Chapter 4

Findings and Results

1. Preparation of plant extracts

The ethanolic extracts and water extracts of Prasaprohyai preparation and its ingredients were prepared as described in Chapter 3 (section 2.1). The crude ethanolic extracts and water extracts were obtained either in the forms of sticky mass or powder. The percentage of yields of Prasaprohyai preparation and its ingredients were shown as percentage by weight in Table 4.1.

Table 4.1

Percentage of yields of the ethanolic extracts and water extracts of Prasaprohyai preparation and its ingredients

Plant species	Extract	Code	% Yield (w/w)
Amomum testaceum Ridl.	Et	AmTEt	2.42
(Krawan)	EW	AmTEW	9.32
	HW	AmTHW	2.41
Anethum graveolens L.	Et	AnGEt	4.34
(Thian ta takkataen)	EW	AnGEW	6.67
	HW	AnGHW	9.66
Angelica dahurica Benth.	Et	AnDEt	5.12
(Kot so)	EW	AnDEW	9.78
	HW	AnDHW	2.54
Angelica sinensis (Oliv.) Diels	Et	AnSEt	15.05
(Kot chiang)	EW	AnSEW	17.83
	HW	AnSHW	11.39

Table 4.1 (Continued)

Plant species	Extract	Code	% Yield (w/w)
Artemisia annua L.	Et	ArAEt	4.27
(Kot chula lampha)	EW	ArAEW	11.16
	HW	ArAHW	12.14
Atractylodes lancea (Thunb.) DC.	Et	AtLEt	16.89
(Kot kamao)	EW	AtLEW	19.29
	HW	AtLHW	18.22
Cuminum cyminum L.	Et	CuCEt	8.73
(Thian khao)	EW	CuCEW	10.83
	HW	CuCHW	7.31
Dracaena loureiri Gagnep.	Et	DrLEt	17.87
(Chan daeng)	EW	DrLEW	0.58
	HW	DrLHW	0.79
Foeniculum vulgare Mill. var. *dulce*	Et	FoVEt	6.69
(Mill.) Thell.	EW	FoVEW	11.09
(Thian khao plueak)	HW	FoVHW	6.46
Kaempferia galanga L.	Et	KaGEt	6.39
(Proh hom)	EW	KaGEW	21.51
	HW	KaGHW	3.38
Lepidium sativum L.	Et	LeSEt	9.20
(Thian daeng)	EW	LeSEW	0.87
	HW	LeSHW	6.37
Ligusticum sinense Oliv. cv. Chuanxiong	Et	LiSEt	12.19
(Kot hua bua)	EW	LiSEW	7.94
	HW	LiSHW	7.24
Mammea siamensis Kosterm.	Et	MaSEt	32.78
(Saraphi)	EW	MaSEW	8.76
	HW	MaSHW	10.42

Table 4.1 (Continued)

Plant species	Extract	Code	% Yield (w/w)
Mesua ferrea L.	Et	MeFEt	23.17
(Bunnak)	EW	MeFEW	15.45
	HW	MeFHW	4.65
Mimusops elengi L.	Et	MiEET	8.82
(Phikul)	EW	MiEEW	4.34
	HW	MiEHW	3.97
Myristica fragrans Houtt.	Et	MyFEt	7.07
(Chan thet)	EW	MyFEW	0.79
	HW	MyFHW	0.44
Myristica fragrans Houtt.	Et	MyFEt(A)	18.97
(Mace)	EW	MyFEW(A)	2.62
	HW	MyFHW(A)	2.63
Myristica fragrans Houtt.	Et	MyFEt(S)	13.67
(Nutmeg)	EW	MyFEW(S)	7.14
	HW	MyFHW(S)	4.70
Nelumbo nucifera Gaertn.	Et	NeNEt	10.59
(Kasorn bua luang)	EW	NeNEW	6.11
	HW	NeNHW	3.04
Nigella sativa L.	Et	NiSEt	32.29
(Thian dam)	EW	NiSEW	6.78
	HW	NiSHW	6.02
Syzygium aromaticum (L.) Merr. et Perry	Et	SyAEt	31.24
(Kan phlu)	EW	SyAEW	6.53
	HW	SyAHW	6.42
Prasaprohyai formula	Et	PSPYEt	18.66
	EW	PSPYEW	3.75
	HW	PSPYHW	5.15

Extract: Et = Maceration, EW = Residue of maceration and then decoction,
HW = Decoction

2. Assay for antioxidant activity

2.1 DPPH radical scavenging assay

The DPPH assay was employed to determine the ability of samples to capture free radicals. The results of antioxidant activity using DPPH assay of Prasaprohyai preparation and its ingredient are shown in Table 4.2 and Figure 4.1-4.3. A lower value of EC_{50} indicates high antioxidant activity. The results found that seven ethanolic extracts (*Dracaena loureiri*, *Mammea siamensis*, *Mesua ferrea*, *Mimusops elengi*, *Myristica fragrans* (Mace), *Myristica fragrans* (Nutmeg) and *Syzygium aromaticum*) and seven water extracts (*Dracaena loureiri* (HW), *Mammea siamensis* (HW), *Mesua ferrea* (EW, HW), *Nelumbo nucifera* (HW) and *Syzygium aromaticum* (EW, HW)) showed high antioxidant activity with EC_{50} in the range of 6.57-18.02 μg/ml and 4.73-23.01 μg/ml, respectively. Interestingly, the ethanolic extracts of *Mammea siamensis*, *Mimusops elengi*, *Myristica fragrans* (Nutmeg) and *Syzygium aromaticum*, and the water extracts of *Mammea siamensis* (HW), *Mesua ferrea* (EW, HW), *Nelumbo nucifera* (HW) and *Syzygium aromaticum* (EW, HW) showed an antioxidative effect higher than BHT which is the reference standard, especially the water extract of *Syzygium aromaticum* (EW) which showed the highest antioxidant activity in this test with EC_{50} value of 4.73 μg/ml.

Table 4.2

EC_{50} (µg/ml) of plant extracts by DPPH assay (n = 3)

Plant species	**Code**	**% Inhibition at conc. 100 µg/ml**	**EC_{50}±SEM (µg/ml)**
Amomum testaceum Ridl.	AmTEt	69.69±0.43	64.45±1.71
(Krawan)	AmTEW	35.22±3.91	>100
	AmTHW	-11.33±1.28	>100
Anethum graveolens L.	AnGEt	32.02±0.85	>100
(Thian ta takkataen)	AnGEW	68.31±2.59	65.45±3.88
	AnGHW	18.28±2.96	>100
Angelica dahurica Benth.	AnDEt	42.47±1.36	>100
(Kot so)	AnDEW	28.21±1.95	>100
	AnDHW	-20.75±0.93	>100
Angelica sinensis (Oliv.) Diels	AnSEt	44.17±4.86	>100
(Kot chiang)	AnSEW	26.75±2.51	>100
	AnSHW	-4.24±0.64	>100
Artemisia annua L.	ArAEt	64.06±2.73	72.40±3.95
(Kot chula lampha)	ArAEW	80.80±1.75	32.75±1.78
	ArAHW	44.01±2.84	>100
Atractylodes lancea (Thunb.)	AtLEt	46.67±1.20	>100
DC.	AtLEW	16.47±1.22	>100
(Kot kamao)	AtLHW	-1.88±0.83	>100
Cuminum cyminum L.	CuCEt	36.49±1.21	>100
(Thian khao)	CuCEW	31.76±1.96	>100
	CuCHW	20.48±3.36	>100
Dracaena loureiri Gagnep.	DrLEt	91.26±1.12	17.28±1.53
(Chan daeng)	DrLEW	81.85±3.37	44.97±4.66
	DrLHW	89.89±1.58	23.01±1.72

