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APPENDICES

Appendix A

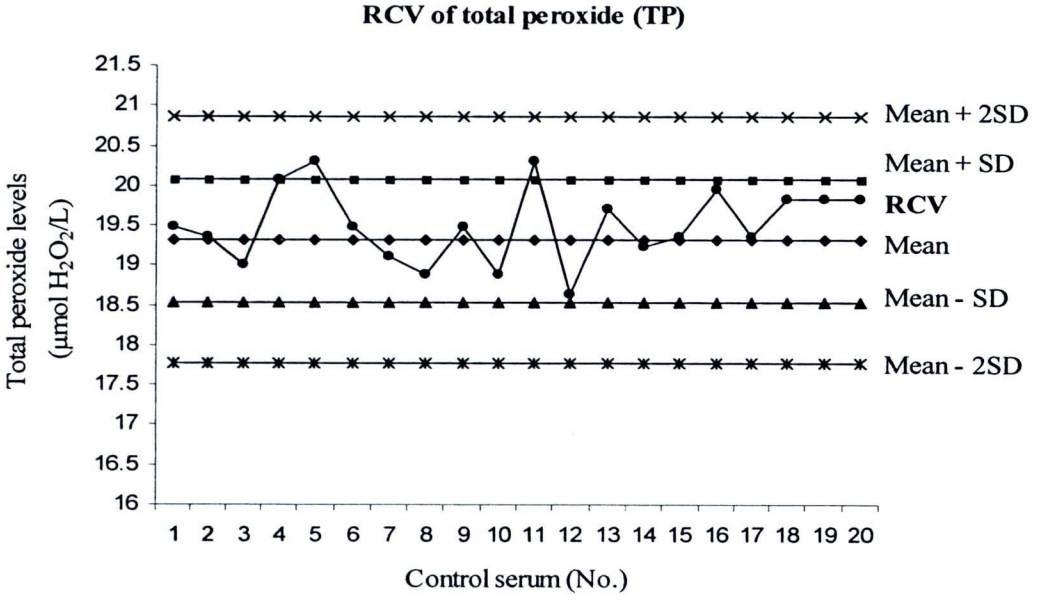
Precision assay: Optimal Condition Variance (OCV) and Routine Condition Variance (RCV)

Table A1 Optimal condition variance (OCV) precisions of the assay for determination of total peroxide (TP)

OCV (No.)	**Absorbance (560 nm)**	**Calculation* (μmol/L)**
1	0.287	19.00
2	0.289	19.24
3	0.281	18.29
4	0.291	19.48
5	0.289	19.24
6	0.291	19.48
7	0.296	20.07
8	0.294	19.83
9	0.280	18.17
10	0.289	19.24
11	0.286	18.88
12	0.279	18.05
13	0.293	19.71
14	0.298	20.31
15	0.280	18.17
16	0.290	19.36
17	0.298	20.31
18	0.297	20.19
19	0.284	18.64
20	0.300	20.55
Mean (μmol/L)		19.31
SD		0.77
OCV (%)		4.01

*Total peroxide (TP) concentration of each control sample was determined from standard curve by hydrogen peroxide (H_2O_2) which was used to make a standard curve. The correlation was shown by R^2 was 0.9989.

Figure A1 Routine condition variance (RCV) precisions of the assay for determination of total peroxide (TP)



RCV (No.)	Absorbance (560 nm)	Calculation*(μmol/L)
1	0.291	19.48
2	0.290	19.36
3	0.287	19.00
4	0.296	20.07
5	0.298	20.31
6	0.291	19.48
7	0.288	19.12
8	0.286	18.88
9	0.291	19.48
10	0.286	18.88
11	0.298	20.31
12	0.284	18.64
13	0.293	19.71
14	0.289	19.24
15	0.290	19.36
16	0.295	19.95
17	0.290	19.36
18	0.294	19.83
19	0.294	19.83
20	0.294	19.83

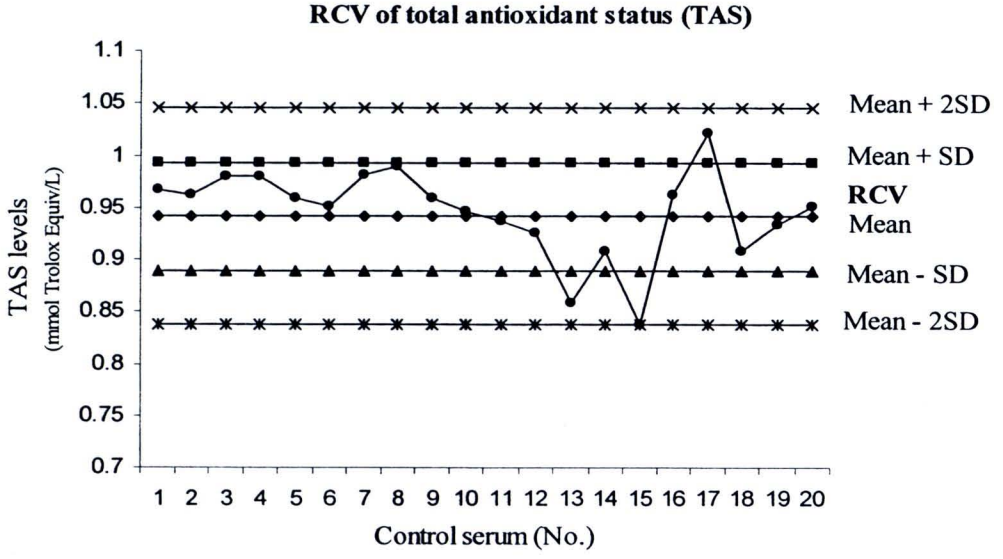
The data were shown as mean =19.50 μmol/L, SD = 0.47 and RCV = 2.42.

Table A2 Optimal condition variance (OCV) precisions of the assay for determination of total antioxidant status (TAS)

OCV (No.)	Absorbance (660 nm)	Calculation* (mmol Trolox equiv./L)
1	0.742	1.00
2	0.749	0.98
3	0.750	0.98
4	0.753	0.97
5	0.746	0.99
6	0.744	1.00
7	0.766	0.94
8	0.769	0.93
9	0.775	0.92
10	0.771	0.93
11	0.764	0.94
12	0.745	0.99
13	0.764	0.94
14	0.746	0.99
15	0.749	0.98
16	0.785	0.89
17	0.781	0.90
18	0.812	0.82
19	0.802	0.85
20	0.786	0.89
Mean (mmol Trolox equiv./L)		0.94
SD		0.05
OCV (%)		5.53

*Total antioxidant status (TAS) concentration of each control sample was determined from standard curve by 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) which was used to make a standard curve. The correlation was shown by R^2 was 0.9986.

