CHAPTER 4

RESULTS AND DISCUSSION

4.1 Quality control of plant materials of Ridsidaungmahakan preparation

4.1.1 Results of loss on drying, total ash, acid insoluble ash and extractive values

For quality control of plant materials of Ridsiduangmahakan preparation, physical standardized was investigated as follow: loss on drying (moisture content), total ash and acid insoluble ash for inorganic contamination and extractive value for quality of plant material. Procedure of loss on drying, total ash and acid insoluble ash method, and calculation of extractive value method found in Section 3.2.2.1, 3.2.2.2 and 3.2.2.3, respectively. Results of loss on drying, total ash, insoluble ash and extractive value is shown in Table 4-1.

Table 4-1

Results of loss on drying, total ash, acid insoluble ash and extractive values of plant materials of Ridsiduangmahakan preparation.

Plant species	%Loss on drying	%Ash content		%Extractive value	
		Total ash	Acid insoluble ash	Ethanol soluble	Water soluble
A. pyrethrum	10.13 ± 0.99	10.56±0.0026	1.62± 0.0020	27.01±0.5	22.92±1.0
A. graveolens	9.19 ± 2.92	9.47±0.0025	2.24±0.0014	13.79±0.7	2.69±0.3
A. sylvestris	11.44 ± 0.54	4.05±0.0005	0.96±0.0002	22.21±0.9	5.10±0.4
A. vulgaris	10.03 ± 0.91	4.31±0.0021	1.49±0.0007	17.22±0.8	13.17±0.5
C. bejolghota	11.67 ± 0.18	1.19±0.0001	0.12±0.0000	16.42±0.6	28.83±0.9
C. zeylanicum	12.44 ± 0.60	3.64±0.0008	0.13±0.0036	10.49±0.2	15.75±0.4
C. abyssinica	12.42 ± 0.68	22.34±0.0324	16.10±0.0009	9.90±0.2	24.98±0.8
C. cyminum	10.22 ± 2.80	7.72 ±0.0021	0.74±0.0030	22.30±1.1	10.55±0.4
F. vulgare	10.02 ± 0.71	7.39 ±0.0002	0.42±0.0004	22.77±0.8	8.49±0.4
L. sativum	9.48 ± 1.06	4.69±0.0029	0.26±0.0034	9.10±0.7	13.54±0.7
M. fragrans (nutmeg)	11.28 ± 0.40	1.88±0.0004	0.03±0.0000	4.73±1.0	15.06±0.8
M. fragrans (mace)	10.83 ± 0.47	8.63±0.0156	0.78±0.0027	3.74±0.8	38.36±1.2

Table 4-1 (Continued)

Plant species	% Loss on drying	%Ash content		%Extractive value	
		Total ash	Acid insoluble ash	Ethanol soluble	Water soluble
N. sativa	9.56 ± 0.78	3.63 ±0.0074	0.05±0.0000	13.93±0.9	32.30±1.3
P. kurroa	9.32 ± 0.22	1.62±0.0023	0.02±0.0000	31.94±1.0	26.67±0.9
P. chaba	13.12 ± 0.66	5.70±0.0004	0.96±0.0005	15.67±0.9	13.09±0.5
P. nigrum	9.25 ± 0.88	3.63 ±0.0074	0.05±0.0000	3.97±0.4	1.55±0.1
P. ribesioides	11.13 ± 0.60	8.45±0.0291	1.72±0.0036	8.19±0.3	2.89±0.1
P. orientalis	8.67 ± 0.31	1.11±0.0002	0.27±0.0034	5.45±0.4	4.40±0.2
P. pentandra (r)	10.05 ± 0.46	8.23±0.0009	1.62±0.0004	4.12±0.7	6.73±0.5
P. pentandra (w)	11.24 ± 0.63	22.45±0.0054	8.25±0.0028	11.24±1.0	2.08±0.2
T. chebula	10.76 ± 0.54	1.74±0.0004	0.03±0.0000	46.98±1.5	40.41±1.2
Z. officinale	10.91 ± 0.87	4.04± 0.0004	0.13± 0.0000	21.54±0.8	8.28±0.9
RSD Preparation	9.68 ± 0.39	7.40±0.0015	1.93±0.0039	16.89±0.6	16.30±0.4

From Table 4-1 showed that all values of raw material of RSD preparation were accepted by standard values (loss on dry < 10% , total ash <10% amd acid insoluble <2%) . It also showed high extractive value more than 10% on both extract (water and ethanol).

4.1.2 Results of determination for the presence of heavy metals by inductively coupled plasma-mass spectrometry (ICP-MS)

Another physical standardization of quality control is to determine toxic heavy metals. Method of determine toxic heavy metal is in Section 3.2.2.4. Results of the presence of heavy metals of Ridsiduangmahakan preparation and its ingredients are shown in Table 4-2.

In summary, RSD preparation which was qualified standardization with moisture content (less than 10%) and contaminants by heavy metal was accepted.

Table 4-2

Results of the presence of heavy metals of Ridsiduangmahakan preparation and its ingredients

Plant species	Arsenic mg/kg	Cadmium mg/kg	Lead mg/kg
A. pyrethrum	0.358±0.015	ND	0.509±0.264
A. graveolens	0.330±0.031	ND	0.503±0.055
A. sylvestris	0.347±0.002	ND	0.423±0.005
A. vulgaris	0.484±0.029	ND	0.557±0.063
C. bejolghota	0.410±0.029	ND	0.577±0.115
C. zeylanicum	0.350±0.060	ND	0.050±0.001
C. abyssinica	0.423±0.015	ND	0.275±0.003
C. cyminum	0.660±0.046	ND	0.169±0.034
F. vulgare	0.320±0.014	ND	0.045±0.001
L. sativum	0.313±0.030	ND	0.145±0.048
M. fragrans (nutmeg)	0.349±0.015	ND	0.144±0.064
M. fragrans (mace)	0.310±0.020	ND	0.140±0.070
N. sativa	0.337±0.045	ND	0.140±0.016
P. kurroa	0.881±0.153	ND	1.148±0.026
P. chaba	0.411±0.044	ND	0.038±0.002
P. nigrum	0.515±0.107	ND	0.120±0.086
P. ribesioides	0.350±0.020	ND	0.090±0.002
P. orientalis	0.410±0.029	ND	0.988±0.028
P. pentandra (r)	1.540±0.270	ND	0.420±0.070
P. pentandra (w)	0.613±0.060	ND	0.088±0.004
T. chebula	0.423±0.007	ND	0.180±0.011
Z. officinale	0.520±0.060	ND	0.210±0.020
RSD Preparation	2.559±0.412	ND	0.286±0.044

Note: Acceptable standardization values of quantity of contaminants must less than 4 mg/kg for arsenic, 0.3 mg/kg for cadmium and 10 mg/kg for lead. ND stands for not detectable.

4.2 Preparation of plant extracts

The ethanolic extracts of Ridsidaungmahakan preparation and its ingredients were prepared as described in Section 3.2.1. The charisteristic of crude ethanolic extracts was two part (solid and oil) because there are many plants which active ingredients are volatile oil such as *Cuminum cyminum, Myristica fragrans, Nigella sativa, Piper chaba* and *Piper nigrum.* The percentage of yields of Ridsidaungmahakan preparation and its ingredients were shown as percentage by weight in Figure 4-1. From the Figure 4-1, we found that *N. sativa* (37.28%) has the most percentage of yields follow by *T. chebula* (33.96%) and *C. abyssinica* (33.66%). The three least percentage of yields are *P. rebesioides* (1.01%), *P. pentandra (white)* (1.84%) and *P. pentandra* (red) (3.71%) respectively. For the preparation, It was found that the percentage of yields of the ethanolic extract from preparation were 16.30% (it showed of oil 4.68% and solid 11.62%). Its results related with the ethanol extractive value as 16.89%.