Table 4.2 (Continued)

Plant names	Code	% Inhibition at conc. 100 μg/ml	EC_{50}±SEM (μg/ml)
Foeniculum vulgare Mill. var.	FoVEt	21.38±4.06	>100
dulce (Mill.) Thell.	FoVEW	81.85±3.37	88.48±4.10
(Thian khao plueak)	FoVHW	14.12±2.41	>100
Kaempferia galanga L.	KaGEt	44.72±2.00	>100
(Proh hom)	KaGEW	1.53±3.05	>100
	KaGHW	-7.15±0.84	>100
Lepidium sativum L.	LeSEt	35.45±4.12	>100
(Thian daeng)	LeSEW	20.23±1.04	>100
	LeSHW	20.73±1.81	>100
Ligusticum sinense Oliv. cv.	LiSEt	72.16±3.31	56.96±3.85
Chuanxiong	LiSEW	35.09±4.28	>100
(Kot hua bua)	LiSHW	10.18±1.17	>100
Mammea siamensis Kosterm.	MaSEt	95.28±0.23	8.54±0.73
(Saraphi)	MaSEW	85.38±1.69	32.69±1.95
	MaSHW	90.67±0.21	8.70±0.58
Mesua ferrea L.	MeFEt	94.25±0.40	16.12±0.93
(Bunnak)	MeFEW	92.07±0.72	7.49±0.57
	MeFHW	91.64±0.45	6.95±0.27
Mimusops elengi L.	MiEEt	95.40±0.40	8.19±0.40
(Phikul)	MiEEW	76.99±2.93	54.28±3.40
	MiEHW	41.06±1.29	>100
Myristica fragrans Houtt.	MyFEt	82.66±2.10	46.62±2.08
(Chan thet)	MyFEW	77.48±3.08	48.93±2.67
	MyFHW	87.01±2.91	34.82±3.99
Myristica fragrans Houtt.	MyFEt(A)	83.96±0.96	18.02±0.76
(Mace)	MyFEW(A)	25.32±2.59	>100
	MyFHW(A)	1.82±1.83	>100

Table 4.2 (Continued)

Plant names	Code	% Inhibition at conc. 100 μg/ml	EC_{50}±SEM (μg/ml)
Myristica fragrans Houtt.	MyFEt(S)	87.98±1.12	11.38±0.64
(Nutmeg)	MyFEW(S)	31.22±1.10	>100
	MyFHW(S)	8.45±1.24	>100
Nelumbo nucifera Gaertn.	NeNEt	57.12±1.90	83.27±2.98
(Kasorn bua luang)	NeNEW	87.23±1.02	32.72±2.55
	NeNHW	88.45±1.82	8.87±0.01
Nigella sativa L.	NiSEt	5.56±0.47	>100
(Thian dam)	NiSEW	36.62±1.26	>100
	NiSHW	14.26±1.65	>100
Syzygium aromaticum (L.)	SyAEt	93.94±0.86	6.57±0.31
Merr. et Perry	SyAEW	93.30±0.52	4.73±0.18
(Kan phlu)	SyAHW	92.47±0.48	5.30±0.35
Prasaprohyai formula	PSPYEt	70.10±2.01	42.98±2.60
	PSPYEW	24.76±1.02	>100
	PSPYHW	37.11±0.17	>100
BHT (positive control)	-	87.54±0.87	11.66±1.01

n = number of independent experiment

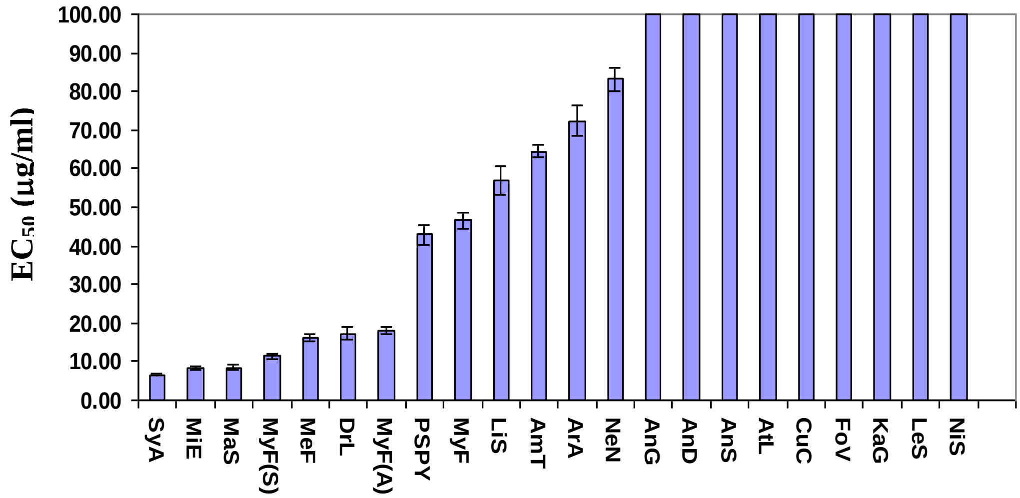

Figure 4.1

Antioxidant activity of ethanolic extract (Et) of Prasaprohyai preparation and its ingredients on DPPH assay, used BHT as positive control

(EC_{50} of BHT = 11.66 μg/ml)

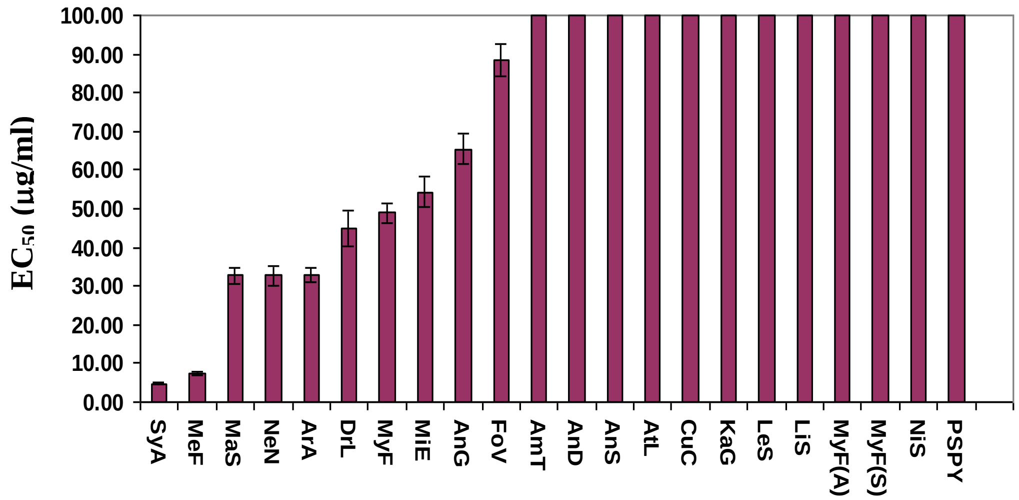

Figure 4.2

Antioxidant activity of water extract (residue) (EW) of Prasaprohyai preparation and its ingredients on DPPH assay, used BHT as positive control

(EC_{50} of BHT = 11.66 μg/ml)