Figure A2 Routine condition variance (RCV) precisions of the assay for determination of total antioxidant status (TAS)



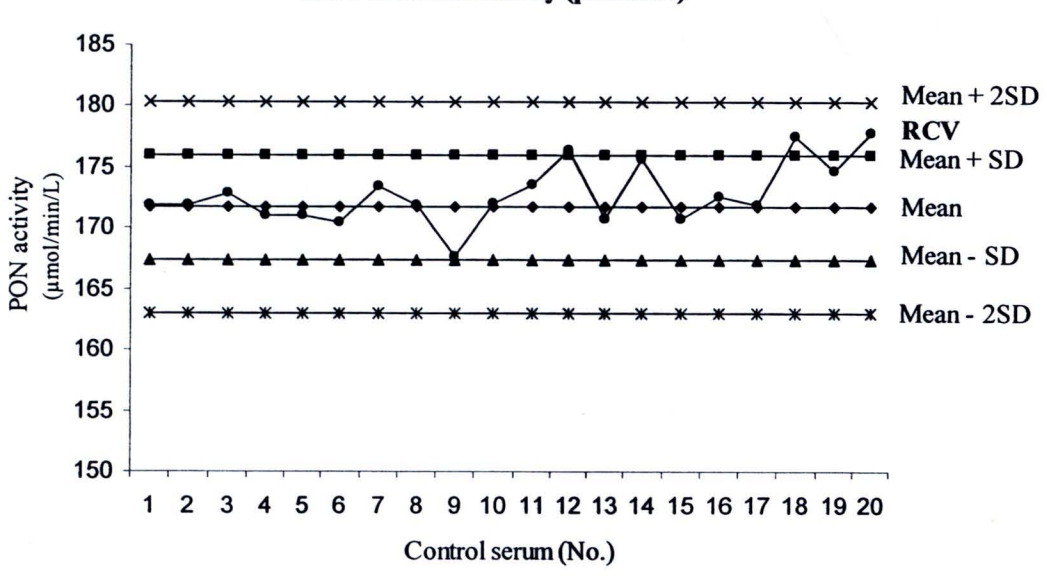
RCV (No.)	Absorbance (660 nm)	Calculation* (mmol Trolox equiv./L)
1	0.755	0.97
2	0.757	0.96
3	0.750	0.98
4	0.750	0.98
5	0.758	0.96
6	0.761	0.95
7	0.749	0.98
8	0.746	0.99
9	0.758	0.96
10	0.763	0.95
11	0.767	0.94
12	0.771	0.93
13	0.797	0.86
14	0.778	0.91
15	0.805	0.84
16	0.757	0.96
17	0.734	1.02
18	0.778	0.91
19	0.768	0.93
20	0.761	0.95

The data were shown as mean = 0.95 mmol Trolox equiv/L, SD = 0.04 and RCV = 4.55.

Table A3 Optimal condition variance (OCV) precisions of the assay for determination of paraoxonase activity (using paraoxon as a substrate)

OCV (No.)	Initial reading	mAbs/Min	Activity
1	0.291	62.18	169.56
2	0.296	63.25	172.48
3	0.286	60.20	164.15
4	0.299	61.30	167.16
5	0.297	61.70	168.25
6	0.300	62.49	170.40
7	0.307	62.61	170.73
8	0.300	63.52	173.23
9	0.305	61.83	168.61
10	0.295	61.06	166.50
11	0.339	66.32	180.85
12	0.317	64.70	176.43
13	0.305	63.61	173.47
14	0.317	62.99	171.76
15	0.324	63.79	173.97
16	0.312	65.78	179.38
17	0.316	64.04	174.62
18	0.306	61.07	166.53
19	0.332	62.54	170.54
20	0.311	64.14	174.91
Mean (µmol/min/L)			171.68
SD			4.33
OCV (%)			2.52

Figure A3 Routine condition variance (RCV) precisions of the assay for determination of paraoxonase activity (using paraoxon as a substrate)



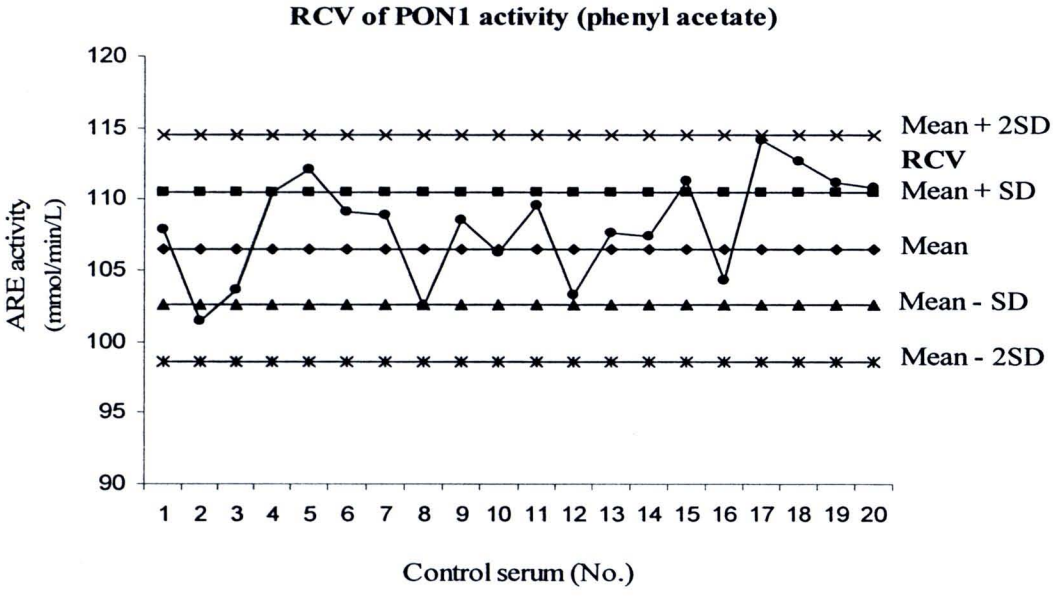
RCV (No.)	Initial reading	mAbs/Min	Activity
1	0.322	63.01	171.83
2	0.310	63.01	171.84
3	0.312	63.40	172.90
4	0.287	62.70	170.97
5	0.304	62.73	171.05
6	0.294	62.51	170.45
7	0.284	63.62	173.49
8	0.286	63.01	171.82
9	0.289	61.48	167.67
10	0.297	63.10	172.09
11	0.294	63.66	173.61
12	0.330	64.68	176.38
13	0.297	62.60	170.71
14	0.276	64.42	175.68
15	0.242	62.61	170.75
16	0.214	63.30	172.61
17	0.302	63.03	171.89
18	0.300	65.10	177.54
19	0.270	64.09	174.77
20	0.237	65.19	177.79

The data were shown as mean = 172.79 μmol/min/L, SD = 2.56 and RCV = 1.48.

Table A4 Optimal condition variance (OCV) precisions of the assay for determination of arylesterase activity (using phenyl acetate as a substrate)

OCV (No.)	Initial reading	mAbs/Min	Activity
1	0.129	135.86	105.84
2	0.122	132.13	102.93
3	0.124	132.20	102.98
4	0.121	129.72	101.05
5	0.127	135.16	105.29
6	0.135	135.13	105.26
7	0.125	131.20	102.20
8	0.122	131.79	102.67
9	0.123	137.24	106.91
10	0.122	135.77	105.77
11	0.123	130.47	101.63
12	0.126	138.44	107.85
13	0.138	137.52	107.13
14	0.148	141.77	110.44
15	0.157	147.01	114.52
16	0.160	142.99	111.39
17	0.146	139.65	108.79
18	0.157	146.02	113.75
19	0.151	141.49	110.22
20	0.160	133.72	104.17
Mean (mmol/min/L)			106.54
SD			3.97
OCV (%)			3.72

Figure A4 Routine condition variance (RCV) precisions of the assay for determination of arylesterase activity (using phenyl acetate as a substrate)