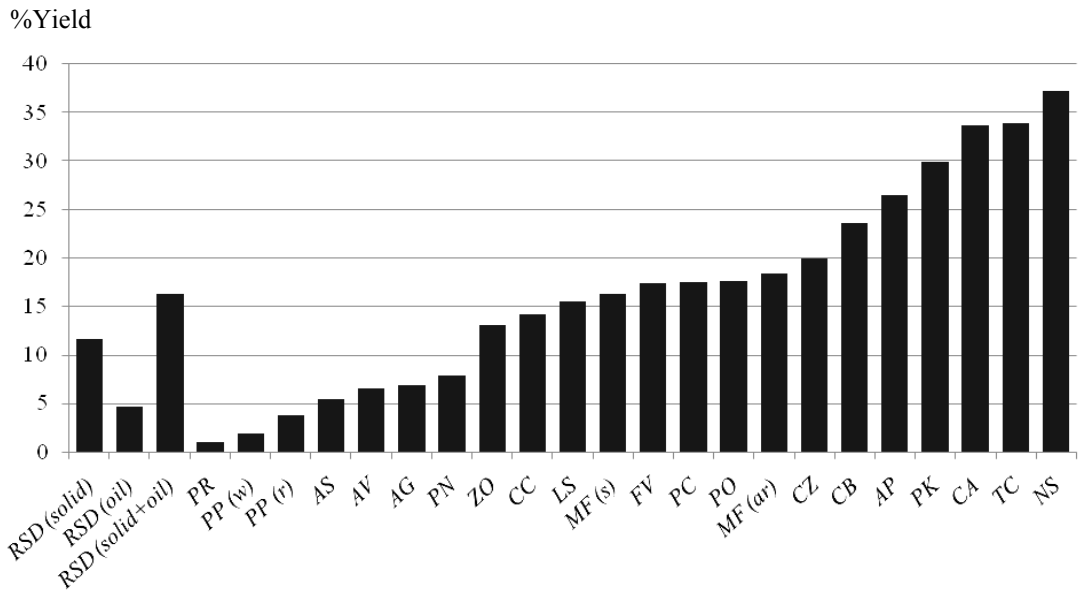

Figure 4-1

Percentage of yields of the ethanolic extracts of Ridsidaungmahakan preparation and its ingredient

4.3 *In vitro* assay for anti-inflammatory activities

4.3.1 Inhibitory effects on LPS-induced Nitric Oxide release from RAW 264.7

Effects of Ridsidaungmahakan preparation and its ingredients on the pro-inflammatory mediator nitric oxide (NO) in activated murine macrophages cell line (RAW 264.7) were measured as anti-inflammatory properties compared with positive control (indomethocin). It was found that lipopolysaccharide (LPS) stimulated the highest NO production by RAW 264.7 cells at concentration of 5 μg/ml. Thus, 5 μg/ml LPS to induce NO production by RAW 264.7 cells was used in this study. The measurement of nitrite accumulation in the culture medium was used to determine NO production. The nitrite concentration was measured by Griess reaction. Method for analyzation of inhibition of NO are in Section 3.2.3.1.

The results of inhibitory activity against LPS induced NO production of Ridsidaungmahakan preparation and its ingredients were shown in Table 4-3 and Figure 4-3. Among the plant species studied, it was found that less than 50% of ethanolic extracts showed inhibition NO production at concentration 100μg/ml, but preparation (oil) and preparation (solid) of showed IC_{50} values as 48.1±1.8 and 56.9±5.1 respectively. It was found that *Z. officinale, P. chaba, T. chebula,* and *A. caulgaris* exhibited high inhibition of NO production. The highest inhibition showed in *Z. officinale* (IC_{50} 21.2±1.8 μg/ml) and they had no cytotoxicity. However these plant extracts exhibited NO production inhibitory effect less than indomethacin (IC_{50} = 56.78 μM or 20.32 μg/ml). It is concluded that the preparation showed less anti-inflammatory by inhibition NO production.

The previous report of ethanolic extract (100 μg/ml) and methanolic extract (200 μg/ml) of *Z. officinale* showed inhibitory effects on the NO production in LPS-stimulated RAW 264.7 macrophagesas 101.9±2.7 and 112.8±0.9 μg/ml respectively compared with positive control, indomethacin, which gave IC_{50} = 25.0 μM or 8.95 μg/ml (Tewtrakul & Subhadhirasakul, 2008; Choi & Hwang, 2005).

Table 4-3

Inhibition of Nitric Oxide (NO) production of ethanolic extracts of Ridsiduangmahakan preparation and its plant ingredients at various concentration with calculation of IC_{50} (n=3)

Extract	Code	%inhibition of NO production (%cytotoxic) (n=3)					IC_{50} (µg/ml)
		3 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	
Z. officinale	ZO	9.2±1.5 (18.5±4.7)	17.1±4.8 (18.7±4.8)	77.2±5.0** (29.3±3.7)	92.9±2.5** (29.7±3.7)	(cytotoxicity)	21.2±1.8**
P. chaba	PC	12.9±6.0 (15.7±9.6)	18.9±8.9 (12.0±4.9)	59.6±8.0** (27.8±4.8)	-	91.1±3.4** (19.9±12.0)	25.9±2.5**
A. vulgaris	AV	8.1±3.1 (2.4±3.2)	16.0±2.0 (7.5±9.0)	54.3±1.1** (15.4±2.2)	-	95.5±2.5** (12.8±0.9)	27.8±0.3**
T. chebula	TC	5.5±3.4 (5.5±0.6)	11.2±2.4 (9.9±1.6)	53.4±7.3** (25.5±2.0)	-	88.3±5.6** (32.7±7.4)	28.7±3.3**
P. nigrum	PN	3.8±3.5 (19.9±1.6)	29.7±3.8** (31.8±3.1)	47.2±4.0** (35.5±2.4)	95.8±3.0** (31.0±0.5)	(cytotoxicity)	32.0±3.1**
C. abyssinica	CA	2.0±1.2** (9.7±7.5)	8.5±5.7 (17.4±3.1)	40.3±1.4** (16.5±3.6)	-	88.5±9.8 (59.7±5.9)	34.5±1.7**
P. ribesioides	PR	8.1±3.7 (23.4±5.2)	19.5±5.5 (25.4±3.4)	37.7±7.8 (33.7±3.5)	96.2±1.2** (46.3±5.3)	(cytotoxicity)	35.4±2.5 **
M. fragrans (aril)	MF (a)	3.7±1.5** (11.9±15.8)	8.5±1.8** (14.9±10.1)	29.7±2.5** (41.4±1.5)	67.9±8.7** (41.4±1.5)	(cytotoxicity)	47.8±5.1**
Preparation (oil)	RSD (o)	2.7±2.6 (1.8±1.5)	16.3±1.1 (7.8±1.2)	52.8±2.6 (8.9±2.1)	-	90.4±1.6 (7.5±3.9)	48.1±1.8**
Preparation (solid)	RSD (s)	6.9±4.2 (9.6±0.7)	13.8±2.7 (3.6±1.7)	43.4±4.4 (12.0±1.9)	-	67.2±1.3 (12.5±1.6)	56.9±5.1**
C. cyminum	CC	3.0±1.8** (11.3±4.6)	10.2±4.3 (12.7±4.7)	16.9±1.8** (17.2±1.2)	-	79.6±3.7 (15.4±4.2)	74.7±4.4

n = number of independent experiment

***p value*< 0.05, compared with the preparation

Table 4-3 (Continued)