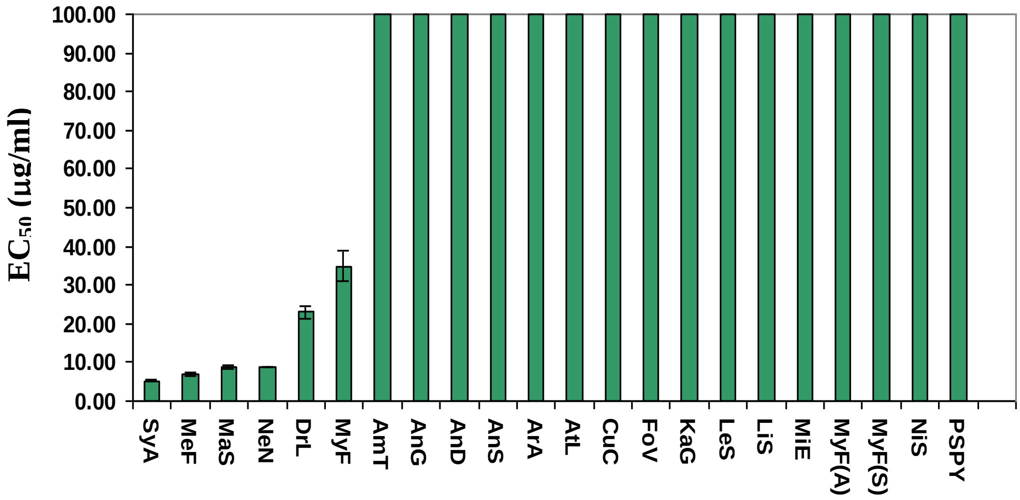

Figure 4.3

Antioxidant activity of water extract (HW) of Prasaprohyai preparation and its ingredients on DPPH assay, used BHT as positive control (EC_{50} of BHT = 11.66 µg/ml)

The results of antioxidant activity of ethanolic extract (Et) of Prasaprohyai and its ingredients showed that the extracts of *Syzygium aromaticum*, *Mimusops elengi*, *Mammea siamensis* and *Myristica fragrans* (Nutmeg) exhibited higher DPPH radical scavenging activity than BHT which is the reference standard (IC_{50} = 6.57, 8.19, 8.54, 11.38, and 11.66 µg/ml, respectively). The results of antioxidant activity of water extract (residue) (EW) of Prasaprohyai and its ingredients showed that the extracts of *Syzygium aromaticum* and *Mesua ferrea* exhibited higher than BHT (IC_{50} = 4.73, 7.49 and 11.66 µg/ml, respectively). Moreover, the results showed that the water extract (HW) of *Syzygium aromaticum*, *Mesua ferrea*, *Mammea siamensis* and *Nelumbo nucifera* displayed higher than BHT too (IC_{50} = 5.30, 6.95, 8.70, 8.87 and 11.66 µg/ml, respectively).

The results of antioxidant activity of all extracts (Et, EW, HW) of Prasaprohyai and its ingredients showed that especially the water extract (residue) (EW) of *Syzygium aromaticum* which showed the highest antioxidant activity in this test (IC_{50} = 4.73µg/ml). It indicated that *Syzygium aromaticum* demonstrated the highest antioxidant effect by acting as a free radical scavenger. Surprisingly, there were no reports on stem of *Dracaena loureiri* and flower of *Mesua ferrea* which showed antioxidant activity. In addition, there have been reports on antioxidant activity by DPPH assay such as the flower of *Mammea siamensis* (Leelapornpisid, Chansakaow, Chiyasut, & Wongwattananukul, 2008), the flower of *Mimusops elengi* (Aromdee, Vorarat, & Benjamapriyagoon, 2005), the aril and seed of *Myristica fragrans* (Khatun, Eguchi, Yamaguchi, Takamura, & Matoba, 2006). The stile and stigma of *Nelumbo nucifera* also showed antioxidant activity by the DPPH and $ONOO^-$ assay (Hyun, Jung, Chung, Jung, & Choi, 2006) and the aroma extract from clove buds (*Syzygium aromaticum*) inhibited the oxidation of hexanal for 30 days at a level of 50 μg/ml and malonaldehyde formation from cod liver oil by 93% at the 160 μg/ml level (Lee & Shibamoto, 2001).

The previous report of phytochemical studies on these plants, except for *Mesua ferrea* and *Mimusops elengi*, have indicated the presence of flavonoids and phenolic compounds (Hyun, Jung, Chung, Jung, & Choi, 2006; Jukic, Politeo, & Milos, 2006; Jung, Kim, Chung, & Choi, 2003; Lee & Shibamoto, 2001; Phuwapraisirisan et al., 2001; Surveswaran et al., 2007). Since the flavonoids and phenolic compounds have also been known to have antioxidant properties, their presence in these species could, therefore, be the basis for the observed antioxidant activity (Abas, Lajis, Israf, Khozirah, & Kalsom, 2006). The strong antioxidant activity of these plants is due to the presence of the flavonoids and phenolic compounds.

3. *In vitro* assay for anti-inflammatory activity

3.1 Assay for NO inhibitory effect

Anti-inflammatory properties of the ethanolic extracts and water extracts of Prasaprohyai preparation and its ingredients and positive control were tested by measuring their effects on the pro-inflammatory mediator nitric oxide (NO) in activated murine macrophages cell line (RAW 264.7). Measurement of nitrtrite accumulation in the culture medium was used to determine NO production. The nitrite concentration was measured by Griess reaction.

To determine the best condition for inducing NO production by murine macrophages cell line (RAW 264.7) were treated with RPMI 1640 complete media with various concentrations of lipopolysaccharide (LPS) as shown in Figure 4.4. Lipopolysaccharide (LPS) stimulated the highest NO production by RAW 264.7 cells at concentration of 5 µg/ml. Therefore, the 5 µg/ml LPS was used to induce NO production by RAW 264.7 cells in this study.

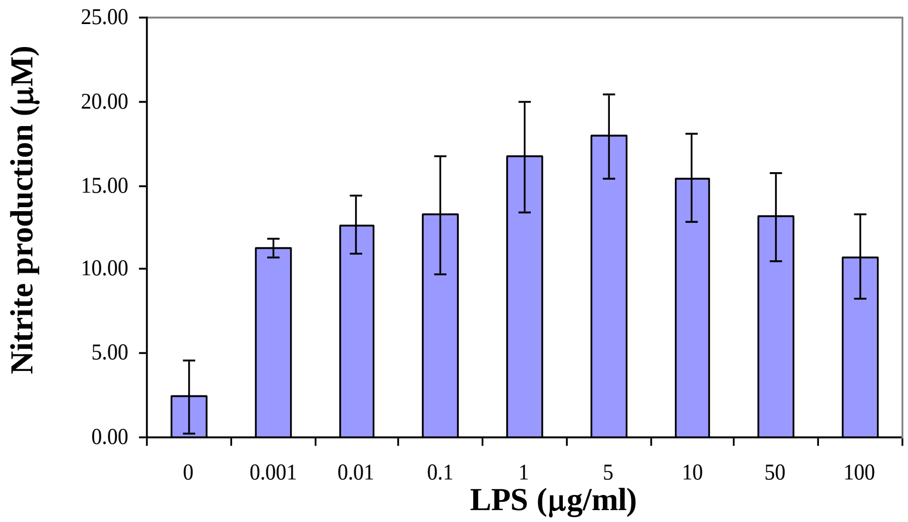

Figure 4.4

Concentration of NO production by RAW 264.7 cells stimulated with LPS (0-100 µg/ml) for 48 h, after which the NO released was measured as nitrite using the Griess reagent (n = 2)

To determine the inhibitory effects of Prasaprohyai preparation and its ingredient on NO production and TNF-α release. RAW 264.7 cells were incubated with the LPS. The extracts induced inhibition of NO production and TNF-α at concentration range of 1-100 μg/ml that had no effect on cell viability (cell viability less than 70-80%).