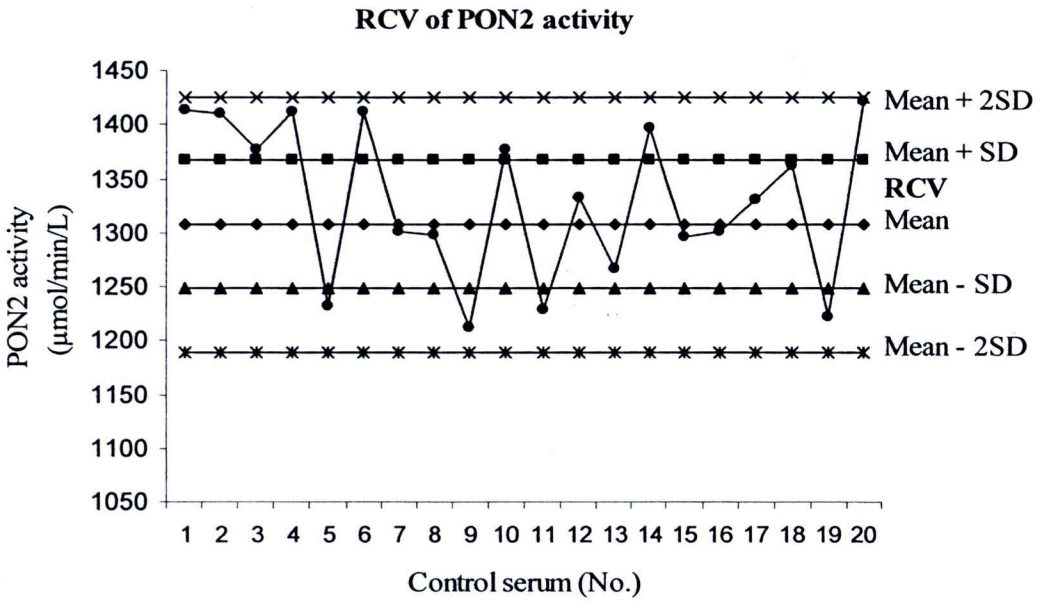
RCV (No.)	Initial reading	mAbs/Min	Activity
1	0.134	138.42	107.83
2	0.122	130.28	101.49
3	0.127	133.02	103.62
4	0.150	141.90	110.54
5	0.144	143.91	112.10
6	0.136	140.08	109.13
7	0.131	139.76	108.87
8	0.029	131.49	102.43
9	0.040	139.39	108.58
10	0.037	136.46	106.30
11	0.047	140.67	109.58
12	0.031	132.65	103.34
13	0.037	138.14	107.61
14	0.041	137.81	107.36
15	0.052	142.91	111.33
16	0.032	133.97	104.36
17	0.169	146.48	114.11
18	0.180	144.58	112.63
19	0.170	142.68	111.15
20	0.171	142.24	110.80

The data were shown as mean = 108.16 mmol/min/L, SD = 3.60 and RCV = 3.33.

Table A5 Optimal condition variance (OCV) precisions of the assay for determination of PON2 activity (using control serum)

OCV (No.)	Initial reading	mAbs/Min	Activity
1	0.200	35.60	1402.10
2	0.197	32.45	1278.00
3	0.224	31.43	1237.81
4	0.161	31.81	1252.77
5	0.165	32.95	1297.54
6	0.162	31.10	1224.90
7	0.157	32.13	1265.37
8	0.150	33.19	1307.15
9	0.155	34.94	1375.95
10	0.199	34.14	1344.59
11	0.182	34.42	1355.37
12	0.177	33.72	1328.01
13	0.153	35.75	1407.75
14	0.140	31.30	1232.51
15	0.177	33.36	1313.61
16	0.169	31.94	1257.95
17	0.132	34.76	1369.06
18	0.129	34.93	1375.44
19	0.121	31.63	1245.50
20	0.124	32.55	1281.90
Mean (µmol/min/L)			1307.67
SD			59.03
OCV (%)			4.51

Figure A5 Routine condition variance (RCV) precisions of the assay for determination of PON2 activity (using control serum)



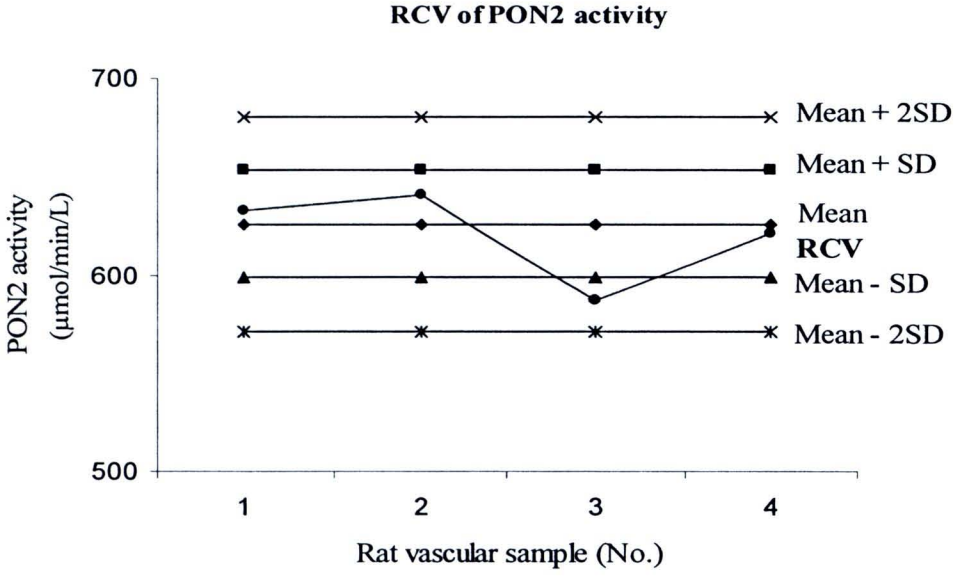
RCV (No.)	Initial reading	mAbs/Min	Activity
1	0.186	35.91	1414.32
2	0.148	35.80	1410.00
3	0.226	34.98	1377.76
4	0.125	35.85	1411.89
5	0.060	31.28	1231.96
6	0.102	35.87	1412.61
7	0.098	33.02	1300.44
8	0.060	32.94	1297.27
9	0.079	30.77	1211.90
10	0.085	34.99	1377.92
11	0.076	31.18	1227.78
12	0.089	33.85	1333.12
13	0.136	32.15	1266.10
14	0.123	35.47	1396.72
15	0.074	32.94	1297.10
16	0.161	33.05	1301.47
17	0.138	33.80	1331.11
18	0.133	34.61	1362.93
19	0.074	31.03	1221.87
20	0.051	36.10	1421.64

The data were shown as mean = 1330.30 µmol/min/L, SD = 72.25 and RCV = 5.43.

Table A6 Optimal condition variance (OCV) precisions of the assay for determination of PON2 activity (using rat vascular sample)

No.	Initial reading	mAbs/Min	Activity
1	0.445	15.89	625.93
2	0.425	16.55	651.71
3	0.456	14.93	587.82
4	0.374	16.18	637.00
Mean (μmol/min/L)			625.61
SD			27.32
OCV (%)			4.37

Figure A6 Routine condition variance (RCV) precisions of the assay for determination of PON2 activity (using rat vascular sample)