Extract	Code	%inhibition of NO production (%cytotoxic) (n=3)					IC_{50} (µg/ml)
		3 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	
P. pentandra (white)	PP (w)	3.1±2.0** (3.2±1.0)	5.5±3.9 (6.4±4.0)	14.4±2.5** (12.0±1.3)	-	63.3±3.6** (17.6±4.1)	83.1±2.8
N. sativa	NS	-	-	-	-	36.9±6.4 (44.9±5.1)	>100
L. sativum	LS	-	-	-	-	8.6±5.5 (22.9±9.2)	>100
Preparation (solid+oil)	RSD	10.2±3.3 (5.8±4.6)	15.1±1.4 (2.9±0.5)	25.0±7.3 (6.7±0.2)	-	73.6±11.4 (4.5±1.9)	78.9±0.6
P. kurroa	PK	-	-	-	-	28.8±4.8 (13.6±4.1)	>100
M. fragrans (seed)	MF (s)	-	-	-	-	44.1±0.7 (21.3±4.0)	>100
P. orientalis	PO	-	-	-	-	42.5±7.1 (5.0±3.7)	>100
C. bejolghota	CB	-	-	-	-	41.6±1.8 (3.6±1.0)	>100
C. zeylanicum	CZ	-	-	-	-	43.2±11.8 (14.7±2.4)	>100
P. pentandra (red)	PP (r)	-	-	-	-	48.8±2.9 (5.2±2.0)	>100

n = number of independent experiment

**p *value*< 0.05, compared with the preparation

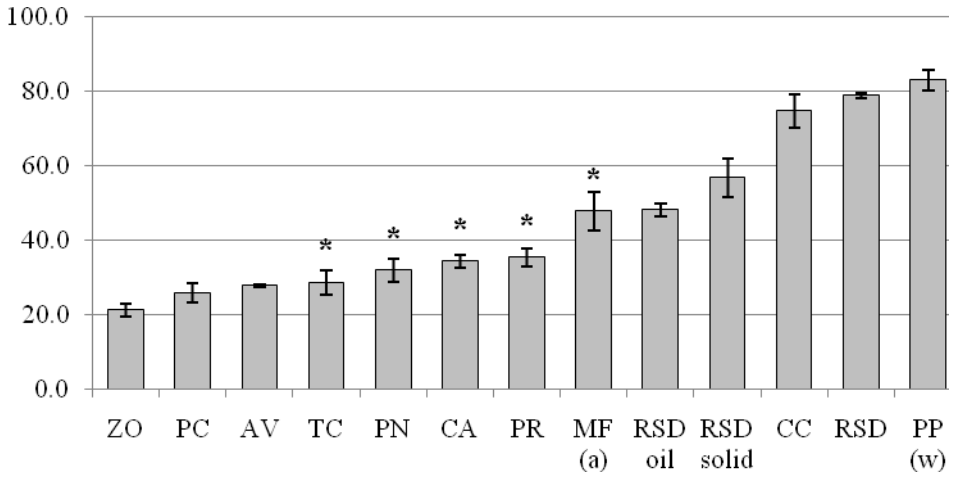

Figure 4-2

IC_{50} of ethanolic extracts of Ridsiduangmahakan preparation and its ingredient on NO inhibitory activity using Griess reagent, used Indomethacin as positive control (IC_{50} of indomethacin = 20.32 μg/ml), *Cytotoxic effect was observed with concentration 100 μg/ml

4.3.2 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 Ridsiduangmahakan preparation and its ingredient were shown in Table 4-4 (methodology is in Section 3.2.3.2). The results revealed that the ethanolic extract of Indomethacin possessed the most potent activity against TNF-α release with IC_{50} value of 51.41±3.58 μg/ml. Three ethanolic extracts *T. chebula, C. abyssinica* and *P. nigrum* exhibited high inhibitory effects on TNF-α release with IC_{50} values of 20.65±2.89, 34.41±5.00 and 40.11±1.53 μg/ml, respectively. For the preparation, preparation (oil) gives the best result with IC_{50} value of 31.86±1.86 μg/ml. Indomethacin was used as positive control, its IC_{50} was 143.69±10.01 μM. or 51.42μg/ml. It was concluded that the RSD preparation possessed the anti-inflammatory by inhibition on TNF-α pathway.

There are only one previous report of *Z. officinale* as a ingredient of RSD preparation showed that [6]-gingerol as its isolated compound can inhibit the

production of pro-inflammatory cytokines, i.e., TNF-α, IL-12, and IL-1β which were produced by LPS stimulated macrophages (Tripathi et al., 2007).

4.3.3 Inhibitory effects on LPS- stimulated PGE_2 release from RAW 264.7 cells

To determine inhibitory effects of Ridsiduangmahakan preparation and its plant ingredients on LPS-stimulated PGE_2 method was described in Section 3.2.3.3. The results are shown in Table 4-5. It was found that *Z. officinale, M. fragrans* (seed), *P. nigrum,* and *P. chaba* exhibited inhibitory effect and *Z. officinale* gave the best IC_{50} value (5.22±1.20 μg/ml). For the ethanolic RSD preparation extracts, all crude extracts (oil, solid, oil+solid) exhibited inhibitory effects, oil part of preparation showed the best IC_{50} value follow by preparation (solid+oil) and preparation (solid) (IC_{50} were 8.85±1.60, 16.80±4.04 and 21.22±3.29 μg/ml, respectively). Indomethacin as positive control showed IC_{50} as 2.80±1.20 μM. or 1.00±0.43 μg/ml. These results concluded that RSD preparation showed anti-inflammation possessing by COX-2 inhibitor. The active plant ingredients in RSD which showed anti-inflammatory activity possessing by COX-2 inhibitor were ginger *(Z. officinale*), black pepper (*P. nigrum*) and long pepper (*P. chaba*).

Many previous reports of RSD ingredients which reported about anti-inflammation by COX-1 and COX-2 pathway. The chloroform fraction which got from partition between water and chloroform from Ginger roots inhibited COX-1 and COX-2 (IC_{50} value of 20.0±0.4 and 7.5±0.6 μg/ml respectively). Its isolated compound such as 10-gingerol, 8-shogaol and 10-shogaol inhibited COX-2 with IC_{50} values of 32, 17.5 and 7.5 μM, respectively. (Breemen et al., 2011). In addition, Tjendraputra et al. 2003 found that [8]-paradol (as isolated compound from *Z. officinale*) exhibited COX-1 inhibitory activity with IC_{50} value of 4.0±1.0 μM.

There is the previous report about *M. fragrans* which showed that Mace lignan potently suppressed the expression of COX-2 and induction of nitric oxide synthase. The consequent resulted in the reduction of nitric oxide in LPS-treated microglial cells (Jin et al., 2005). Another literature showed that the methanolic extracts of black pepper (*P. nigrum*) and its compounds concentration at 200 μg/ml 25 μg/ml inhibited COX enzymes by 31-80% (Yunbao et al., 2010). There is no report for COX-1 and COX-2 inhibitor of *P. chaba*.

Table 4-4

Inhibition on LPS-induced TNF-α release of ethanolic extracts of Ridsiduangmahakan preparation and its plant ingredients at various concentration with calculation of IC_{50}. (n=3)

Extract	Code	%inhibition of NO production (%cytotoxic) (n=3)					IC_{50}
		3 μg/ml	10 μg/ml	30 μg/ml	50 μg/ml	100 μg/ml	(μg/ml)
T. chebula	TC	23.90±1.84*	37.46±6.14*	63.75±3.12*	-	83.34.±3.68*	20.65±2.89*
Preparation (oil)	RSD (o)	4.46±1.20	11.22±3.15	46.87±3.08	-	61.97±0.93	31.86±1.86*
C. abyssinica	CA	15.01±2.58*	32.43±4.27*	48.18±2.89*	-	75.67±4.08*	34.41±5.00*
P. nigrum	PN	0.91±0.27*	11.06±1.95	36.11±2.21	64.61±2.66*	(cytotoxicity)	40.11±1.53*
P. chaba	PC	2.98±0.72	6.12±1.12*	33.95±5.46	-	64.79±5.17*	45.48±10.9*
A. vulgaris	AV	2.58±0.26	8.79±2.56	21.13±2.40*	34.20±4.74	(cytotoxicity)	>100
Z. officinale	ZO	2.80±0.27	5.54±0.92*	17.02±2.93*	31.99±1.92*	(cytotoxicity)	>100
Preparation (solid)	RSD (s)	2.45±1.06	8.52±4.26	25.19±2.00	-	41.26±3.52	>100
Preparation (solid+oil)	RSD	2.16±0.15	11.26±0.59	32.27±1.36	-	40.44±1.72	>100

*p *value*< 0.05, compared with the preparation (solid+oil).