The results of inhibitory activity against LPS induced NO production using Griess reagent of Prasaprohyai preparation and its ingredient were shown in Table 4.3, 4.4 and Figure 4.5. Among the plant species that were studied, the ethanolic extracts of Prasaprohyai preparation and its ingredient exhibited NO production inhibitory activity, whereas the water extracts were apparently inactive. The results found that seven ethanolic extracts namely *Angelica sinensis*, *Artemisia annua*, *Atractylodes lancea*, *Cuminum cyminum*, *Ligusticum sinense, Mesua ferrea* and Prasaprohyai formula showed potent inhibitory activity, with IC_{50} in the range of 9.70-26.23 μg/ml. Interestingly, these plant extracts exhibited a NO production inhibitory effect higher than Indomethacin (IC_{50} = 56.78 μM or 20.32 μg/ml) which is a positive control but not for *Mesua ferrea*. Especially, the ethanolic extract of *Atractylodes lancea* exhibited the highest potent inhibitory activity in this test with IC_{50} value of 9.70 μg/ml.

Table 4.3

Inhibitory effects of the ethanolic extracts of Prasaprohyai preparation and its ingredients on LPS-induced NO production from RAW 264.7 cells and percentage of viable cells at various concentration (n = 3)

Plant species	% Inhibition at various concentration (µg/ml)[a] / (percentage of viable cells at various concentration)							
	1	3	10	30	50	70	80	100
Amomum testaceum Ridl.	-63.80±3.29	-64.37±11.32	-39.40±6.80	-12.33±10.26**	-	-	-	82.50±4.53*
	(107.81±0.75)	(108.92±1.68)	(90.78±4.48)	(96.37±8.0)				(88.99±4.84)
Anethum graveolens L.	-63.50±10.52	-70.97±14.54	-28.80±6.91	-3.87±9.08**	-	-	-	83.13±3.20*
	(110.88±4.17)	(107.07±7.68)	(101.71±9.73)	(98.13±9.01)				(91.92±2.38)
Angelica dahurica Benth.	-26.33±0.88**	-25.00±2.31**	11.67±1.45**	38.33±3.93**	56.33±1.45**	-	-	88.33±1.76[b]
	(107.89±1.63)	(104.06±1.19)	(93.43±2.69)	(85.02±6.79)	(82.00±4.63)			(18.83±0.75)
Angelica sinensis (Oliv.)	-65.27±6.53	-62.63±4.52	32.27±15.57	82.23±4.37	-	-	-	93.60±0.71[b]
Diels	(108.15±2.84)	102.35±3.69)	(98.96±4.28)	(96.75±5.56)				(26.76±7.13)
Artemisia annua L.	-75.40±1.30	-59.47±3.36	9.60±6.82	73.40±7.68	-	-	-	96.47±2.23[b]
	(114.77±3.19)	(114.78±2.74)	(110.74±1.38)	(79.30±2.45)				(19.20±0.89)
Atractylodes lancea	-26.47±11.67**	-26.10±10.55*	56.60±7.94**	84.53±7.14	-	-	-	94.03±3.22[b]
(Thunb.) DC.	(96.84±1.83)	(100.06±2.96)	(94.13±3.62)	(93.85±3.05)				(12.53±2.74)
Cuminum cyminum L.	-92.90±2.84	-101.57±0.85**	3.40±4.75	46.03±4.73**	64.00±3.96**	-	-	92.57±2.05[b]
	(112.47±1.36)	(114.39±0.27)	(99.72±1.19)	(86.84±3.71)	(72.97±1.30)			(66.15±1.87)
Dracaena loureiri Gagnep.	-45.33±6.44**	-20.33±3.48**	-10.67±2.91	33.00±1.53**	59.67±1.20**	-	-	89.00±2.87[b]
	(99.34±0.58)	(101.27±1.34)	(100.86±1.30)	(102.55±1.91)	(98.59±3.24)			(61.16±0.61)

Table 4.3 (Continued)

Plant species	% Inhibition at various concentration (μg/ml)[a] / (percentage of viable cells at various concentration)							
	1	**3**	**10**	**30**	**50**	**70**	**80**	**100**
Foeniculum vulgare Mill.	-32.57±3.28**	-41.90±2.28**	-14.20±0.70	33.13±1.03**	69.70±2.76*	-	-	89.57±1.59^{b}
var. *dulce* (Mill.) Thell.	(99.23±5.37)	(93.89±0.54)	(97.00±5.41)	(79.58±6.38)	(77.55±0.84)			(19.52±0.82)
Kaempferia galanga L.	-59.30±1.39**	-45.93±4.42**	-4.60±2.74	49.27±1.80*	81.93±2.19	-	-	94.60±3.29^{b}
	(113.23±1.29)	(113.59±0.96)	(113.10±2.71)	(109.36±0.31)	(79.59±3.82)			(11.30±0.34)
Lepidium sativum L.	-	-	-	-	-	-	-	44.93±1.67
								(108.41±9.44)
Ligusticum sinense Oliv.	-61.40±12.21	-53.53±15.79	7.60±13.19	79.40±1.53	-	-	-	92.00±4.70^{b}
cv. Chuanxiong	(109.94±5.07)	(107.93±0.38)	(95.36±5.21)	(83.40±5.80)				(56.99±2.22)
Mammea siamensis	-51.97±16.29	-46.23±11.78	-33.67±14.30	-4.1±11.17**	-	-	-	73.07±3.64**
Kosterm.	(111.37±7.21)	(105.44±7.18)	(105.04±6.51)	(89.09±5.37)				(77.88±0.82)
Mesua ferrea L.	-71.30±14.47	-52.20±14.25	-12.40±18.66	54.40±3.65	-	-	-	96.03±1.82^{b}
	(105.63±4.51)	(106.37±1.73)	(80.11±6.62)	(75.83±3.06)				(9.21±0.57)
Mimusops elengi L.	-67.50±4.57	-	-53.63±5.76	-	35.73±5.00	45.93±5.38	67.10±3.23	83.33±2.83^{b}
	(112.78±3.30)		(103.29±2.59)		(97.64±1.65)	(92.79±1.97)	(87.63±1.89)	(63.19±0.43)
Myristica fragrans Houtt.	-64.00±7.39	-54.50±14.34	-7.73±13.18	69.80±8.37	-	-	-	95.57±1.79^{b}
(Chan thet)	(108.44±6.15)	(111.49±3.45)	(103.13±5.76)	(91.86±9.72)				(11.06±0.51)
Myristica fragrans Houtt.	-65.10±10.57	-68.47±16.52	-27.40±8.30	10.70±1.10**	-	-	-	88.97±2.71
(Mace)	(113.21±1.74)	(106.68±4.00)	(101.00±4.36)	(98.21±1.87)				(82.33±6.19)

Table 4.3 (Continued)

Plant species	% Inhibition at various concentration (μg/ml)[a] / (percentage of viable cells at various concentration)							
	1	**3**	**10**	**30**	**50**	**70**	**80**	**100**
Myristica fragrans Houtt. (Nutmeg)	-97.10±2.48 (113.85±2.79)	-89.57±3.57 (108.17±1.14)	-47.47±6.56** (97.90±3.32)	3.00±2.76** (95.33±1.80)	59.27±1.18** (75.49±1.82)	-	-	91.60±3.63[b] (8.56±0.31)
Nelumbo nucifera Gaertn.	-	-	-	-	-	-	-	44.20±2.03 (97.97±1.19)
Nigella sativa L.	-	-	-	-	-	-	-	27.60±7.97 (97.97±0.69)
Syzygium aromaticum (L.) Merr. et Perry	-70.30±6.61 (107.40±6.51)	-69.53±6.72 (103.11±6.78)	-55.40±8.90 (98.75±4.06)	-26.13±3.31** (90.14±0.99)	-	-	-	81.43±1.74* (77.73±5.49)
Prasaprohyai formula	-90.90±4.69 (117.91±0.93)	-85.53±2.28 (115.84±1.04)	-10.50±2.74 (114.70±1.52)	64.20±2.06 (114.04±2.42)	82.30±4.79 (109.89±3.20)	-	-	95.23±0.79[b] (50.84±5.80)
Ketotifen fumarate	-	-32.95±15.70[c] (84.27±11.96)	-	-9.15±3.40[c] (79.66±1.76)	38.60±3.80[c] (82.46±4.87)	-	-	63.70±1.50[c] (91.26±1.15)

