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Appendices

Appendix A

Verification of methods for the determination of alkoxyresorufin O-dealkylation, aniline 4-hydroxylation and erythromycin N-demethylation

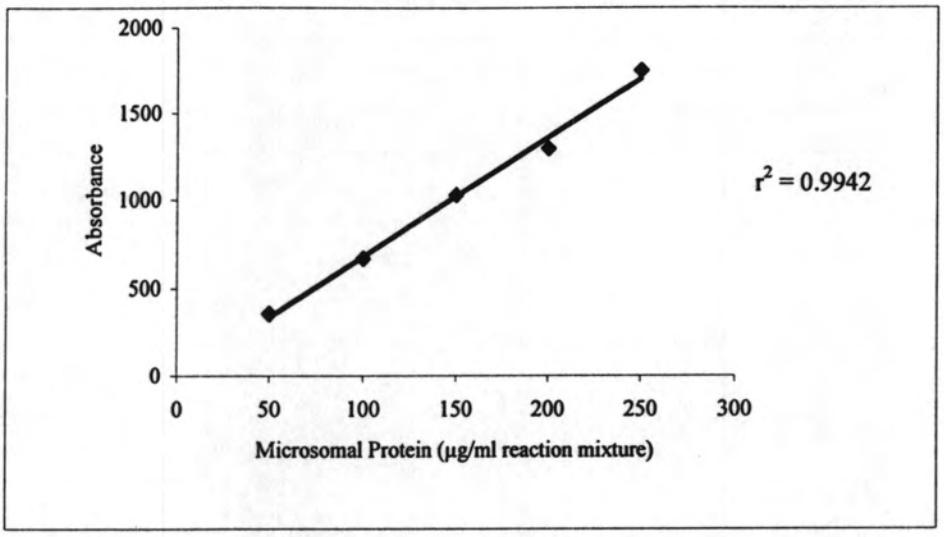

Figure A1 Linearity of the ethoxyresorufin O-dealkylation assay using for determination of CYP1A1 activity.

Correlation between amounts of microsomal protein used in the reaction and the corresponding fluorometric absorbances of the product was shown to possess a coefficient of determination (r^2) of 0.9942. Each point was mean of n=2. (Procedure was demonstrated in the Materials and Methods).

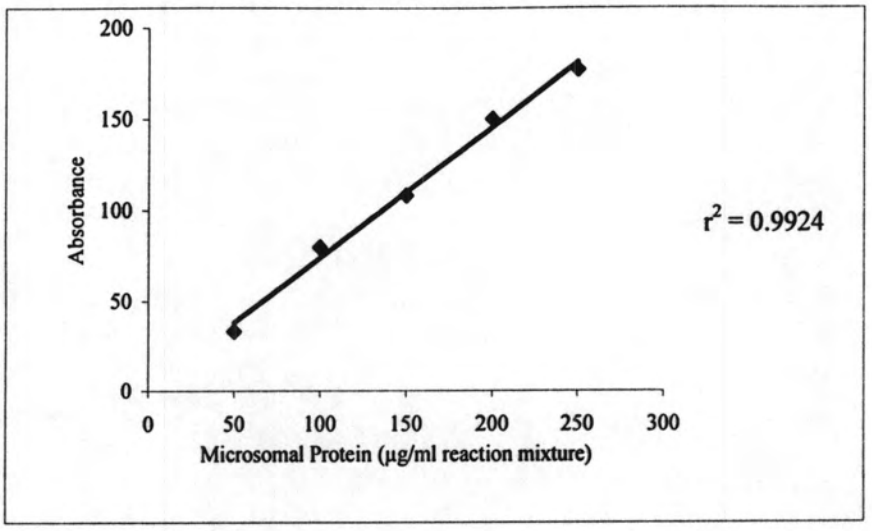

Figure A2 Linearity of the methoxyresorufin O-dealkylation assay using for determination of CYP1A2 activity.

Correlation between amounts of microsomal protein used in the reaction and the corresponding fluorometric absorbances of the product was shown to possess a coefficient of determination (r^2) of 0.9924. Each point was mean of n=2. (Procedure was demonstrated in the Materials and Methods).