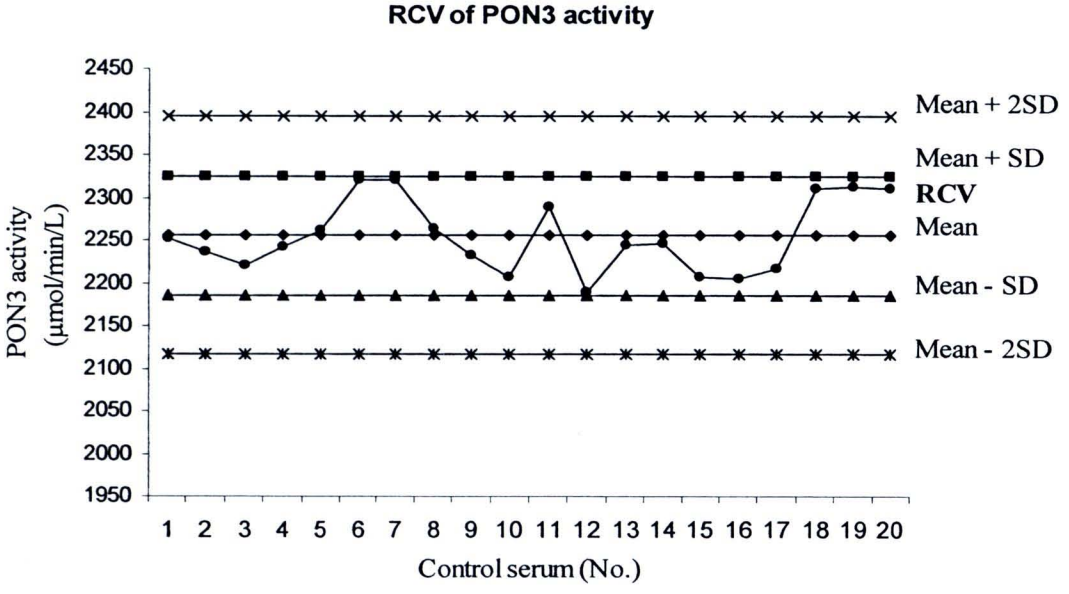
RCV (No.)	Initial reading	mAbs/Min	Activity
1	0.354	16.06	632.39
2	0.413	16.28	641.11
3	0.463	14.90	586.60
4	0.367	15.78	621.37

The data were shown as mean = 620.37 μmol/min/L, SD = 23.92 and RCV = 3.86.

Table A7 Optimal condition variance (OCV) precisions of the assay for determination of PON3 activity

OCV (No.)	Initial reading	mAbs/Min	Activity
1	0.447	438.81	2370.01
2	0.464	424.65	2293.53
3	0.448	440.51	2379.17
4	0.418	424.82	2294.44
5	0.394	432.07	2333.60
6	0.387	429.77	2321.20
7	0.367	424.90	2294.87
8	0.341	426.96	2306.02
9	0.291	421.85	2278.38
10	0.267	423.13	2285.31
11	0.250	415.24	2242.73
12	0.220	412.24	2226.48
13	0.195	410.27	2215.88
14	0.184	400.70	2164.18
15	0.191	405.55	2190.40
16	0.145	396.26	2140.18
17	0.151	410.37	2216.39
18	0.115	406.24	2194.09
19	0.101	403.00	2176.60
20	0.102	404.74	2186.00
Mean (μmol/min/L)			2255.47
SD			69.63
OCV (%)			3.09

Figure A7 Routine condition variance (RCV) precisions of the assay for determination of PON3 activity



RCV (No.)	Initial reading	mAbs/Min	Activity
1	0.513	417.07	2252.60
2	0.520	414.18	2237.01
3	0.489	411.20	2220.91
4	0.391	415.02	2241.51
5	0.344	418.75	2261.65
6	0.441	429.50	2319.74
7	0.361	429.66	2320.61
8	0.264	419.12	2263.66
9	0.195	413.49	2233.29
10	0.116	408.70	2207.41
11	0.563	432.91	2289.52
12	0.370	405.31	2189.08
13	0.333	415.56	2244.42
14	0.571	415.91	2246.34
15	0.423	408.45	2206.02
16	0.374	408.22	2204.82
17	0.306	410.44	2216.79
18	0.539	427.71	2310.05
19	0.408	428.38	2313.69
20	0.279	427.91	2311.12

The data were shown as mean = 2254.51 μmol/min/L, SD = 42.76 and RCV = 1.90.

Appendix B

Standard curve for the determination of Total peroxide (TP), Total antioxidant status (TAS) and Protein assay

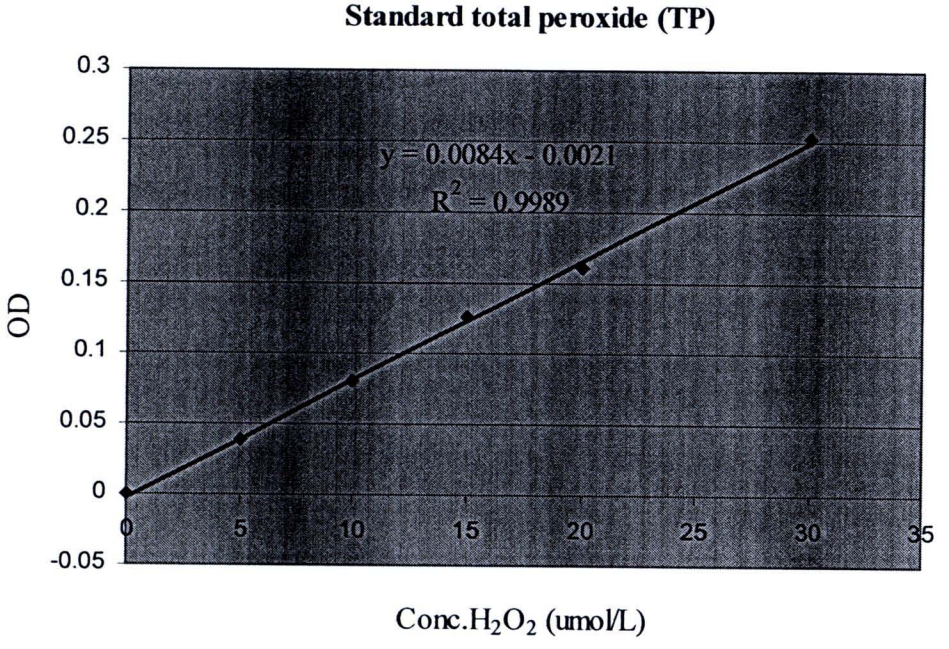


Figure B1 Standard curve of total peroxide (TP) was determined by using hydrogen peroxide (H_2O_2). The correlation was shown by R^2 was 0.9989.

Working standard (μmol/l)	Absorbance (560 nm)	Correct OD
0	0.057	0.000
5	0.096	0.039
10	0.138	0.081
15	0.183	0.126
20	0.218	0.161
30	0.310	0.253

Data shown were absorbance of the reactions.

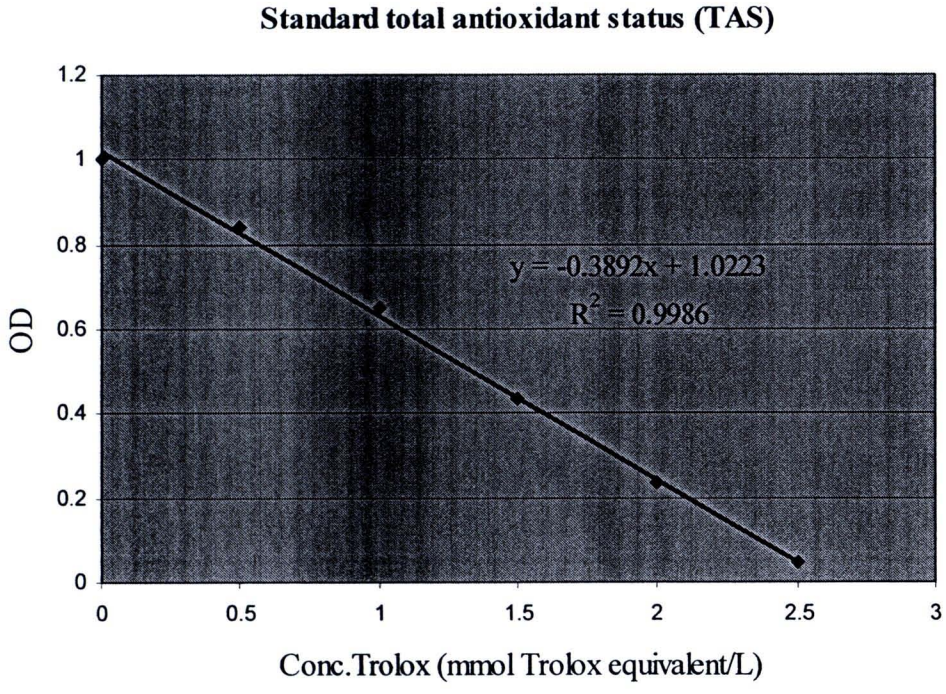


Figure B2 Standard curve of total antioxidant status (TAS) was determined by using 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox). The correlation was shown by R^2 was 0.9986.