Table 4-5

Inhibition on LPS- stimulated PGE_2 release of ethanolic extracts of Ridsiduangmahakan preparation and its plant ingredients at various concentration with calculation of IC_{50}. (n=3)

Extract	Code	% Inhibitory effects on LPS- stimulated PGE_2 (n=3)							IC_{50}
		0.1 µg/ml	1 µg/ml	3 µg/ml	10 µg/ml	30 µg/ml	50 µg/ml	100 µg/ml	(µg/ml)
Z. officinale	ZO	0.51±1.10	15.05±1.21	51.35±0.63	53.71±2.18 **	81.64±5.41	-	(cytotoxicity)	5.22±1.20*
Preparation (oil)	RSD (o)	-	-	23.57±4.59	35.46±4.02	60.08±4.42	-	70.34±3.90	8.85±1.60
Preparation (solid+oil)	RSD	-	-	25.89±4.43	53.13±4.28	77.92±5.23	-	75.48±5.57	16.80±4.04
M. fragrans (seed)	MF (s)	-	-	11.66±0.88**	35.04±4.07*	66.32±2.11	66.20±2.69	(cytotoxicity)	16.99±1.93
P. nigrum	PN	-	-	19.81±2.27	37.30±3.44	63.60±4.68	65.27±6.23	(cytotoxicity)	17.70±2.48
Preparation (solid)	RSD (s)	-	-	26.71±4.38	40.95±4.72	62.51±5.23	-	75.36±5.94	21.22±3.29
P. chaba	PC	-	-	19.89±2.74	31.51±2.99*	58.51±5.04	68.03±3.73	-	23.08±3.09
P. ribesioides	PR			16.55±1.02	21.06±1.42	40.40±1.65	57.54±3.69	-	40.93±3.18**
M. fragrans (aril)	MF (a)			5.63±0.18**	10.10±0.91 **	33.92±1.76 **	62.32±4.71	-	41.46±1.83**
P. pentandra (red)	PP (r)	-	-	-	-	-	45.18±2.20	-	
C. zeylanicum	CZ	-	-	-	-	-	38.17±5.14	-	
C. abyssinica	CA	-	-	-	-	-	36.97±4.12	-	
P. pentandra (white)	PP (w)	-	-	-	-	-	35.63±1.98	-	
T. chebula	TC	-	-	-	-	-	34.76±3.86	-	
F. vulgare	FV	-	-	-	-	-	33.29±3.82	-	

*p *value* < 0.1, ** p *value* < 0.05, compared with the preparation

Table 4-5 Continued)

Extract	Code	% Inhibitory effects on LPS- stimulated PGE_2 (n=3)							IC_{50}
		0.1 μg/ml	1 μg/ml	3 μg/ml	10 μg/ml	30 μg/ml	50 μg/ml	100 μg/ml	(μg/ml)
P. orientalis	PO	-	-	-	-	-	31.29±4.55	-	
A. vulgaris	AV	-	-	-	-	-	28.87±1.30	-	
P. kurroa	PK	-	-	-	-	-	27.73±4.26	-	
A. graveolens	AG	-	-	-	-	-	25.93±4.29	-	
A. sylvestris	AS	-	-	-	-	-	23.89±1.34	-	
A. pyrethrum	AP	-	-	-	-	-	22.94±3.72	-	
C. cyminum	CC	-	-	-	-	-	18.96±1.88	-	
C. bejolghota	CB	-	-	-	-	-	17.18±2.25	-	
N. sativa	NS	-	-	-	-	-	10.51±3.83	-	
L. sativum	LS	-	-	-	-	-	9.77±3.82	-	

*p *value* < 0.1, ** p *value* < 0.05, compared with the preparation

Table 4-6

Comparision of three type of anti-inflammatory activities on NO production, TNF-α release and COX-2 inhibition

Extract	IC_{50} of anti-inflammatory effect stimulated by LPS (μg/ml)		
	NO Production	TNF-α release	COX-2 inhibition
A. pyrethrum	>100	>100	>100
A. graveolens	>100	>100	>100
A. sylvestris	>100	>100	>100
A. vulgaris	27.8±0.3*	>100	>100
C. bejolghota	>100	>100	>100
C. zeylanicum	>100	>100	>100
C. abyssinica	34.5±1.7*	34.41±5.00*	>100
C. cyminum	74.7±4.4	>100	>100
F. vulgare	>100	>100	>100
L. sativum	>100	>100	>100
M. fragrans (nutmeg)	>100	>100	16.99±1.93
M. fragrans (mace)	47.8±5.1*	>100	41.46±1.83**
N. sativa	>100	>100	>100
P. kurroa	>100	>100	>100
P. chaba	25.9±2.5*	45.48±10.91*	23.08±3.09
P. nigrum	32.0±3.1*	40.11±1.53*	17.70±2.48
P. ribesioides	35.4±2.5	>100	40.93±3.18**
P. orientalis	>100	>100	>100
P. pentandra (r)	>100	>100	>100
P. pentandra (w)	83.1±2.8	>100	>100
T. chebula	28.7±3.3*	>100	>100
Z. officinale	21.2±1.8*	>100	5.22±1.20
RSD Preparation (solid+oil)	78.9±0.6	>100	16.80±4.04
RSD Preparation (solid)	56.9±5.1*	>100	21.22±3.29
RSD Preparation (oil)	48.1±1.8*	31.86±1.86*	8.85±1.60
indomethocin	56.78 μM or 20.32 μg/ml	143.69±10.01 μM. or 51.42μg/ml	2.80±1.20 μM. or 1.002 μg/ml

* *p value* < 0.05, compared with the preparation

From Table 4-6 showed comparison of anti-inflammatory on three pathway were found that RSD preparation (all parts) showed the highest anti-inflammatory activity on COX-2 inhibitor but they showed less activity than indomethocin. However oil part of RSD preparation also showed inhibition TNF-α release activity more than indomethocin. The ingredients of this preparation such as *P.nigrum, P.chaba and Z. officinale* showed the highest anti-inflamation on three pathway. Interestingly these three plants were combined as Trikratuk in Thai traditional medicine.

4.4 *In vitro* assay for cytotoxic activity by SRB assay

To investigate effect of Ridsiduangmahakan preparation and its plant ingredients on cytotoxic activity by SRB assay, the method described in Section 3.2.4. Calculations of the IC_{50} values of all plants were shown in Table 4-7. This data showed that the ethanolic extracts of Ridsiduangmahakan preparation exhibited cytotoxic activity against LS174T followed by the American National Cancer Institue (NCI) standard (IC_{50} < 20 μg/ml for crude extract) (Boyde,1997). In this work there is only one extract that has IC_{50} less than 20 μg/ml, so all plants that have IC_{50} < 30 μg/ml were chose to test cytotoxicity against normal cell (MRC5). The selected plants such as *C. abyssinica P. nigrum Z. officinale* including the preparation (solid+oil) were also tested. The results were shown in Table 4-6. It was found that *Z. officinale, C. abyssinica* and *P. nigrum* exhibited cytotoxicity on LS174T. *Z. officinale* gave the best IC_{50} value (12.53±0.13 μg/ml) followed by *C. abyssinica* and *P. nigrum* (27.17±0.65 and 27.65±0.70 μg/ml) . For the preparation, we found that preparation (oil), preparation (solid+oil) and preparation (solid) showed cytotoxic activity against LS174- T IC_{50} values as 66.62±3.08, 35.44±1.07, and 46.54±2.92 μg/ml, respectively. This preparation was specific cytotoxic against colon cancer only LS174-T cell line, not cytotoxic against SW480. *Z. offinale* showed the highest cytotoxic activity against two types of colon cancer and it is only one ingredient in this preparation showed cytotoxic activity against SW480. It also showed specific cytotoxic activity against LS174T more than SW480 and less cytotoxic activity against normal cells. These results related with the previous reports which found that [6]-shogaol, [10]-shogaol

and 1,7-bis(4-hydroxy-3-methoxyphenyl) hept-4-en-3-one which were isolated from *Z. officinale*, possessed significant cytotoxicity against HL-60 cells (with IC_{50} < 50 μM) and they also showed apoptosis mechanism (Wei et al., 2005). [6]-shogaol exhibited potent cytotoxicity against human A549, SK-OV-3, SK-MEL-2, and HCT15 tumor cells and also inhibited proliferation of the transgenic mouse ovarian cancer cell lines, (Kim et al., 2008).