- = Not test

[a] Each value represents the mean ± SEM of three determinations. Significantly different from Prasaprohyai formula, *p<0.05, **p<0.01

[b] Cytotoxic effect was observed; [c] Value in μM

Table 4.4

IC_{50} (µg/ml) of plant extracts on LPS-induced NO production from RAW 264.7 cells (n = 3)

Plant species	Code	Inhibition of NO production	
		% Inhibition at conc. 100 µg/ml	IC_{50}±SEM (µg/ml)
Amomum testaceum Ridl.	AmTEt	82.50±4.54	81.42±3.48
(Krawan)	AmTEW	40.93±5.11	>100
	AmTHW	37.20±3.18[a]	>100
Anethum graveolens L.	AnGEt	83.13±3.20	84.97±4.48
(Thian ta takkataen)	AnGEW	27.93±2.26	>100
	AnGHW	53.80±9.93[a]	-
Angelica dahurica Benth.	AnDEt	88.33±1.76[a]	44.23±2.71
(Kot so)	AnDEW	13.17±0.72	>100
	AnDHW	13.50±0.35[a]	>100
Angelica sinensis (Oliv.) Diels	AnSEt	95.60±0.71[a]	12.52±2.31
(Kot chiang)	AnSEW	6.17±0.23	>100
	AnSHW	42.13±4.56	>100
Artemisia annua L.	ArAEt	96.47±2.23[a]	17.06±2.69
(Kot chula lampha)	ArAEW	16.50±0.80	>100
	ArAHW	35.17±3.08[a]	>100
Atractylodes lancea (Thunb.) DC.	AtLEt	94.03±3.22[a]	9.70±0.54
(Kot kamao)	AtLEW	49.30±0.35[a]	>100
	AtLHW	39.27±4.41[a]	>100
Cuminum cyminum L.	CuCEt	92.57±2.05[a]	13.56±0.59
(Thian khao)	CuCEW	62.50±0.20[a]	-
	CuCHW	27.40±7.31	>100
Dracaena loureiri Gagnep.	DrLEt	89.00±2.89[a]	38.37±1.66
(Chan daeng)	DrLEW	18.97±1.84	>100
	DrLHW	47.63±2.87[a]	>100

Table 4.4 (Continued)

Plant species	Code	Inhibition of NO production	
		% Inhibition at conc. 100 μg/ml	IC_{50}±SEM (μg/ml)
Foeniculum vulgare Mill. var.	FoVEt	89.57±1.59[a]	40.81±0.59
dulce (Mill.) Thell.	FoVEW	48.00±1.61[a]	>100
(Thian khao plueak)	FoVHW	14.33±3.21	>100
Kaempferia galanga L.	KaGEt	94.60±3.29[a]	30.30±1.23
(Proh hom)	KaGEW	12.13±1.25	>100
	KaGHW	45.77±4.13[a]	>100
Lepidium sativum L.	LeSEt	44.93±1.67	>100
(Thian daeng)	LeSEW	68.07±1.05[a]	-
	LeSHW	5.30±2.66	>100
Ligusticum sinense Oliv. cv.	LiSEt	92.00±4.70[a]	16.48±2.03
Chuanxiong	LiSEW	43.57±1.78[a]	>100
(Kot hua bua)	LiSHW	20.43±5.06	>100
Mammea siamensis Kosterm.	MaSEt	73.07±3.65	74.62±8.77
(Saraphi)	MaSEW	12.77±2.37	>100
	MaSHW	43.77±2.59[a]	>100
Mesua ferrea L.	MeFEt	96.03±1.82[a]	26.23±3.42
(Bunnak)	MeFEW	24.33±2.25	>100
	MeFHW	41.30±6.90	>100
Mimusops elengi L.	MiEEt	83.33±2.83[a]	69.24±5.30
(Phikul)	MiEEW	35.90±2.03	>100
	MiEHW	43.40±6.05[a]	>100
Myristica fragrans Houtt.	MyFEt	95.57±1.80[a]	30.42±3.58
(Chan thet)	MyFEW	25.40±1.83	>100
	MyFHW	40.50±4.40[a]	>100

Table 4.4 (Continued)

Plant species	Code	Inhibition of NO production	
		% Inhibition at conc. 100 μg/ml	IC_{50}±SEM (μg/ml)
Myristica fragrans Houtt.	MyFEt(A)	88.97±2.71	78.38±1.82
(Mace)	MyFEW(A)	46.37±2.28	>100
	MyFHW(A)	44.13±4.06	>100
Myristica fragrans Houtt.	MyFEt(S)	78.38±1.84[a]	47.23±0.32
(Nutmeg)	MyFEW(S)	38.37±3.03	>100
	MyFHW(S)	36.30±2.87	>100
Nelumbo nucifera Gaertn.	NeNEt	44.20±2.03	>100
(Kasorn bua luang)	NeNEW	48.57±0.37	>100
	NeNHW	42.23±1.38[a]	>100
Nigella sativa L.	NiSEt	27.60±7.97	>100
(Thian dam)	NiSEW	7.20±0.53	>100
	NiSHW	78.20±3.02[a]	-
Syzygium aromaticum (L.) Merr. et	SyAEt	81.43±1.74	81.34±2.62
Perry	SyAEW	27.37±1.59	>100
(Kan phlu)	SyAHW	24.43±1.64	>100
Prasaprohyai formula	PSPYEt	95.23±0.79[a]	18.40±0.43
	PSPYEW	24.77±1.59	>100
	PSPYHW	65.57±2.04[a]	-
Indomethacin	-	63.70±1.50[b]	56.78±3.28[b]