Working standard (mmol/L)	Absorbance (660 nm)
0	1.003
0.5	0.842
1.0	0.650
1.5	0.437
2.0	0.236
2.5	0.047

Data shown were absorbance of the reactions.

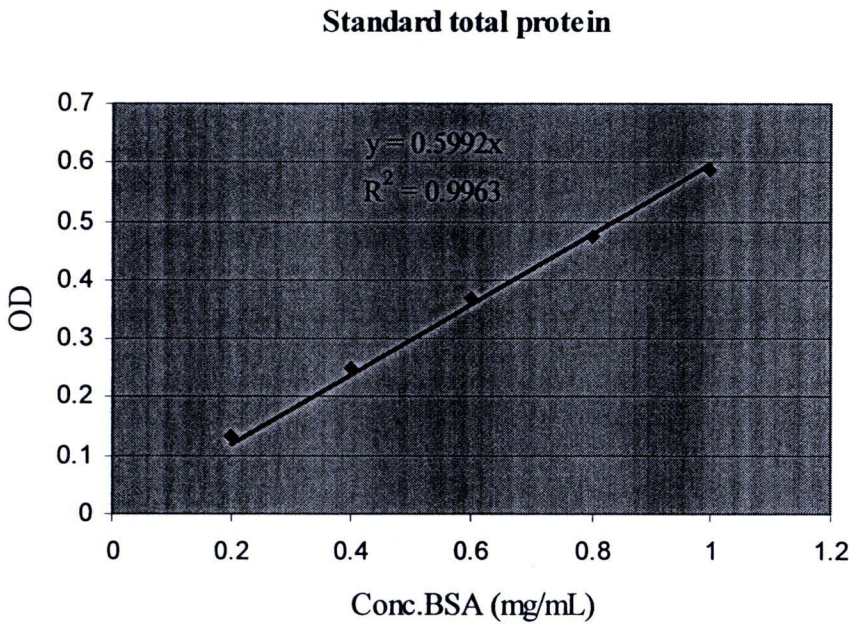


Figure B3 Standard curve of total protein assay was determined by using bovine serum albumin (BSA). The correlation was shown by R^2 was 0.9963.

Working standard (mg/ml)	Absorbance (595 nm)
0.2	0.132
0.4	0.250
0.6	0.370
0.8	0.476
1.0	0.589

Data shown were absorbance of the reactions.

Appendix C

Effects of *C. comosa* and simvastatin on oxidative stress parameters and paraoxonase activities

Table C1 Oxidative stress parameters of individual rabbit

Group.	No.	Oxidative stress parameters (mean)		
		TP (μmol/L)	**TAS** (mmol Trolox equiv./L)	**OSI**
Cholesterol	1	3.35	1.06	0.32
	2	5.97	1.10	0.55
	3	5.24	1.21	0.44
	4	5.17	1.16	0.45
	Mean	4.93	1.13	0.44
	SEM	0.56	0.03	0.05
Cholesterol + simvastatin	1	7.12	0.95	0.75
	2	8.11	0.82	0.99
	3	8.76	0.89	0.99
	4	8.51	0.83	1.03
	Mean	8.13	0.87	0.94
	SEM	0.36	0.03	0.07
Cholesterol + *C. comosa*	1	7.24	0.98	0.74
	2	7.87	0.98	0.80
	3	5.85	1.16	0.51
	4	6.01	0.91	0.66
	Mean	6.74	1.01	0.68
	SEM	0.49	0.05	0.06

Values are mean ± SEM obtained from 4 rabbits.

The experiment was performed in triplicate for 4 rabbits in each group.

Table C2 Paraoxonase activities of individual rabbit

Group.	No.	Activity (mean)			
		PON1		PON2	PON3
		Phenyl acetate (mmol/min/L)	**Paraoxon** (μmol/min/L)	(U/mg protein)	(μmol/min/L)
Cholesterol	1	441.08	2553.38	200.13	4860.73
	2	384.45	1802.42	228.84	8944.05
	3	373.38	1706.46	287.18	5595.59
	4	318.56	1889.63	245.81	4824.20
	Mean	379.37	1987.98	240.49	6056.14
	SEM	25.11	192.15	18.20	978.90
Cholesterol + simvastatin	1	322.22	2696.89	131.39	3969.93
	2	284.66	1209.58	129.12	5920.80
	3	341.96	1563.09	113.52	4886.01
	4	323.17	1857.37	219.89	4961.40
	Mean	318.00	1831.73	148.48	4934.54
	SEM	12.00	317.33	24.13	398.57
Cholesterol + *C. comosa*	1	285.61	2464.47	144.26	4158.99
	2	345.64	1890.12	205.98	9141.35
	3	372.75	2341.53	163.16	6081.74
	4	367.11	2132.84	286.61	6501.03
	Mean	342.78	2207.24	200.00	6470.78
	SEM	19.93	125.93	31.62	1025.86

Values are mean ± SEM obtained from 4 rabbits.

The experiment was performed in triplicate for 4 rabbits in each group.

Appendix D

Clinical blood chemistry

Lipid parameters, Renal function and Liver function

Table D1 Lipid parameters at baseline of individual rabbit

Group.	No.	Lipid parameters (mg/dL)			
		TC	TG	HDL-C	LDL-C
Cholesterol	1	53	50	46	14
	2	71	52	55	24
	3	51	84	38	13
	4	40	119	27	7
	Mean	53.75	76.25	41.50	14.50
	SEM	6.42	16.24	5.95	3.52
Cholesterol + simvastatin	1	69	64	53	21
	2	43	53	31	9
	3	53	76	39	16
	4	54	38	49	11
	Mean	54.75	57.75	43.00	14.25
	SEM	5.36	8.09	4.97	2.69
Cholesterol + *C. comosa*	1	55	91	40	15
	2	62	60	48	18
	3	35	76	29	4
	4	45	93	37	5
	Mean	49.25	80.00	38.50	10.50
	SEM	5.89	7.67	3.93	3.52

Values shown were mean ± SEM obtained from 4 rabbits.

TC=total cholesterol, TG=triglyceride, HDL-C=high density lipoprotein-cholesterol, LDL-C=low density lipoprotein-cholesterol.

Table D2 Lipid parameters at 4 months of individual rabbit

Group.	No.	Lipid parameters (mg/dL)			
		TC	TG	HDL-C	LDL-C
Cholesterol	1	2,584	144	419	2,587
	2	1,877	124	357	1,711
	3	1,870	99	355	1,758
	4	2,210	190	416	1,981
	Mean	2,135.25	139.25	386.75	2,009.25
	SEM	169.32	19.26	17.77	201.39
Cholesterol + simvastatin	1	1,680	121	347	1,534
	2	1,924	89	622	1,615
	3	1,243	99	229	1,155
	4	1,453	43	338	1,329
	Mean	1,575.00	88.00	384.00	1,408.25
	SEM	146.61	16.42	83.74	103.67
Cholesterol + *C. comosa*	1	1,659	92	315	1,534
	2	1,553	219	287	1,370
	3	1,463	110	365	1,328
	4	1,896	444	301	1,622
	Mean	1,642.75	216.25	317.00	1,463.50
	SEM	93.44	80.94	16.99	69.04

Values shown were mean ± SEM obtained from 4 rabbits.