Table 4-7

Cytotoxicity activity at 50 μg/ml concentration and IC_{50} (μg/ml) of Ridsiduangmahakan preparation and its plant ingredients extract against two type of colon cancer cell lines (LS174T and SW480) and one type of normal cell line (MRC5) (n=3)

Extract	Colon adenocarcinoma (LS174T)		Colon, colorectal adenocarcinoma (SW480)		Normal cell line (MRC5)
	%Cytotoxicty (50 μg/ml)	IC_{50}	%Cytotoxicty (50 μg/ml)	IC_{50}	IC_{50}
A. pyrethrum	9.78±0.79	>100	9.61±1.81	>100	NT
A. vulgaris	28.52±2.56	>100	6.09±0.59	>100	NT
A. graveolens	8.72±0.86	>100	8.82±2.18	>100	NT
A. sylvestris	4.59±1.50	>100	43.81±5.87	>100	NT
C. bejolghota	94.09±3.66	32.91±0.72*	16.94±4.14	>100	NT
C. cyminum	34.69±1.78	>100	15.25±4.86	>100	NT
C. abyssinica	90.48±5.49*	27.17±0.65* **(2.25)**	47.87±3.13	>100	61.25±1.73*
C. zeylanicum	31.45±1.89	>100	20.17±1.60	>100	NT
F. vulgare	7.61±0.57	>100	7.83±0.77	>100	NT
L. sativum	5.00±2.37	>100	2.36±2.55	>100	NT

* *p value*< 0.05, compared with the preparation

NT : not tested (IC_{50} for colon cancer <30 μg/ml)

Table 4-7 (Continued)

Extract	Colon adenocarcinoma (LS174T)		Colon, colorectal adenocarcinoma (SW480)		Normal cell line (MRC5)
	%Cytotoxicty (50 μg/ml)	IC_{50}	%Cytotoxicty (50 μg/ml)	IC_{50}	IC_{50}
M. fragrans (ar)	22.67±2.04	>100	20.50±3.22	>100	NT
M. fragrans (s)	31.92±1.32	>100	9.97±0.49	>100	NT
N. sativa	8.63±2.23	>100	6.05±1.16	>100	NT
P. chaba	91.68±2.12*	31.12±.088* **(2.95)**	39.46±0.81	>100	91.71±0.50
P. kurroa	13.86±2.57	>100	4.04±1.56	>100	NT
P. nigrum	96.80±0.51*	27.65±0.70* **(1.63)**	40.90±1.69	>100	45.15±3.01*
P. pentandra (w)	41.72±2.10	>100	49.32±1.41	>100	NT
P. ribesioides	65.47±4.59	35.00±0.92* **(0.98)**	9.87±0.33	>100	34.44±1.61*
P. orientalis	14.60±1.95	>100	17.36±5.82	>100	NT
P. pentandra (r)	39.01±2.69	>100	43.20±2.84	>100	NT

* *p value*< 0.05, compared with the preparation

NT : not tested (IC_{50} for colon cancer <30 μg/ml)

Table 4-7 (Continued)

Extract	Colon adenocarcinoma (LS174T)		Colon, colorectal adenocarcinoma (SW480)		Normal cell line (MRC5)
	%Cytotoxicty (50 μg/ml)	IC_{50}	%Cytotoxicty (50 μg/ml)	IC_{50}	IC_{50}
T. chebula	95.36 ± 2.54*	30.42 ± 1.07* **(>3.29)**	97.52±1.17*	32.11±0.81* **(>3.11)**	>100
Z. officinale	98.50±1.14*	12.54±0.13* **(4.19)**	98.39±0.62*	30.75±1.31* **(1.71)**	52.53±4.93*
RSD (oil)	78.38 ± 1.55	35.44 ± 1.07 **(>2.82)**	13.18±1.22	>100	>100
RSD (solid)	34.49 ± 3.33	66.62 ± 3.08 **(>1.50)**	8.32±1.42	>100	>100
RSD (solid+oil)	54.93±4.45	46.54±2.92 **(>2.14)**	9.55±2.16	>100	>100

* *p value*< 0.05, compared with the preparation

NT : not tested (IC_{50} for colon cancer <30 μg/ml)

4.5 Nitric Oxide inhibitory effect of bioassay-guided fractionation

The results of Ridsiduangmahakan ethanolic extracts and its ingredient extract showed in Section 4.1 gave the evidences of the presence of active constituents in the ethanolic extracts of Ridsiduangmahakan preparation, so the separation of these active extracts was carried out by bioassay guide fractionation described in Section 3.5. Six fractions from the ethanolic extracts of Ridsiduangmahakan preparation (RSD1, RSD2, RSD3, RSD4, RSD5 and RSD6) were tested for inhibitory effects on LPS-induced Nitric Oxide release from RAW 264.7. The purpose of this test was found in preliminary results which did for selecting the fraction which showed the best inhibitory effects and has high percentage of yield. Then the best fraction was selected to separated pure compound.

Table 4-8

IC_{50} (μg/ml) of the fractions from Ridsiduangmahakan preparation separated by vacuum liquid chromatography on LPS-induced NO release

Fraction	%Yield	IC_{50} (μg/ml)
RSD1 (Hexane)	0.18	Cytotoxic
RSD2 (Hexane:$CHCl_3$)	0.22	>100
RSD3 ($CHCl_3$)	19.30	37.73
RSD4 ($CHCl_3$:MeOH)	33.59	27.41
RSD5 ($CHCl_3$:MeOH)	11.57	62.18
RSD6 (MeOH)	17.45	86.51

RSD1, RSD2, RSD3, RSD5 and RSD6 showed less NO release compared with the crude ethanolic extract of Ridsiduangmahakan preparation (Table 4-8). RSD4 was only one fraction which showed high percentage of yield and also has the least IC_{50} value. From these results, RSD4 was chosen to isolate antiinflammatory compounds because it showed the best inhibitory effect on NO release.

4.6 Isolation of chemical constituents from Ridsiduangmahakan preparation

An aliquot of the ethanolic extract of Ridsiduangmahakan preparation (50gm) was separated by vacuum liquid chromatography (VLC) using silica gel and a gradient elution of hexane (4×500 ml), hexane:chloroform (1:1) (4×500 ml), chloroform (4×500 ml), chloroform:methanol (1:1) (4×500 ml) and methanol (4×500 ml) Drying and evaporation of each fraction yield residues showed on Table 4-9 These six fractions were tested for Nitric Oxide inhibitory effect on Table 4-9. It was found that RSD4 exhibited the best anti-inflammation on Nitric Oxide inhibitory effect. Thus RSD4 was continue isolated active anti-inflammatory compound by column chromatography described in chapter 3, **3.2.6. The pure compound was identified as piperine** by comparison on TLC and HPLC chromatography with authentic Piperine Standard purchased from Merck followed by Sakpakdeejaoen (2008) and It was a major component isolated. The ingredients of Ridsiduangmahakan preparation found in *Piper* species such as *Piper nigrum* , *Piper chaba* and *P. ribesioides* were component , so this preparation should have piperine, which was found as the main compound of *Piper* species such as *Piper nigrum* and *Piper chaba* (Wu et al*.,* 2004; Park et al*.,* 2007).