n = number of independent experiment

\- = Not test

[a] Cytotoxic effect was observed

[b] Value in μM

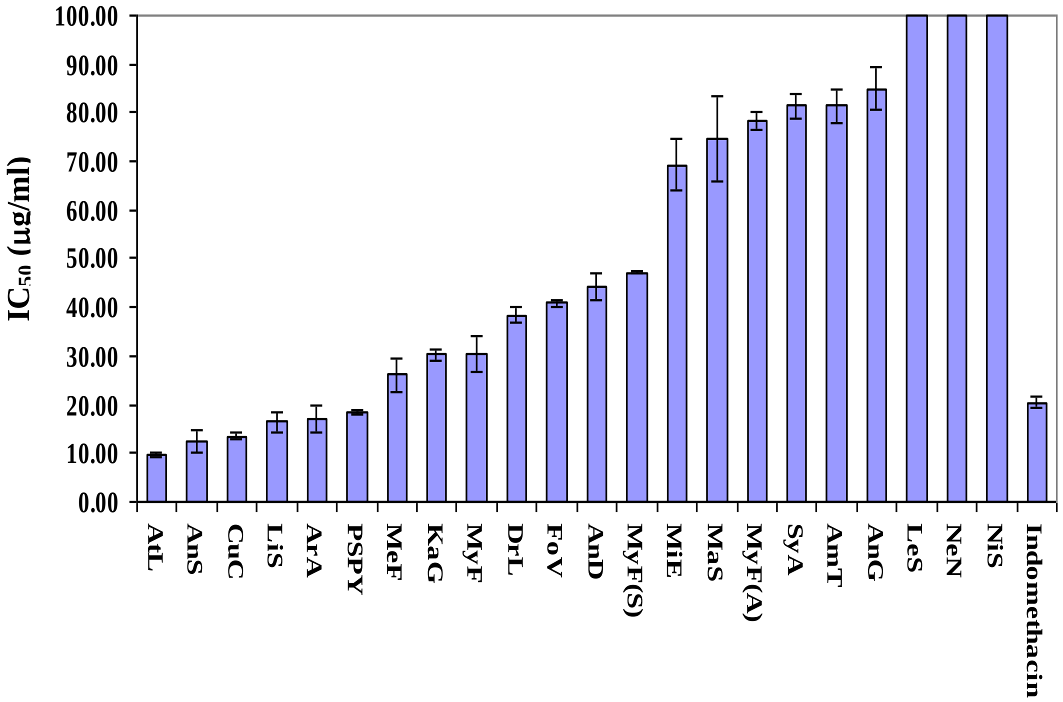

Figure 4.5

IC_{50} of ethanolic extracts of Prasaprohyai preparation and its ingredients on NO inhibitory activity using Griess reagent, used Indomethacin as positive control (IC_{50} of Indomethacin = 20.32 μg/ml)

3.2 Inhibitory effects on LPS-induced TNF-α release from RAW 264.7 cells

The results of inhibitory effects on LPS-induced TNF-α release in RAW 264.7 cells using Quantikine mouse TNF-α ELISA test kit of Prasaprohyai preparation and its ingredient were shown in Table 4.5. The result revealed that ethanolic extract of *Cuminum cyminum* demonstrated the most potent activity against TNF-α release with IC_{50} value of 7.95 μg/ml, followed by ethanolic extracts of Prasaprohyai formula and *Atractylodes lancea* with IC_{50} values of 20.34 and 24.35 μg/ml, respectively.

Table 4.5

Inhibitory effects of plant extracts on LPS-induced TNF-α release from RAW 264.7 cells

Plant species	Code	Inhibition of TNF-α release	
		% Inhibition at conc. 100 μg/ml	IC_{50}±SEM (μg/ml)
Angelica sinensis (Kot chiang)	AnSEt	86.59±0.17[a]	>30
Artemisia annua (Kot chula lampha)	ArAEt	87.68±0.92[a]	>30
Atractylodes lancea (Kot kamao)	AtLEt	81.62±0.69[a]	24.35±1.19
Cuminum cyminum (Thian khao)	CuCEt	71.75±3.88[a]	7.95±0.62
Ligusticum sinense (Kot hua bua)	LiSEt	3.66±1.15[a]	>100
Prasaprohyai formula	PSPYEt	91.88±1.76[a]	20.34±0.61

[a] Cytotoxic effect was observed

Nitric oxide (NO), the highly unstable gas, appears to be a major macrophage mediator of tumor cell killing. However, high level of NO production may induce host cell death and inflammatory tissue damage (Wibuloutai, 2006). Numerous cytokines and microbial product, often acting in synergistic pairs, stimulate production of NO. The effective agents and combinations depend on cell type and species. With the RAW 264.7 macrophage-like cell lines, bacterial LPS has been the only agent to be effective when tested alone (MacMicking, Xie, & Nathan, 1997). Therefore, bacterial LPS alone were used in this study to activate RAW 264.7 cells for 48 h to induce NO production. Direct measurement of NO is difficult, because NO is a short-lived free radical and a very small compound that diffuses freely within cells from its site of formation to its site of action (Aktan, 2004). The rapid diffusion of NO and its transient nature require further analytical methods for spatial detection that have rapid response times. Nowadays, the primary methods for detecting NO include spectroscopic methods, such as absorbance, fluorescence, chemiluminescence, and electron paramagnetic resonance (EPR), and electrochemical techniques, such as permselective, and electrocatalytic (Hetrick & Schoenfisch, 2009).

One of the most common spectroscopic methods for detecting NO from a wide variety of samples and matrices is the diazotization assay, also known as the Griess reaction. The Griess reaction actually measures nitrite (NO_2^-). Fortunately, NO's reactivity results in the formation of nitrite (NO_2^-) in oxygenated media. The reaction involved reacting nitrite (NO_2^-) with sulfaniliamide and *N*-(1-naphthyl)ethylenediamine under acidic conditions to yield an azo dye, whose concentration could then be used as an indirect indicator for nitrite (NO_2^-) (and NO) concentration in the sample (Hetrick & Schoenfisch, 2009). The present study used the Griess reaction to determine NO production since the method is simple, inexpensive, and sensitive enough to detect the induced form of NO production.

In the anti-inflammatory activity tests of this study, the results showed that the ethanolic extracts of *Angelica sinensis, Artemisia annua*, *Atractylodes lancea*, *Cuminum cyminum*, *Ligusticum sinense* and Prasaprohyai formula demonstrated higher inhibitory activity against LPS induced NO production using Griess reagent than Indomethacin which is a positive control, especially the ethanolic extract of *Atractylodes lancea* which showed the highest inhibitory activity in this test. Moreover, the potent inhibitory activity of seven ethanolic extracts were also tested for the inhibitory effects on LPS-induced TNF-α release in RAW 264.7 cells using Quantikine mouse TNF-α ELISA test kit. The result showed that the *Atractylodes lancea*, *Cuminum cyminum* and Prasaprohyai formula extracts demonstrated potent activity against TNF-α release, especially the *Atractylodes lancea* extract which showed the highest inhibitory activity in this test. These results related with the previous investigation which found that the water and ethanol extracts of *Angelica sinensis* inhibited NO production in LPS activated RAW 264.7 macrophages (Huang, Chen, Lin, & Chiang, 2008). Atractylenolide I isolated from *Atractylodes lancea* which inhibited LPS-induction of NO and TNF-α production (Wang, He, Wang, & Liu, 2009). Although the extract of *Nigella sativum* had no anti-inflammatory activity or inhibitory activity on NO production, there have been previous reports about anti-inflammatory activity, because the extract is pure compound (El-Mahmoudy et al., 2002).

In addition, there have been reports on anti-inflammatory activity of the extracts. For example, the root extract of *Angelica dahurica* showed anti-inflammatory activity by COX-2, COX-1, 5-LOX and PGE2 assay (Ban et al., 2003; Hua et al., 2008; Moon, Jin, Son, & Chang, 2008). The root extract of *Angelica sinensis* (Ozaki, 1992) and seed extract of *Lepiduim sativum* (Al-Yahya, Mossa, Ageel, & Rafatullah, 1994) showed anti-inflammatory activity by *in vivo* assay. The rhizome extract of the *Atractylodes lancea* showed inhibitory activity on COX-1 and 5-LOX (Resch, Steigel, Chen, & Bauer, 1998). The stilbene isolated from *Dracaena loureiri* showed inhibitory activity on COX-2 (Sawasdee, 2001). The aril extract of *Myristica fragrans* showed anti-inflammatory activity by *in vivo* assay while the seed extract showed anti-inflammatory activity by *in vitro* assay (Jin, Lim, Hwang, Ha, & Han, 2005). The seed extract of *Nigella sativum* showed activitity in the *in vivo* inflammatory model of carrageenan-induced paw edema in rats (Al-Ghamdi, 2001), *in vitro* inflammatory assay of COX-2 (El-Mezayen et al., 2006; Landa, Marsik, Vanek, & Kokoska, 2007; Marsik et al., 2005), COX-1 assay (Landa et al., 2007).