TC=total cholesterol, TG=triglyceride, HDL-C=high density lipoprotein-cholesterol, LDL-C=low density lipoprotein-cholesterol.

Table D3 Renal function at 4 months of individual rabbit

Group.	No.	Renal function	
		BUN (mg/dL)	Cr (mg/dL)
Cholesterol	1	20	1.3
	2	17	0.8
	3	20	1.2
	4	25	1.5
	Mean	20.50	1.20
	SEM	1.66	0.15
Cholesterol + simvastatin	1	20	1.1
	2	20	1.3
	3	16	1.0
	4	20	1.2
	Mean	19.00	1.15
	SEM	1.00	0.07
Cholesterol + *C. comosa*	1	27	1.4
	2	25	1.1
	3	20	1.1
	4	23	1.2
	Mean	23.75	1.20
	SEM	1.49	0.07

Values are mean ± SEM obtained from 4 rabbits.
BUN = blood urea nitrogen, Cr = creatinine.

Table D4 Liver function at 4 months of individual rabbit

Group.	No.	Liver function					
		SGOT (U/L)	**SGPT** (U/L)	**Alk** (U/L)	**TPro** (g/dL)	**TB** (mg/dL)	**DB** (mg/dL)
Cholesterol	1	56	24	62	6.6	0.4	0
	2	44	28	49	5.7	0.2	0
	3	31	40	44	6.7	0.2	0
	4	73	44	108	6.5	0.2	0
	Mean	51.00	34.00	65.75	6.38	0.25	0
	SEM	8.93	4.76	14.59	0.23	0.05	0
Cholesterol + simvastatin	1	53	40	138	7.3	0.3	0
	2	87	139	65	6.3	0.3	0
	3	69	79	52	7.2	0.2	0
	4	83	152	70	6.6	0.2	0
	Mean	73.00	102.50*	81.25	6.85	0.25	0
	SEM	7.70	26.21	19.29	0.24	0.03	0
Cholesterol + *C. comosa*	1	58	27	42	6.8	0.3	0
	2	65	27	47	6.1	0.3	0
	3	50	31	57	7.1	0.3	0
	4	42	27	45	7.2	0.4	0
	Mean	53.75	28.00	47.75	6.80	0.33	0
	SEM	4.97	1.00	3.25	0.25	0.03	0

Values are mean ± SEM obtained from 4 rabbits.

*$p<0.05$ significant difference from cholesterol-fed control and cholesterol-fed with *C. comosa* groups.

SGOT = serum glutamic-pyruvic transaminase, Alk = alkaline phosphatase,

SGPT = serum glutamic-oxaloacetic transaminease, TPro = total protein,

TB = total bilirubin, DB = direct bilirubin.

Appendix E

Experimental diets

Food

1. Standard food for rabbits was purchased from Charoen Pokphand Foods Public Company Limited, Thailand. The composition of the standard food was as following:

Raw protein	17.00%
Raw fiber	17.00%
Raw ash	7.00%
Raw fat	2.50%
Methionine	0.35%
Lysine	0.95%
Calcium	0.85%
Phosphate	0.55%
Sodium	0.30%
Magnesium	0.25%
Vitamin A	30.00 IU/kg
Vitamin D_3	1.00 IU/kg
Vitamin E	100.00 IU/kg

2. High-cholesterol diet was the standard food combined with 1.0% cholesterol or 0.5% cholesterol by Charoen Pokphand Foods Public Company Limited, Bangkok, Thailand.

Appendix F

- Study Protocol Approval by Ethic Committee of the Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand
- Study Protocol Approval by Ethic Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

62 หมู่ 7 อำเภอองครักษ์
จังหวัดนครนายก 26120
โทร.0-3739-5085 ต่อ 10513

ใบอนุญาตให้ใช้สัตว์

ในงานวิจัย งานทดสอบ งานผลิตชีววัตถุ และงานสอน

เลขที่หนังสือรับรอง 10/2550

ชื่อโครงการวิจัย	การศึกษาผลของสารสกัดว่านชักมดลูกต่อการยับยั้งโรคหลอดเลือดแข็งตัวในกระต่าย
	Inhibition of atherosclerosis development in rabbits by crude extracts of *Curcuma Comosa Roxb.*
ชื่อหัวหน้าโครงการ / หน่วยงานที่สังกัด	ผศ.ดร.ลัดดาวัลย์ ผิวทองงาม / ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
รหัสโครงการ	10/2550
สถานที่ทำการวิจัย	คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
เอกสารรับรอง	- แบบเสนอโครงการวิจัยเพื่อขอรับการพิจารณา - แบบฟอร์มขออนุญาตใช้สัตว์
รับรองโดย	คณะกรรมการรับผิดชอบและดูแลการใช้สัตว์ทดลอง
วันที่รับรอง	8 พฤศจิกายน 2550
วันหมดอายุ	7 พฤศจิกายน 2551

ใบอนุญาตนี้ให้ไว้เพื่อแสดงว่าคณะกรรมการรับผิดชอบและดูแลการใช้สัตว์ทดลอง คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ ซึ่งมีหน้าที่กำกับดูแลการใช้สัตว์ ในงานวิจัย งานทดสอบ งานผลิตชีววัตถุ และงานสอนให้เป็นไปตามจรรยาบรรณการใช้สัตว์ของสภาวิจัยแห่งชาติ

ลงนาม.....

(ผู้ช่วยศาสตราจารย์ ดร.ปัทมา ลิ้วนิช)

ประธานคณะกรรมการ ฯ

ลงนาม.....

(ศาสตราจารย์ นายแพทย์สมเกียรติ วัฒนศิริชัยกุล)

คณบดีคณะแพทยศาสตร์

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☑ Original ☐ Renew
Animal Use Protocol No. 08-33-002	**Approval No. 08-33-002**
Protocol Title Effects of *Curcuma comosa* rhizome on paraoxonase activity and oxidative stress in rabbits fed with high-cholesterol diet	
Principal Investigator Somsong Lawanprasert, Ph.D.	
Certification of Institutional Animal Care and Use Committee (IACUC) This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.	
Date of Approval March 24, 2008	**Date of Expiration** March 24, 2009
Applicant Faculty/Institution **Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Rd., Pathumwan BKK-THAILAND. 10330**	
Signature of Chairperson Withaya Janthasoot	**Signature of Authorized Official**
Name and Title **WITHAYA JANTHASOOT** **Chairman**	**Name and Title** **RUNGPETCH SAKULBUMRUNGSIL, Ph.D.** **Associate Dean (Research and Academic Service)**
The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals. *This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.*	

Appendix G

Publication

Thai Journal of Pharmacology

www.phartherst.org

Official Publication of
Pharmacological and Therapeutic Society of Thailand

Proceedings of 30th Pharmacological and Therapeutic Society of Thailand Meeting

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P06 Effect of *Curcuma comosa* Powder on Serum Paraoxonase Activities in Cholesterol-Diet Fed Rabbits

Cheerana Yomchot[1,*], Somsong Lawanprasert[1], Laddawal Phivthong-ngam[2], Yupin Sanvarinda[3], Sureerut Porntadavity[4].