4.7 Anti-inflammatory activities of the isolated compound of RSD preparation

Piperine was isolated from Ridsiduangmahakan preparation. Then it was tested for anti-inflammatory activities, such as inhibitory effects on LPS-induced NO, TNF-α release and inhibitory effects on LPS- stimulated PGE_2 release. The results are shown in Table 4-9. Piperine exhibited as all proinflammation mediators on this study. The best inhibitory effect was on NO inhibition followed by Inhibitory effects on LPS- stimulated PGE_2 release and LPS-induced TNF-α release IC_{50} = 17.0±4.6 , 20.36±1.89 and 63.05±7.86 μg/ml, respectively. This study related with the previous report , it was found that piperine inhibited the expression of IL6 and MMP13 and reduced the production of PGE_2 in a dose dependant manner at concentrations of 10 to 100 μg/ml (Bang et al., 2009). Piperine (1 or 5 mg/kg of body weight) also inhibited LPS-induced endotoxin shock in TNF-α knockout (KO) mice and type 1

IFN *in vivo* (Bae et al., 2010). From this results was concluded that piperine can inhibit TNF-α. However it also is anti-inflammatory compound possessing by NO, COX-2 and TNF-α. It also was a compound which increased the bioavailability of the other compounds or some drugs (Atal et al., 1985). Thus it might be a compound to help increasing bioavailability of compound in RSD preparation extract for increasing anti-inflammatory effect.

Table 4-9

Anti-inflammatory activities of piperine on LPS-induced from RAW 264.7 cells

Method	%inhibition of proinflammatory mediators release (n=3)				IC_{50} (μg/ml, μM)
	3 μg/ml	10 μg/ml	30 μg/ml	100 μg/ml	
Inhibitory effects on LPS-induced NO release (%cytotoxic)	18.43±3.5 (18.1±4.2)	39.03±6.3 (23.3±6.1)	61.08±3.4 (17.7±2.5)	76.24±1.1 (22.5±4.8)	17.0±4.6, 59.57±16.12
Inhibitory effects on LPS-induced TNF-α release	5.54±1.43	11.54±0.72	27.31±2.59	71.70±1.38	63.05±7.86, 220.9±27.54
Inhibitory effects on LPS- stimulated PGE_2 release	20.17±4.16	28.63±4.35	72.27±4.47	74.09±6.04	20.36±1.89, 71.35±6.62

Indomethacin as positive control showed 56.78, 143.69 and 2.80 μM on LPS-induced NO and TNF-α release and LPS- stimulated PGE_2 release .

4.8 Study on chemical fingerprint of Ridsiduangmahakan preparation using Gas Chromatography - Mass Spectrometry

Ethanolic extract of Ridsiduangmahakan preparation was studied on chemical fingerprint by Gas Chromatography - Mass Spectrometry is show in Section 3.7. GC-MS conditions for analysis of ethanolic extract of Ridsiduangmahakan preparation are as follow.

GC-MS from Fortune Scientific, co. ltd. has following specification: ZB-5ms capillary column, 30 m. in length, internal diameter is 0.25 mm, non-polar stationary phase with thickness 0.25 μm.

Column	
Stationary phase	5%phenyl&95%dimethylpolysiloxane
Length	30 meters
Thickness of stationary phase	0.25 μm
Diameter	0.25 mm
Dynamic phase	Helium
Flow rate of stationary phase	1.0 ml/min
Initial temperature	60.0°C for 5 mins.
Raising temperature rate	3.0°C/mins
Maximum temperature	250 °C for 15 mins
Injection	
Temperature of injection sample	200°C
Split rate of sample	1 : 50
Quantity of sample per injection	1 μl
Total time for analysis	83.33 mins
Detector	
Detector type	Mass spectrometer
Temperature between-detector and terminal column	275 °C
Temperature of Mass source	240 °C
Energy of electron	70.0 eV: EI

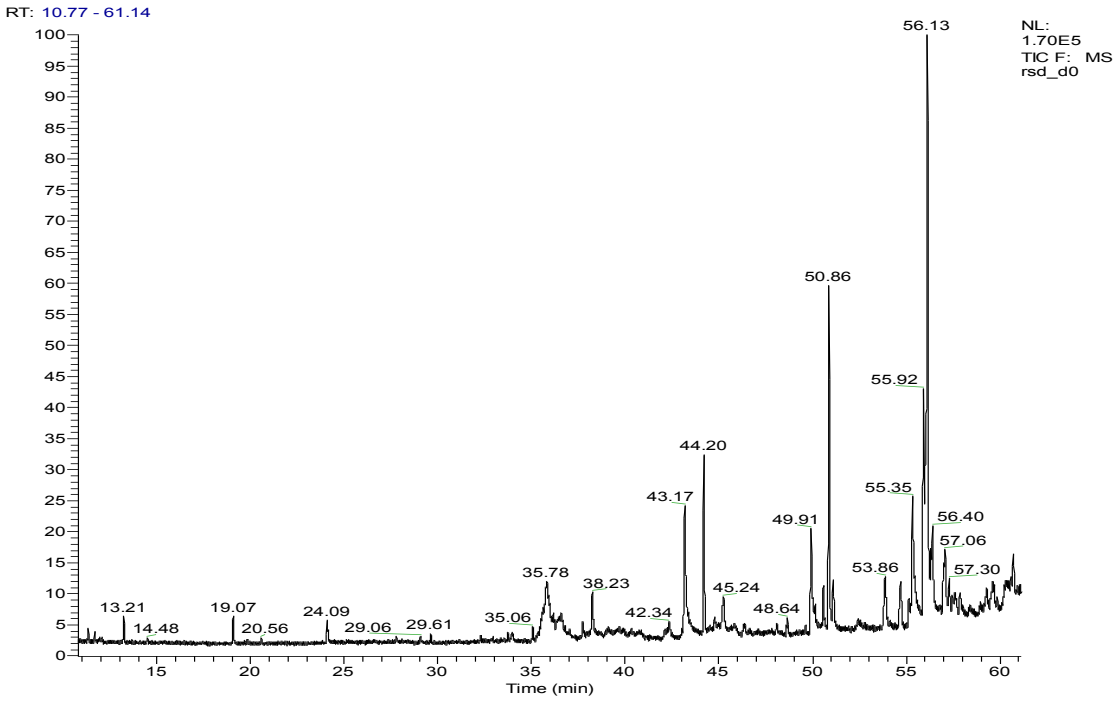

Figure 4-3

Chemical fingerprint using GC MS of Ridsiduangmahakan preparation

Table 4-10

Chemical analysis using GC MS of Ridsiduangmahakan preparation

RT	Peak Area	Area %	Names
9.39	129150	2.99	Alpha pinene
11.31	10022	0.23	Terpinolene
11.68	8610	0.20	P cymene
13.22	22355	0.52	Gamma terpinene
19.06	26879	0.62	Terpinene 4-ol
24.08	32295	0.75	Safrole
35.79	603793	13.96	Diethyl phthalate
38.23	60828	1.41	1,4-bis(methoxy)-triquinacene
43.18	273767	6.33	Isopropyl myristate
44.20	179045	4.14	Ethyl myristate
45.24	74334	1.72	Isoproplymyristate
49.91	114545	2.64	Palmitic acid
50.86	787988	18.22	Ethyl palmitate
51.10	44239	1.02	Astratone
55.34	298498	6.90	Olic acid
56.13	1433619	33.14	Unknown
57.06	186432	4.31	Unknown

4.9 The stability study on RSD extract

The stability of Ridsiduangmahakan preparation extracts were evaluated and described in section 3.9. It was determined chemical fingerprint contents with method in section 3.7. The results of stability testing are shown in Table 4-11 and Figure 4-7. The purpose of investigation of stability was to analyze chemical fingerprint of preparation extracts which altered under accelerated condition (45 ± 2°C with 75 ± 5% RH) during 120 days. The samples were taken on the beginning (day 0), day 15, day 30, day 60, day 90 and day 120. Nevertheless, chemical quantity was determined by relative area as comparison between peak area with overall area.

