ± 0.00	0.13 ± 0.00	0.13 ± 0.00
7	Dill-apiol	21.97	1.04 ± 0.03	1.04 ± 0.01	1.02 ± 0.00	0.98 ± 0.05	1.04 ± 0.00	1.03 ± 0.00	1.06 ± 0.02	1.04 ± 0.01	1.00 ± 0.00
8	Valencene	22.55	0.17 ± 0.01	0.17 ± 0.00	0.15 ± 0.00	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.17 ± 0.00	0.17 ± 0.01	0.17 ± 0.00
9	2-Propenoic acid, 3-(3-methoxyphenyl)-, ethyl ester	22.84	1.65 ± 0.02	1.70 ± 0.00	1.57 ± 0.00	1.44 ± 0.11	1.67 ± 0.00	1.67 ± 0.03	1.75 ± 0.01	1.70 ± 0.01	1.52 ± 0.01
10	Hexadecane	23.87	0.23 ± 0.00	0.21 ± 0.00	0.21 ± 0.00	0.20 ± 0.02	0.22 ± 0.02	0.21 ± 0.00	0.24 ± 0.02	0.22 ± 0.02	0.24 ± 0.00
11	Allyl phenoxyacetate	24.16	0.14 ± 0.01	0.14 ± 0.00	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.00	0.14 ± 0.00	0.14 ± 0.01	0.13 ± 0.00	0.14 ± 0.01
12	3 N Butyl phathalide	24.48	0.84 ± 0.02	0.82 ± 0.01	0.82 ± 0.00	0.78 ± 0.04	0.81 ± 0.00	0.81 ± 0.00	0.80 ± 0.01	0.79 ± 0.01	0.82 ± 0.00
13	Ethyl p-methoxycinnamate	25.19	40.50 ± 0.32	41.14 ± 0.03	41.38 ± 0.35	39.12 ± 1.87	41.37 ± 0.17	41.30 ± 0.05	41.47 ± 0.35	41.58 ± 0.70	42.69 ± 0.27
14	Ethyl myristate	25.87	3.23 ± 0.07	3.29 ± 0.00	3.21 ± 0.00	3.06 ± 0.16	3.19 ± 0.03	3.17 ± 0.00	3.19 ± 0.03	3.22 ± 0.09	3.23 ± 0.01
15	Ethyl 9-hexadecenoate	29.36	0.45 ± 0.06	0.47 ± 0.00	0.50 ± 0.00	0.46 ± 0.02	0.44 ± 0.03	0.47 ± 0.00	0.49 ± 0.01	0.48 ± 0.00	0.48 ± 0.02
16	Octadecanoic acid	29.76	7.30 ± 0.08	7.28 ± 0.06	7.25 ± 0.01	6.89 ± 0.34	7.25 ± 0.01	7.14 ± 0.00	7.22 ± 0.09	7.11 ± 0.19	7.21 ± 0.08
17	Unknown	31.23	0.44 ± 0.01	0.48 ± 0.01	0.49 ± 0.01	0.47 ± 0.04	0.52 ± 0.00	0.53 ± 0.01	0.54 ± 0.00	0.56 ± 0.05	0.63 ± 0.03
18	9, 12-Octadecadienoic acid, ethyl ester	32.00	9.25 ± 0.23	9.14 ± 0.07	9.04 ± 0.13	8.73 ± 0.55	9.13 ± 0.03	9.19 ± 0.03	9.18 ± 0.17	8.88 ± 0.03	8.94 ± 0.32
19	Oleic acid ethyl ester	32.07	12.38 ± 0.16	12.14 ± 0.08	12.24 ± 0.05	11.48 ± 0.59	12.04 ± 0.01	11.97 ± 0.03	12.04 ± 0.06	11.59 ± 0.17	21.84 ± 9.90

Table 4 Compounds identified in PPY ethanolic extract by GC-MS analysis (n = 2)