[1]*Department of Pharmacology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand*
[2]*Department of Pharmacology, Faculty of Medicine, Srinakharinwirot University, Thailand*
[3]*Department of Pharmacology, Faculty of Sciences, Mahidol University, Thailand*
[4]*Department of Clinical Chemistry, Faculty of Medical Technology, Mahidol University, Thailand*
E-mail address: mtspy@mahidol.ac.th

Abstract
Introduction: Serum paraoxonase, PON1 and PON3, is HDL-associated antioxidant enzymes. The role of PON1 and PON3 as anti-atherosclerosis has been clearly demonstrated both *in vitro* and *in vivo*. The activity of PON1 is modulated by various factors including hypolipidemic agents. *Curcuma comosa* Roxb. (Zingiberaceae) is an indigenous plant of Thailand and has been widely used in Thai traditional medicine for treatment of abnormal uterine symptoms. Recently, the hypolipidemic effect of *C. comosa* was extensively investigated.
Objective: We investigated effects of *C. comosa* on PON1 and PON3 activities in cholesterol-diet fed rabbits.
Materials and Methods: Twelve male New Zealand White (NZW) rabbits were treated with 1.0% cholesterol for 1 month and subsequently treated with either 0.5% cholesterol or 0.5% cholesterol combined simvastatin at the dosage of 5 mg/day or 0.5% cholesterol combined *C. comosa* powder at the dosage of 400 mg/kg/day for 3 months.
Results: At 4 months after treatment, lipid parameters and PON1 and PON3 activities were determined. The results showed that *C. comosa* powder significantly decreased levels of total cholesterol and LDL similarly to simvastatin. We found that both *C. comosa* powder and simvastatin did not affect PON1 and PON3 activities.
Conclusion: *C. comosa* powder has hypolipidemic similarly to simvastatin but did not affect PON1 and PON3 activities.
Keywords: Paraoxonase; *Curcuma comosa*; Simvastatin; Cholesterol

Introduction

Serum paraoxonase (PON) consists of two members: PON1 and PON3 which are located adjacent to one another on chromosome 7q21.3-22.1 (1). PON1 and PON3 are expressed primarily in the liver and then secreted into the serum where they are closely associated with HDL (2). Increasing evidences demonstrated that PON1 and PON3 are involved in anti-atherosclerosis. PON1 inhibits copper-induced lipid peroxidation (3). The human PON1 transgenic mice have been found to reduce atherosclerotic lesion (4,5) whereas the PON1 knock out mice accelerated atherosclerosis process and increased lipid peroxidation (6). The information of PON3 is scarcely but has a promising evidence of anti-atherosclerosis properties. Rabbit PON3 is significantly more potent than rabbit PON1 in protecting LDL against oxidative modification (7). Recently, it was found that over-expression of human PON3 in mice reduced atherosclerotic lesion (8). Some clinical data suggest that treatment with hypolipidemic drugs such as simvastatin modulate PON1 activity (9,10). However, at present, it is not known whether simvastatin, might influence PON3 activity.

Curcuma comosa Roxb. is a plant in family Zingiberaceae. It is an indigenous plant of Thailand with a common name in Thai as Waan Chak Mod Look. Rhizomes of *C. comosa* has been used extensively in Thai traditional medicine as an anti-inflammatory agent particularly for the treatment of postpartum uterine bleeding, peri-menopausal bleeding and uterine inflammation. The choleretic effect of *C. comosa* rhizome extract has been recently investigated. It remarkably stimulated bile secretion and enhanced biliary excretion of bile salt and cholesterol which consequently led to a decrease in plasma cholesterol (11).The hypolipidemic effect of *C. comosa* from ethyl acetate extract has been shown to effectively decreased LDL, triglycerides but increased HDL (12,13). The anti-oxidative effect of crude ethanol extract of *C. comosa* has been revealed past year (14). The aim of this study was to investigate the effect of *C. comosa* powder on PON1 and PON3 activities in cholesterol-diet fed rabbits, which was compared with simvastatin, the known medicine using in cardiovascular disease.

Materials & Methods

Materials

Diethyl *p*-nitrophenyl phosphate (paraoxon) and *p*-nitrophenyl butyrate were purchased from Sigma-Aldrich (St.Louis, MO, USA). Phenyl acetate was purchased from Merck (Darmstadt, Germany). Simvastatin was purchased from an accredited drug store (Bangkok, Thailand). *C. comosa* powder was kindly provided by Professor Dr. Apichart Suksamrarn, Faculty of Sciences, Ramkamhaeng University.

Animals and treatment

Twelve male NZW rabbits of body weight between 1.5 – 2.0 kg were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were housed one per cage at the Faculty of Medicine, Srinakharinwirot University, Thailand. All animals were in a controlled humidify room at a constant temperature of 25 ± 2 °C and maintained on a 12-hour alternate light-dark cycle. They were allowed to freely access to food (C.P. Company, Thailand) and drinking water. Prior to the experiment, they were randomly divided into three treatment groups of 4 rabbit each. All treatment groups were given orally with 1.0% cholesterol for 1 month. After 1 month, rabbits in group 1, 2 and 3 were given orally for 3 months with 0.5% cholesterol, 0.5% cholesterol combined simvastatin at the dosage of 5 mg/day and 0.5% cholesterol combined *C. comosa* at the dosage of 400 mg/kg/day, respectively.

Blood sample collection

Blood were collected from 12 hours fasted rabbit at the end of treatment. Plasma were separated and analyzed for lipid profile, liver function and kidney function using auto-analyzer (Hitachi 917) at by Professional Laboratory Management Corp Co., Ltd., Bangkok. Serum were separated and stored at −80 °C until analysis of PON1 and PON3 activities.

Determination of serum PON1 activity

PON1 activity toward paraoxon was measured in 100 mM Tris-HCl buffer pH 8.0 containing 2 mM $CaCl_2$ and 1.1 mM paraoxon at 37 °C (15). The rate of *p*-nitrophenol generation was monitored at 405 nm, and a molar extinction coefficient of 18,700 was used to calculate the enzyme activity. PON1 arylesterase activity was determined in 10 mM Tris-HCl buffer pH 8.0 and 0.9 mM $CaCl_2$ with 1.0 mM phenyl acetate at 37 °C (15). Reaction was monitored at 270 nm, and as extinction coefficient of 1,310 was used for activity calculation.

Determination of serum PON3 activity

PON3 activity was measured in 50 mM Tris-HCl buffer pH 8.0 and 1 mM $CaCl_2$ with 1 mM *p*-nitrophenyl butyrate at 37 °C (16). The rate of of *p*-nitrophenol generation was monitored at 405 nm, and a molar extinction coefficient of 18,700 was used to calculate the enzyme activity.

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). Differences between groups were analyzed using one-way analysis of variance (ANOVA) by Student-Newman-Keuls and Kruskal-Wallis tests for normally and non-normally distributed parameters, respectively. Changes form baseline outcomes were analyzed using Student's *t*-test. Values of $p<0.05$ were considered to be statistically significant.

Results

Table 1 shows no significant different in the levels of lipid parameters among groups at the baseline. The supplement feeding of cholesterol to rabbits successfully raised the level of lipid parameters at 4 months. The levels of TC, HDL and LDL were highly significant increased at 4 months of treatment in all groups ($p<0.001$) while the level of TG significantly increased at 4 months of treatment in all groups with $p<0.05$ as compared to the levels at baseline.