4. *In vitro* assay for anti-allergic activity

4.1 Inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cells

The ethanolic and water extracts of Prasaprohyai preparation and its ingredients were determined for their anti-allergic effect. Inhibitory effects of the ethanolic and water extracts on the release of β-hexosaminidase in RBL-2H3 cells are shown in Table 4.6, 4.7 and Figure 4.6.

The results found that twelve ethanolic extracts showed potent activities in this study, whereas the water extracts were apparently inactive. As shown in Table 4.7, *Mammea siamensis* exhibited the most potent anti-allergic activity with an IC_{50} value of 7.90 μg/ml, followed by *Dracaena loureiri* (10.67 μg/ml), *Myristica fragrans* (Mace) (11.65 μg/ml), *Angelica dahurica* (12.81 μg/ml), *Mimusops elengi* (13.51 μg/ml), *Atractylodes lancea* (13.60 μg/ml), *Myristica fragrans* (Nutmeg) (13.89 μg/ml), *Mesua ferrea* (14.07 μg/ml), *Kaempferia galanga* (14.91 μg/ml), *Artemisia annua* (15.67 μg/ml), Prasaprohyai formula (16.59 μg/ml) and *Syzygium aromaticum* (23.69 μg/ml). The results also indicated that the anti-allergic effect of these plants were higher than that of Ketotifen fumarate, a positive control (IC_{50} = 94.9 μM or 40.41 μg/ml).

Table 4.6

Inhibitory effects of the ethanolic extracts of Prasaprohyai preparation and its ingredients on the release of β-hexosaminidase in RBL-2H3 cells at various concentration (n = 3)

Plant species	% Inhibition at various concentration (μg/ml)[a]				
	1	**3**	**10**	**30**	**100**
Amomum testaceum Ridl. (Krawan)	-	-	-	-	21.60±0.04
Anethum graveolens L. (Thian ta takkataen)	-	-5.52±13.13	18.10±0.91	31.81±3.00**	66.81±4.85**
Angelica dahurica Benth. (Kot so)	-	-70.47±17.63	27.03±3.98	50.56±0.09	88.89±1.43
Angelica sinensis (Oliv.) Diels (Kot chiang)	-	-	-	-	39.75±2.36
Artemisia annua L. (Kot chula lampha)	-	-44.63±9.87	20.40±3.78	62.83±4.62	99.64±0.85
Atractylodes lancea (Thunb.) DC. (Kot kamao)	-	-39.13±13.60	29.56±7.52	53.96±5.79	91.41±2.23
Cuminum cyminum L. (Thian khao)	-	-31.25±0.74	12.24±2.46	39.89±2.86	82.12±1.00
Dracaena loureiri Gagnep. (Chan daeng)	-	-89.94±10.24*	48.31±18.57*	90.16±11.84**	110.00±7.73**
Foeniculum vulgare Mill. var. *dulce* (Mill.) Thell. (Thian khao plueak)	-	-10.05±11.78	14.27±1.84	25.77±1.37**	70.45±4.08**
Kaempferia galanga L. (Proh hom)	-	-30.00±4.13	25.86±4.12	65.26±5.82	89.18±3.39
Lepidium sativum L. (Thian daeng)	-	-49.36±19.11	4.55±2.43	16.68±1.65**	60.56±4.05**

Table 4.6 (Continued)

Plant species	% Inhibition at various concentration (μg/ml)[a]				
	1	3	10	30	100
Ligusticum sinense Oliv. cv. Chuanxiong (Kot hua bua)	-	0.43±4.55	14.66±3.38	40.20±1.92	87.07±1.93
Mammea siamensis Kosterm. (Saraphi)	-	-45.07±19.54	79.90±4.93**	92.20±3.00**	96.74±1.49
Mesua ferrea L. (Bunnak)	-	-18.07±3.99	33.55±5.50	67.83±4.43	93.32±6.53
Mimusops elengi L. (Phikul)	-	-34.89±14.48	31.34±1.09	51.37±5.31	80.48±6.93
Myristica fragrans Houtt. (Chan thet)	-	-10.76±5.54	9.19±2.76	30.48±3.96**	84.31±1.63
Myristica fragrans Houtt. (Mace)	-	-30.59±5.63	38.97±2.45	77.43±1.64	94.41±1.91
Myristica fragrans Houtt. (Nutmeg)	-	-21.69±8.63	30.73±1.25	76.06±7.48	98.23±3.68
Nelumbo nucifera Gaertn. (Kasorn bua luang)	-	-	-	-	26.01±8.36
Nigella sativa L. (Thian dam)	-	-	-	-	6.75±3.44
Syzygium aromaticum (L.) Merr. et Perry (Kan phlu)	-	-30.98±12.11	9.03±5.57	60.26±4.46	85.22±2.79
Prasaprohyai formula	-	-35.14±9.55	22.38±2.02	61.09±3.51	92.21±1.41
Ketotifen fumarate	-48.40±7.16[b]	-41.30±8.00[b]	3.17±5.96[b]	23.30±8.07[b]	55.16±0.91[b]

- = Not test

[a] Each value represents the mean ± SEM of three determinations. Significantly different from Prasaprohyai formula, *p<0.05, **p<0.01; [b] Value in μM

Table 4.7

IC_{50} (μg/ml) of plant extracts on the release of β-hexosaminidase in RBL-2H3 cells (n = 3)

Plant species	Code	Anti-allergic activity	
		% Inhibition at conc. 100 μg/ml	**IC_{50}±SEM (μg/ml)**
Amomum testaceum Ridl.	AmTEt	21.60±0.04	>100
(Krawan)	AmTEW	-1.40±1.97	>100
	AmTHW	5.79±1.73	>100
Anethum graveolens L.	AnGEt	66.81±4.85	76.81±3.97
(Thian ta takkataen)	AnGEW	-19.95±5.66	>100
	AnGHW	-14.23±3.55	>100
Angelica dahurica Benth.	AnDEt	88.89±1.43	12.81±0.56
(Kot so)	AnDEW	13.54±8.91	>100
	AnDHW	7.72±1.57	>100
Angelica sinensis (Oliv.) Diels	AnSEt	39.75±2.36	>100
(Kot chiang)	AnSEW	13.51±6.49	>100
	AnSHW	-57.30±3.82	>100
Artemisia annua L.	ArAEt	99.64±0.85	15.67±0.82
(Kot chula lampha)	ArAEW	-31.30±2.50	>100
	ArAHW	-9.80±2.13	>100
Atractylodes lancea (Thunb.) DC.	AtLEt	91.41±2.23	13.60±1.32
(Kot kamao)	AtLEW	15.72±3.09	>100
	AtLHW	6.05±4.27	>100
Cuminum cyminum L.	CuCEt	82.12±1.00	63.59±5.50
(Thian khao)	CuCEW	-35.85±8.19	>100
	CuCHW	-9.51±2.91	>100
Dracaena loureiri Gagnep.	DrLEt	110.00±7.74	10.67±1.44
(Chan daeng)	DrLEW	-66.08±0.40	>100
	DrLHW	0.48±1.34	>100

Table 4.7 (Continued)