Peak	Compounds	Retention time (RT)	%Peak area ± SEM								
			Day 0	Day 15	Day 30	Day 45	Day 60	Day 90	Day 120	Day 150	Day 180
20	Eicosanoic acid	32.37	1.22 ± 0.00	1.24 ± 0.03	1.22 ± 0.00	6.17 ± 4.93	1.23 ± 0.00	1.22 ± 0.01	1.25 ± 0.00	1.20 ± 0.02	1.23 ± 0.00
21	Ethyl 3, 7, 11, 15-tetramethyl-2-hexadecenoate	34.17	0.82 ± 0.00	0.85 ± 0.02	0.82 ± 0.01	0.76 ± 0.07	0.82 ± 0.00	0.82 ± 0.00	0.82 ± 0.00	0.80 ± 0.01	0.81 ± 0.00
22	Docosanoic acid (CAS)	34.42	0.40 ± 0.02	0.40 ± 0.01	0.41 ± 0.01	0.38 ± 0.03	0.40 ± 0.01	0.40 ± 0.01	0.40 ± 0.00	0.40 ± 0.01	0.39 ± 0.01
23	Unknown	35.46	0.35 ± 0.03	0.39 ± 0.02	0.47 ± 0.04	0.45 ± 0.06	0.47 ± 0.02	0.51 ± 0.03	0.52 ± 0.03	0.46 ± 0.09	0.50 ± 0.00
24	Unknown	35.97	1.65 ± 1.34	0.31 ± 0.00	0.31 ± 0.00	0.29 ± 0.02	0.29 ± 0.01	0.30 ± 0.01	0.30 ± 0.00	0.30 ± 0.01	0.29 ± 0.01
25	Unknown	36.14	0.75 ± 0.01	0.81 ± 0.01	0.83 ± 0.00	0.77 ± 0.04	0.80 ± 0.00	0.82 ± 0.01	0.83 ± 0.01	0.79 ± 0.02	0.82 ± 0.01
26	Unknown	36.24	0.77 ± 0.03	0.81 ± 0.01	0.78 ± 0.00	0.76 ± 0.03	0.80 ± 0.00	0.81 ± 0.01	0.82 ± 0.00	0.79 ± 0.02	0.81 ± 0.03
27	Austrobailignan-6	36.82	5.41 ± 0.14	5.75 ± 0.17	5.72 ± 0.14	5.47 ± 0.16	5.59 ± 0.04	5.64 ± 0.05	4.80 ± 0.82	5.54 ± 0.12	5.78 ± 0.10
28	Unknown	36.97	3.04 ± 0.20	3.43 ± 0.17	3.57 ± 0.02	3.50 ± 0.25	3.80 ± 0.01	3.80 ± 0.05	3.96 ± 0.02	3.51 ± 0.34	3.70 ± 0.09
29	Unknown	38.02	0.95 ± 0.02	1.09 ± 0.03	1.12 ± 0.00	1.06 ± 0.06	1.09 ± 0.03	1.11 ± 0.01	1.14 ± 0.00	1.02 ± 0.09	1.08 ± 0.09