Table 1 Lipid parameters at baseline and 4 months of the 3 treatment groups

Parameters	Baseline			4 months		
(mg/dl)	Cholesterol	Cholesterol+ Simvastatin	Cholesterol+ *C. comosa*	Cholesterol	Cholesterol+ Simvastatin	Cholesterol+ *C. comosa*
TC	53.8 ± 6.4	54.8 ± 5.4	49.3 ± 5.9	2135.3 ± 169.3**	1575.0 ± 146.6**	1642.8 ± 93.4**
TG	76.3 ± 16.2	57.8 ± 8.1	80.0 ± 7.7	139.3 ± 19.3*	88.0 ± 16.4*	216.3 ± 80.9*
HDL	41.5 ± 6.0	43.0 ± 5.0	38.5 ± 3.9	386.8 ± 17.8**	384.0 ± 83.7**	317.0 ± 17.0**
LDL	14.5 ± 3.5	14.3 ± 2.7	10.5 ± 3.5	2009.3 ± 201.4**	1408.3 ± 103.7**	1463.5 ± 69.0**

Values are mean ± SEM obtained from 4 rabbits. **$p<0.001$ significant difference from baseline. *$p<0.05$ significant difference from baseline. TC=total cholesterol, TG=triglyceride, HDL=high density lipoprotein, LDL=low density lipoprotein.

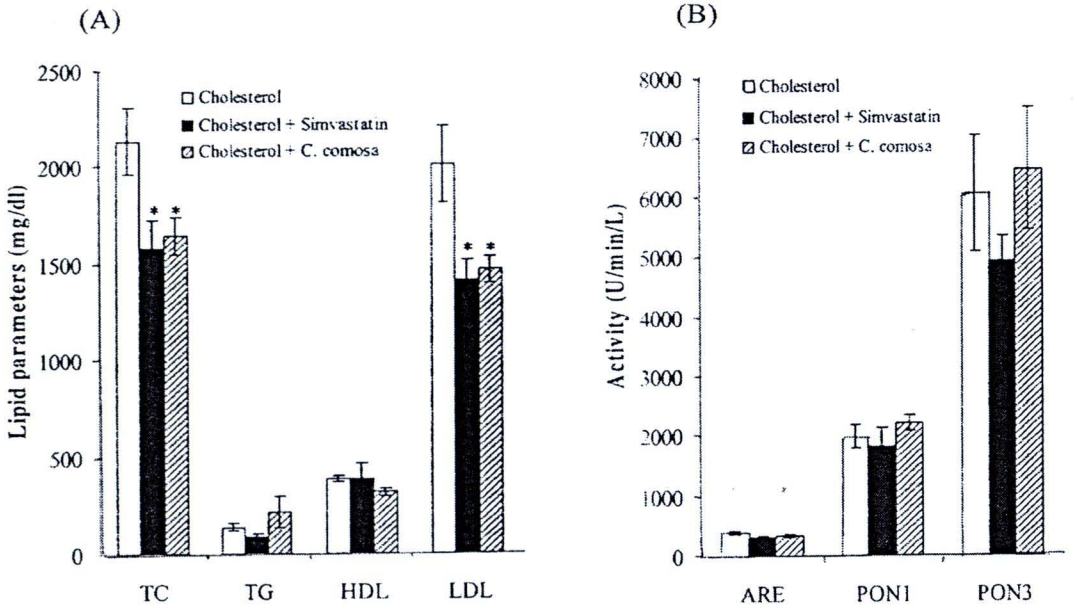

Figure1 Effects of *C. comosa* powder and simvastatin (A) on lipid parameters and (B) on PON1 (using phenyl acetate (ARE) and paraoxon (PON1) as substrates) and PON3 activities at 4 months. Animals were cholesterol-fed, cholesterol-fed with simvastatin and cholesterol-fed with *C. comosa*. Values are mean ± SEM obtained from 4 rabbits. *$p<0.05$ significant difference from cholesterol-fed control.

At 4 months of treatment, both TC and LDL levels in the cholesterol-fed with *C. comosa* and cholesterol-fed with simvastatin groups were significantly decreased than that in the cholesterol-fed control group ($p<0.05$). Remarkably, the decreasing of both TC and LDL levels in the cholesterol-fed with simvastatin group was the same extent as in the cholesterol-fed with *C. comosa*. There were no significant differences in the TG and HDL levels among groups (Fig. 1A).

PON1 activity toward paraoxon and PON3 activities were increased in the cholesterol-fed with *C. comosa* when compared to the cholesterol-fed control, but did not reach statistical significant. The lowest in PON1 activity toward paraoxon and PON3 activities were observed in cholesterol-fed with simvastatin. However, the PON1 activity toward arylesterase was similar in all three groups (Fig. 1B).

Discussion

This study mainly focused on the anti-atherosclerotic effects of *C. comosa* in cholesterol-diet fed rabbit groups by monitoring lipid parameters and serum paraoxonase activity at 4 months of treatment. At 4 months of treatment, the levels of biomarkers for liver function and kidney function were in the reference values in all treatment which indicated that the side effects from the treatment was unlikely occurred (data not shown). Simvastatin decreased TC and LDL levels, but HDL and TG levels remained unchanged. These finding are consistent with the observation found in human (9,10). In our cholesterol-diet fed rabbits, we found that long term treatment with *C. comosa* powder decreased TC and LDL levels as seen in the short term treatment in hypercholesterolemic hamsters (12,13). However, our long term treatment with *C. comosa* powder did not result in the decreased of TG levels. The mechanism of *C. comosa* to lipid parameters is still unknown, it might be associated with interference with the synthesis as well as secretion of lipoprotein into plasma and/or with acceleration of removal of the circulating cholesterol for excretion.

Accumulated data indicated that both PON1 and PON3 are closely associated with HDL and are involved in the prevention of atherosclerosis (7). Hence, the factors influence PON1 activity has been intensively investigated. In addition to reduce plasma lipid, simvastatin with short term treatment has been found to increase the PON1 activity (9,17). In this study, we found that the long term treatment with *C. comosa* powder and simvastatin in cholesterol-diet fed rabbit did not affect PON1 and PON3 activities.

Conclusion

In conclusion, this study demonstrated that long term treatment with *C. comosa* powder decreased total cholesterol and LDL in cholesterol-diet fed rabbits similarly to simvastatin. Both *C. comosa* powder and simvastatin did not affect PON1 and PON3 activities. Our data is limited, thus, true difference between treatment that should present in larger samples might be missed in our study group of 4 rabbits or modulation of PON1 and PON3 might not be associated with the hypolipidemic effect of *C. comosa* powder.

Acknowledgements

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สมาคมเภสัชวิทยาแห่งประเทศไทย

ร่วมกับ

ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ คณะเภสัชศาสตร์

คณะสัตวแพทยศาสตร์ คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ขอมอบประกาศนียบัตรนี้ไว้เพื่อแสดงว่า

จีรณา ยมโชติ

เป็นผู้ได้รับรางวัลชนะเลิศอันดับ 3

การประกวดผลงานวิจัยในการประชุมวิชาการประจำปี ครั้งที่ 30 สมาคมเภสัชวิทยาแห่งประเทศไทย

From Pharmacology to National Drug Policy

วันที่ 27-28 มีนาคม 2551 ณ ห้องประชุม สี สิริสิงห อาคารสมเด็จย่า คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

(ดร.อุดม จันทรารักษ์ศรี)
นายกสมาคมเภสัชวิทยาแห่งประเทศไทย

(รศ.พญ.สุมนา ชมพูทวีป)
ประธานการจัดงานประชุมวิชาการประจำปี ครั้งที่ 30

VITAE

Miss Cheerana Yomchot was born in November 25, 1979 in Chumporn, Thailand. She graduated with a Bachelor of Pharmacy in 2002 from the Faculty of Pharmaceutical Sciences, Khonkaen University, Khonkaen, Thailand.