Plant species	Code	Anti-allergic activity	
		% Inhibition at conc. 100 μg/ml	IC_{50}±SEM (μg/ml)
Foeniculum vulgare Mill. var.	FoVEt	71.16±4.04	80.50±2.31
dulce (Mill.) Thell.	FoVEW	-11.44±7.96	>100
(Thian khao plueak)	FoVHW	-13.30±1.32	>100
Kaempferia galanga L.	KaGEt	89.18±3.39	14.91±0.86
(Proh hom)	KaGEW	7.55±2.29	>100
	KaGHW	10.76±1.58	>100
Lepidium sativum L.	LeSEt	60.56±4.06	93.95±0.49
(Thian daeng)	LeSEW	-34.38±4.35	>100
	LeSHW	-22.66±2.83	>100
Ligusticum sinense Oliv. cv.	LiSEt	87.07±1.93	41.69±1.41
Chuanxiong	LiSEW	-38.57±4.75	>100
(Kot hua bua)	LiSHW	-43.07±2.63	>100
Mammea siamensis Kosterm.	MaSEt	96.74±1.49	7.90±0.58
(Saraphi)	MaSEW	-36.47±2.43	>100
	MaSHW	7.63±1.75	>100
Mesua ferrea L.	MeFEt	86.65±13.07	14.07±1.79
(Bunnak)	MeFEW	-62.97±7.35	>100
	MeFHW	-4.15±1.65	>100
Mimusops elengi L.	MiEEt	80.48±6.93	13.51±0.63
(Phikul)	MiEEW	-20.78±3.86	>100
	MiEHW	-7.56±1.77	>100
Myristica fragrans Houtt.	MyFEt	84.31±1.63	59.89±4.04
(Chan thet)	MyFEW	-63.08±1.45	>100
	MyFHW	-10.31±3.17	>100

Table 4.7 (Continued)

Plant species	Code	Anti-allergic activity	
		% Inhibition at conc. 100 μg/ml	**IC_{50}±SEM (μg/ml)**
Myristica fragrans Houtt.	MyFEt(A)	94.41±1.91	11.65±0.54
(Mace)	MyFEW(A)	4.24±4.58	>100
	MyFHW(A)	-13.30±2.65	>100
Myristica fragrans Houtt.	MyFEt(S)	98.23±3.68	13.89±0.65
(Nutmeg)	MyFEW(S)	1.39±5.52	>100
	MyFHW(S)	-83.62±1.83	>100
Nelumbo nucifera Gaertn.	NeNEt	26.01±8.36	>100
(Kasorn bua luang)	NeNEW	-36.46±7.76	>100
	NeNHW	-24.17±1.37	>100
Nigella sativa L.	NiSEt	6.75±3.44	>100
(Thian dam)	NiSEW	-23.71±4.78	>100
	NiSHW	-63.21±2.82	>100
Syzygium aromaticum (L.) Merr. et	SyAEt	85.22±2.79	23.69±2.34
Perry	SyAEW	-28.30±2.88	>100
(Kan phlu)	SyAHW	-129.22±4.04	>100
Prasaprohyai formula	PSPYEt	92.21±1.41	16.59±1.68
	PSPYEW	-39.18±4.84	>100
	PSPYHW	-33.40±2.81	>100
Ketotifen fumarate	-	55.16±0.91[a]	94.98±1.71[a]

n = number of independent experiment

[a] Value in μM

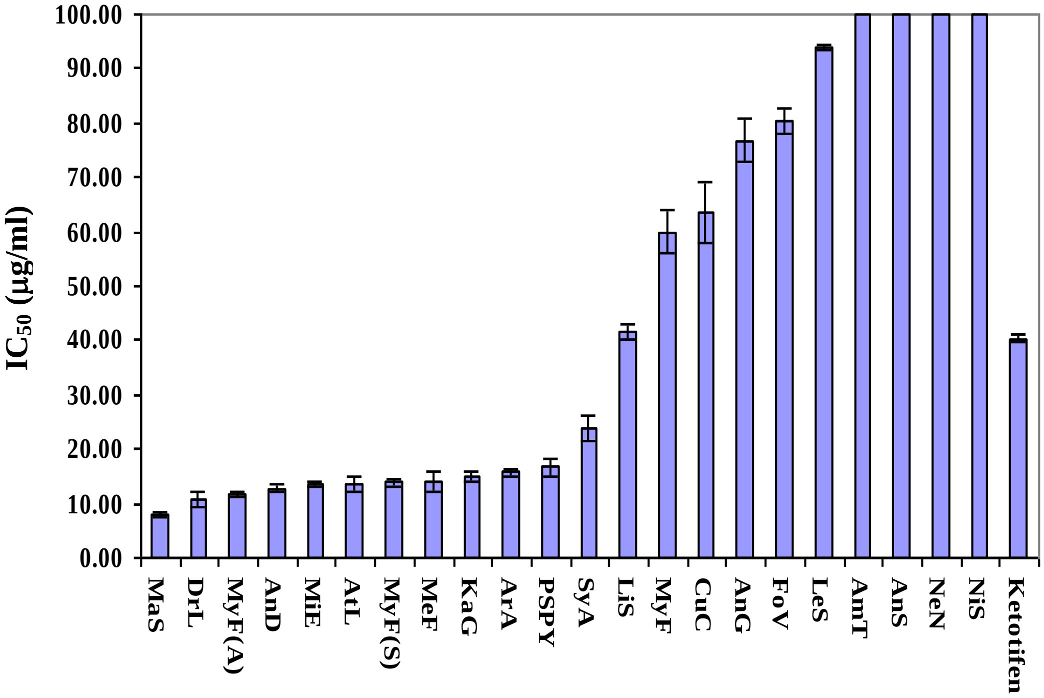

Figure 4.6

IC_{50} of ethanolic extracts of Prasaprohyai preparation and its ingredients on the release of β-hexosaminidase in RBL-2H3 cells, used Ketotifen fumarate as positive control (IC_{50} of Ketotifen fumarate = 40.41 µg/ml)

An allergic reaction is initiated when binding of multivalent allergens or antigens to specific IgE bound to the high-affinity IgE receptor (FcεRI) on the mast cells or basophils. The immediate reaction, taking effect within minutes of allergen or antigen provocation, results in the release of mediators such as histamine that lead to symptoms of allergy including dermatitis and asthma (Gould et al., 2003; Yamashita et al., 2000). β-Hexosaminidase, the highly enzyme, appears when the granules in mast cells or basophils degranulate. Moreover, enzyme β-hexosaminidase is released along with histamine when the allergy occurs.

Rat basophilic leukemia RBL-2H3 cells are mucosal mast cell type that is major model for the study of IgE-mediated degranulation. Therefore, stimulation of RBL-2H3 cells with IgE and specific antigen cam mimic cell activation by allergens under physiological conditions. FcεRI triggers under a cascade of events that induce degranulation, lipid mediator release, cytokine secretion, contributing to allergic reaction (Yamashita et al., 2000). Measurement of histamine is difficult, so the method for detecting anti-allergic activity from a wide variety of samples and matrices is the inhibitory effect on the release of β-hexosaminidase.

In the anti-allergic activity test of this study, the results showed that the ethanolic extracts of *Angelica dahurica*, *Artemisia annua*, *Atractylodes lancea*, *Dracaena loureiri*, *Kaempferia galanga*, *Mammea siamensis*, *Mesua ferrea*, *Mimusops elengi*, *Myristica fragrans* (Mace and Nutmeg), *Syzygium aromaticum* and Prasaprohyai formula demonstrated the higher inhibitory activity against antigen-induced β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells than Ketotifen furmurate which is a positive control, especially the ethanolic extract of *Mammea siamensis* which showed the highest inhibitory activity in this test.

In addition, there have been few reports on anti-allergic activity of the extracts. For example, the root extract of *Angelica dahurica* showed anti-histamine activity (Kimura, Okuda, & Baba, 1997). The water extract of *Kaempferia galanga* showed moderate anti-allergic activity, whereas the ethanolic extract and volatile oil showed mild anti-allergic activity (Tewtrakul & Subhadhirasakul, 2007). The flower-bud extract of *Syzygium aromaticum* have been report for immediate hypersensitivity activity in several systems such as compound 48/80-induced systemic anaphylaxis in at, and IgE-mediated passive cutaneous anaphylactic reaction (Kim, Lee, et al., 1998).