After bioassay-guided fractionation, piperine was found as a main component of the preparation. Piperine is an alkaloid found naturally in plants belonging to the Piperaceae family, such as *Piper nigrum* Linn, also known as black pepper (Chung Soo Lee et al., 2006). Hence, the quality of piperine was not analyzed by the GC-MS technique, but its quantity was determined quantity by HPLC technique followed by Sakpakdeejaroen (2009).

A C18 reversed phase HPLC column for quantification of piperine. A moderately to highly polar mobile phase composed of water and acetronitrile with gradient elution as follows: 0 min, 60:40; 30 min, 50:50; 50 min, 5:95; 60 min, 0:100, was eluted with 1 ml/min flow rate. Detection wavelength was determined at wavelenge 256 nm. (See also standard curve of piperine in Appendix C).

This method was analyzed quantity of piperine of ethanolic extract of Ridsiduangmahakan preparation. The amount of piperine of Ridsiduangmahakan preparation was between 0 to 120 days showed in Table 4-11. It was found that the amount of piperine at day 0, 15, 30, 60, 90, 120 were 100, 105.2, 99.50, 84.33,77.44 and 61.33%, respectively. It was interpreted that this extract was not stable when kept in normal condition in 2 years because the amount of main ingredients decreased more than 20%.

Table 4-11

Amount of piperine, IC_{50} (μg/ml) of Nitric Oxide (NO) production and PGE_2 release of the ehtanolic extract of Ridsiduangmahakan preparation under accelerated condition (45 ± 2°C with 75 ± 5% RH) for 120 days

Day	Amount of Piperine		Anti-inflammatory activity	
	mg/g	%content	IC_{50} of inhibitory effect of NO production (μg/ml)	IC_{50} of inhibitory effect of PGE_2 release (μg/ml)
0	9.32	100	73.50±4.68	17.41
15	9.81	105.23	79.52±3.32	18.32
30	9.28	99.50	86.74±1.95	19.53
60	7.86*	84.33*	86.87±3.74	22.13
90	7.22*	77.44*	87.28±4.77	29.16
120	5.72*	61.33*	95.59±1.71*	29.64

*p *value* < 0.05, compared with Day 0

The stability of anti-inflammation testing, inhibitory effects on LPS-stimulated PGE_2 release and Nitric Oxide production from RAW 264.7 cells, of the preparation. The results are shown in Table 4-11, figure 4-6 and 4-7. Inhibitory effect of NO production was no significant changed at day 0 to day 90. At day 120, IC_{50} of NO production value increased to 95.59±1.71 μg/ml which showed significant values between day 0 and day 120 but IC_{50} of stimulated PGE_2 release, was increased a little bit and non significant when compared with day 0 to day 120. IC_{50} values from 17.41 μg/ml at day 0 to 29.64 μg/ml at day 120. It was concluded that the accelerated condition might reduced only anti-inflammation possessing by inhibition NO production but could reduced inhibitory activity on LPS- stimulated PGE_2 release. Piperine content showed significant different at day 60. The previous report found that piperine showed anti-inflammation possessing by reduced the production of PGE_2 in a dose dependant (Hong et al., 2002). Thus, not only piperine reduced the production of PGE_2 but also another compounds in this preparation might be reduced. Thus it was concluded that this preparation stable on inhibitory activity on LPS-

stimulated PGE_2 release or as COX-2 inhibitor within 2 years. If the RSD extract will be analyzed for drug from this preparation should use testing inhibitory activity on LPS- stimulated PGE_2 release or COX-2 inhibitory activity as biological finger print for standardization product. Chemical finger print of GCMS on Table 4-10 showed that the chemical degradation of peak at retention time 9-24 min, 35-44 min at accelerated condition these peak at this time was disappeared but they changed as peak at more than 50 min. This chemical finger print will be used as leading guild of anti-inflammatory activity.

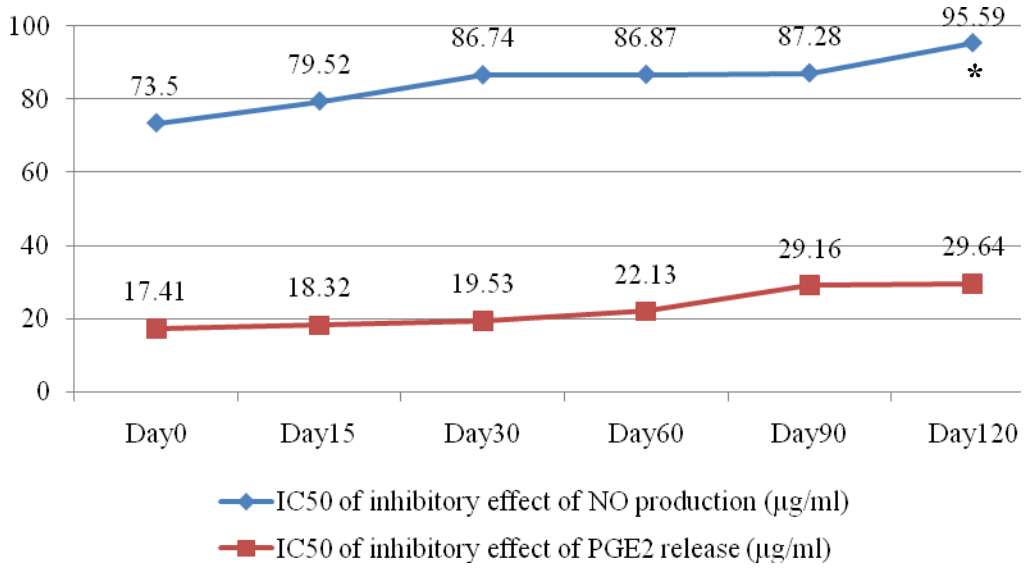

Figure 4-4

The stability of biological activity test for inhibition of Nitric Oxide production and PGE_2 release (IC_{50}) of Ridsiduangmahakan preparation under accelerated condition (45 ± 2°C with 75 ± 5% RH)

* *p value*< 0.05, compared with day 0

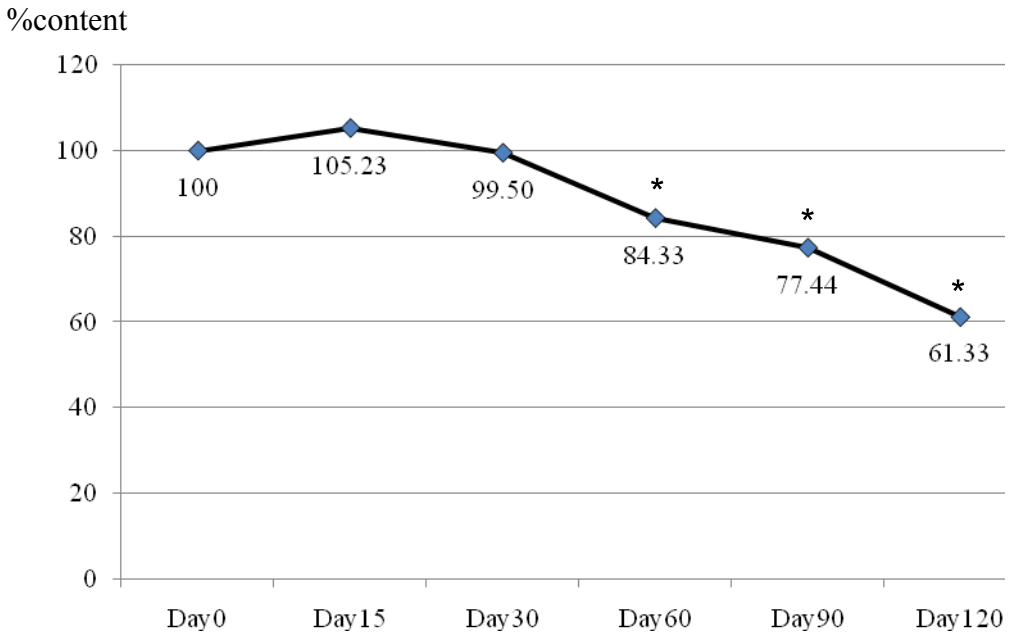

Figure 4-5

The stability of piperine (%content) in the ehtanolic extract of Ridsiduangmahakan preparation under accelerated condition (45 ± 2°C with 75 ± 5% RH)