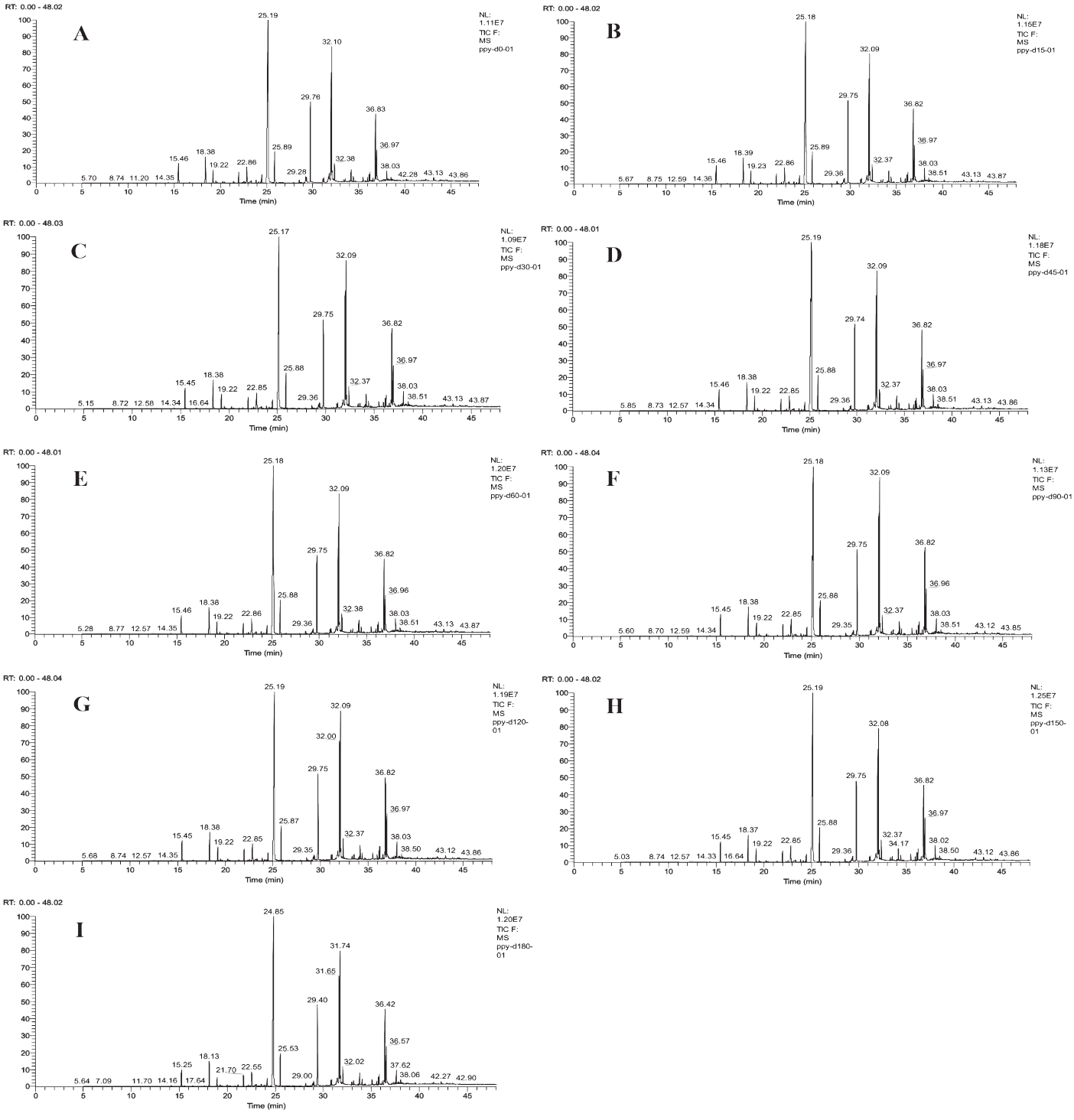

Figure 3 GC-MS chromatogram of PPY ethanolic extract under accelerated conditions at the various stored times; Day 0 (A), Day 15 (B), Day 30 (C), Day 45 (D), Day 60 (E), Day 90 (F), Day 120 (G), Day 150 (H) and Day 180 (I).

Discussion

This is the first report of *in vitro* anti-allergic and anti-inflammatory activities of PPY ethanolic extract under forced degradation and accelerated conditions. In the present study, PPY remedy extract was unstable regarding anti-allergic activity under stress conditions of moisture hydrolysis, acid hydrolysis, alkaline hydrolysis, thermal degradation and oxidation degradation because the IC_{50} values of β-hexosaminidase release increased significantly when compared with normal condition. However, it was stable regarding anti-inflammatory activity determined as NO production inhibition under alkaline hydrolysis but it was unstable upon exposure to moisture, acid, heat and oxidation. It is possible for this extract which had volatile oil from plant in Zingiberaceae composed more than 50% of remedy, such that volatile oil of these plant extract was volatile at high temperature (80°C). It is possible that the active ingredients as volatile oil in PPY may be volatized at high temperature for every condition so the anti-allergic and anti-inflammatory activities of extract were reduced. However, these results can be used for preparing PPY dosage form. Dosage forms which are suitable with this extract have to avoid moisture, oxidation, acid, alkaline and high temperature condition because it is unstable under all these conditions. Tablet dosage form preparing by dry method should be suitable for oral drug administration.

On the other hand, the stability testing of PPY ethanolic under accelerated storage conditions exhibited no change in both activities i.e., inhibitory effect on the release of β-hexosaminidase and inhibitory effect on the production of nitric oxide. The chemical contents of the extract were also stable. The major chemical components obtained from PPY ethanolic extract were identified to be ethyl p-methoxycinnamate; oleic acid ethyl ester; 9, 12-octadecadienoic acid, ethyl ester; octadecanoic acid; austrobailignan-6 and ethyl myristate. Among these compounds, ethyl p-methoxycinnamate is a member of the class of compounds known as cinnamic acid esters which is the highest component in PPY ethanolic extract. Moreover, ethyl p-methoxycinnamate was also the main component isolated from *Kaempferia galanga*[14], which is the main plant ingredient in PPY remedy. In previous research, ethyl p-methoxycinnamate showed inhibitory effects on IL-1 and TNF-α using both *in vivo* and *in vitro* experiments, and exhibited inhibition of NO production using *in vitro* experiment[15]. It also showed inhibitory activities against COX-1 and COX-2 using an *in vitro* experiment[16]. Accordingly, in this study, the results show that PPY ethanolic extract is stable on anti-allergic and anti-inflammatory activities under accelerated conditions, and ethyl p-methoxycinnamate as a main content of PPY remedy did not change during storage under accelerated conditions. Therefore, ethyl p-methoxycinnamate from PPY as an active anti-allergic and anti-inflammatory agent is stable under accelerated condition. This result can conclude that PPY ethanolic extract is stable regarding the chemical content and both activities, as it can be kept in a tight container protected from light and heat for two years at room temperature.

Force degradation test is beneficial for pre-formulation or for selecting dosage form of PPY. This result showed that the dosage form of PPY should avoid moisture, high temperature, oxidation and acid conditions. For accelerated storage conditions, PPY ethanolic extract showed to be stable on chemical content and both biological activities related allergy. Thus, it can be concluded that PPY ethanolic extract is stable for at least two years at room temperature without loss of *in vitro* anti-allergic and anti-inflammatory activities when it is stored in a tight container and protected from light. This study supports the preparing of PPY dosage form for treatments fever, cold and allergy in the future. However, further study needs to isolate the active anti-allergic compounds which lead to the development of anti-allergic drugs.