* *p value*< 0.05, compared with day 0

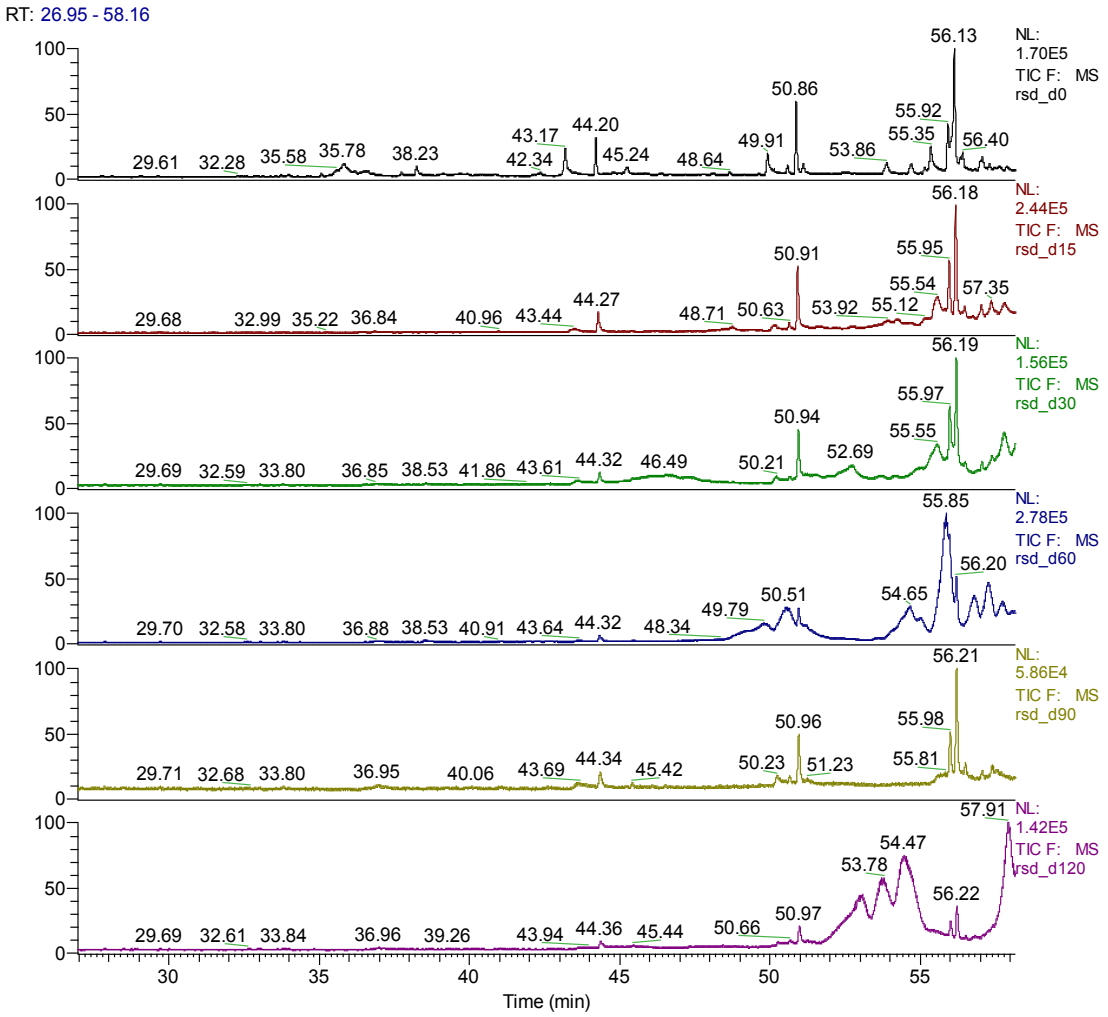

Figure 4-6

Stability of Chemical fingerprint using GC MS of Ridsiduangmahakan preparation on Day0, 15, 30, 60, 90 and 120 (from top to bottom panels) under accelerated condition (45 ± 2°C with 75 ± 5% RH)

Table 4-12

Chemical analysis using GC MS of RSD preparation under accelerated condition (45 ± 2°C with 75 ± 5% RH) for 120 days

RT	Chemical compound of various days using GC MS					
	Day0	**Day15**	**Day30**	**Day60**	**Day90**	**Day120**
9.39	Alpha pinene (2.99%)	-	-	-	-	-
11.31	Terpinolene (0.23%)	-	-	-	-	-
11.68	P cymene (0.20%)	-	-	-	-	-
13.22	Gamma terpinene (0.52%)	-	-	-	-	-
19.06	Terpinene 4-ol (0.62%)	-	-	-	-	-
24.08	Safrole (0.75%)	-	-	-	-	-
27.84	NI	Caryophyllene (0.07%)	Caryophyllene (0.12%)	Caryophyllene (0.02%)	Caryophyllene (0.16%)	-
29.69	NI	Junipene (0.64%)	Junipene (0.22%)	Junipene (0.03%)	-	Junipene (0.06%)
33.77	NI	Guaiene (0.09)	Guaiene (0.31%)	Guaiene (0.03%)	Guaiene (0.56%)	Guaiene (0.23%)
36.80	NI	Cedrenol (0.87%)	Cedrenol (1.57%)	Cedrenol (0.20%)	Cedrenol (5.30%)	Cedrenol (0.77%)
35.79	Diethyl phthalate (13.96%)	-	-	-	-	-
38.23	1,4-bis(methoxy)-triquinacene (1.41%)	-	-	-	-	-
43.18	Isopropyl myristate (6.33%)	-	-	-	-	-
44.20	Ethyl myristate (4.14%)	-	-	-	-	-
44.28	-	Palmitic acid (7.32%)	Palmitic acid (1.71%)	Palmitic acid (0.42%)	Palmitic acid (12.13%)	Palmitic acid (0.96%)

Table 4-12 (Continued)

RT	Chemical compound of various days using GC MS					
	Day0	**Day15**	**Day30**	**Day60**	**Day90**	**Day120**
45.24	Isoproplymyristate (1.72%)	-	-	-	-	-
48.70	NI	Unknown (2.88%)	-	-	-	-
49.91	Palmitic acid (2.64%)	-	-	-	-	-
50.60	NI	(-)-sclareol (1.11%)				
50.86	Ethyl palmitate (18.22%)	-	-	-	-	-
50.92	-	Hexadecanoic acid (13.70%)	Hexadecanoic acid (10.68)	Hexadecanoic acid (15.12%)	Hexadecanoic acid (27.33%)	-
51.10	Astratone (1.02%)	-	-	-	-	-
52.69	-	-	Unknown (12.58%)	-	-	-
54.65	-	-	-	Unknown (4.30%)	-	Unknown (38.46%)
55.34	Oleic acid (6.90%)	-	-	-	-	-
55.90	-	ethyl linoleate (7.74%)	ethyl linoleate (7.80%)	ethyl linoleate (24.03%)	-	-
56.13	Unknown (33.14%)	-	-	-	-	-
56.18	-	Oleic acid (42.01%)	Oleic acid (71.34%)	-	Unknown (53.53%)	-
57.06	Unknown (4.31%)	-	-	-	-	-
58.73	-	Unknown (23.45%)	-	-	-	-
59.16	-	-	-	Unknown (55.31%)	-	Unknown (59.36%)