Potential conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgements

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บทคัดย่อ

การศึกษาฤทธิ์ต้านการแพ้และต้านการอักเสบของสารสกัดตำรับประสะเปราะใหญ่ชั้นเอทานอลภายใต้สภาวะเครียดและสภาวะเร่ง

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*** ศูนย์ความเป็นเลิศทางการวิจัยแพทย์แผนไทยประยุกต์ คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์

บทนำ: ตำรับยาประสะเปราะใหญ่เป็นตำรับยาไทยอยู่ในบัญชียาหลักแห่งชาติ ใช้สำหรับรักษาอาการไข้ อาการหวัด และโรคหอบหืด ซึ่งยังไม่เคยมีรายงานการศึกษาองค์ประกอบทางเคมีและความคงตัวของฤทธิ์ทางชีวภาพของสารสกัดตำรับประสะเปราะใหญ่ชั้นเอทานอลมาก่อน ดังนั้นการศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านการแพ้และต้านการอักเสบของสารสกัดตำรับประสะเปราะใหญ่ชั้นเอทานอลภายใต้สภาวะเครียดและสภาวะเร่ง

วิธีการศึกษา: ศึกษาสารสกัดตำรับประสะเปราะใหญ่ในสภาวะเครียดตามข้อกำหนดของไอซีเอช ได้แก่ ผ่านทางความชื้นไฮโดรไลซีสด้วยกรด ไฮโดรไลซีสด้วยด่าง ความร้อน และออกซิเดชั่น นอกจากนั้นศึกษาสารสกัดตำรับประสะเปราะใหญ่ในสภาวะเร่ง (อุณหภูมิ 40 องศาเซลเซียส ความชื้นสัมพัทธ์ 75 เปอร์เซ็นต์ เป็นเวลา 6 เดือน) และศึกษาองค์ประกอบทางเคมีของตำรับโดยเทคนิคแก๊สโครมาโตรกราฟี ศึกษาฤทธิ์ต้านการแพ้โดยดูการยับยั้งการหลั่งเอนไซม์บีต้า-เฮกโซสามินิเดสจากเซลล์ RBL-2H3 และการยับยั้งการสร้างไนตริกออกไซด์จากเซลล์ RAW 264.7

ผลการศึกษา: การศึกษาภายใต้สภาวะเครียด พบว่าสารสกัดตำรับประสะเปราะใหญ่ไม่คงตัวในฤทธิ์ต้านการแพ้และฤทธิ์ต้านการอักเสบ ยกเว้นสารภายใต้การไฮโดรไลซีสด้วยด่างที่มีความคงตัวในการยับยั้งการสร้างไนตริกออกไซด์เมื่อเทียบกับสภาวะปกติ การศึกษาภายใต้สภาวะเร่ง พบว่าสารสกัดตำรับประสะเปราะใหญ่มีความคงตัวของฤทธิ์ต้านการแพ้ ฤทธิ์ต้านการอักเสบ และองค์ประกอบทางเคมีซึ่งเป็นสารสำคัญหลักของตำรับไม่เปลี่ยนแปลง เมื่อเทียบกับ day 0

สรุปผลการศึกษา: การศึกษาภายใต้สภาวะเครียดมีประโยชน์สำหรับการตั้งสูตรตำรับหรือการเลือกรูปแบบในการเตรียมยาจากสารสกัด จากผลการทดลองพบว่ารูปแบบของการตั้งสูตรตำรับประสะเปราะใหญ่ควรหลีกเลี่ยงความชื้น อุณหภูมิที่สูง การออกซิเดชั่น และในสภาวะที่เป็นกรด สำหรับการศึกษาในสภาวะเร่งสารสกัดตำรับประสะเปราะใหญ่ชั้นเอทานอลมีความคงตัว เก็บไว้ได้อย่างน้อยสองปีที่อุณหภูมิห้อง ในภาชนะที่ปิดสนิทและไม่โดนแสง โดยที่ฤทธิ์ต้านการแพ้และฤทธิ์ต้านการอักเสบไม่เปลี่ยนแปลง การศึกษานี้สามารถสนับสนุนการใช้ยาตำรับประสะเปราะใหญ่ในทางการแพทย์แผนไทยเพื่อรักษาอาการไข้ อาการหวัด และอาการหอบหืดได้

คำสำคัญ: สภาวะเร่ง, ต้านการแพ้, ต้านการอักเสบ, สภาวะเครียด, ตำรับประสะเปราะใหญ่