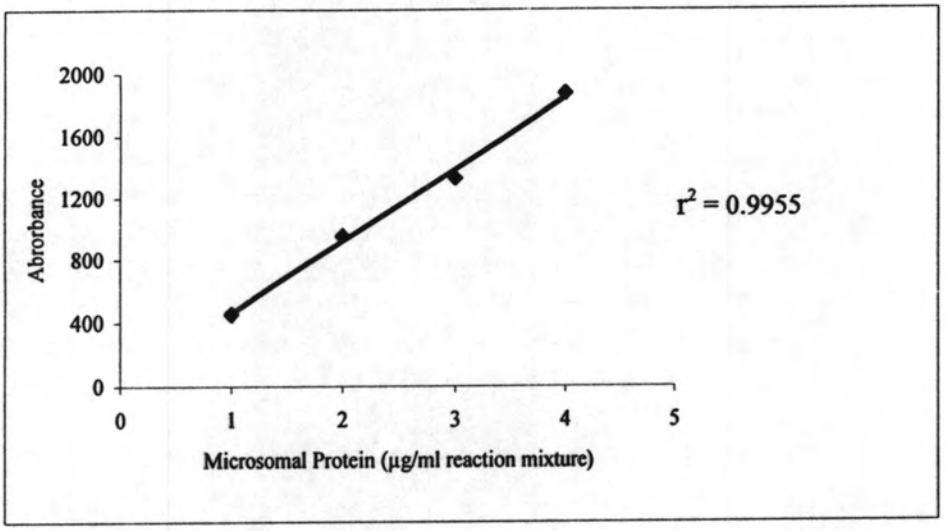

Figure A3 Linearity of the benzyloxyresorufin O-dealkylation assay using for determination of CYP2B1/2B2 activity.

Correlation between amounts of microsomal protein used in the reaction and the corresponding fluorometric absorbances of the product was shown to possess a coefficient of determination (r^2) of 0.9955. Each point was mean of n=2. (Procedure was demonstrated in the Materials and Methods).

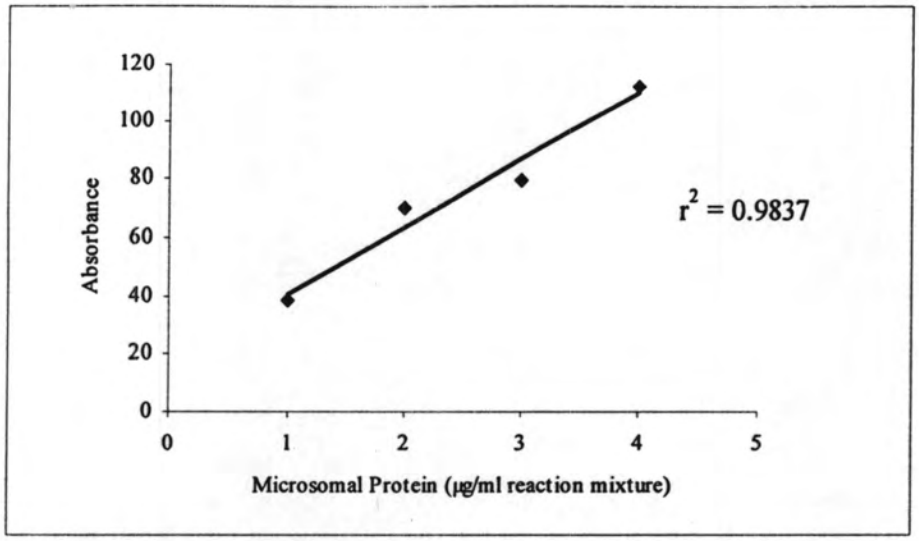

Figure A4 Linearity of the pentoxyresorufin O-dealkylation assay using for determination of CYP2B1/2B2 activity.

Correlation between amounts of microsomal protein used in the reaction and the corresponding fluorometric absorbances of the product was shown to possess a coefficient of determination (r^2) of 0.9837. Each point was mean of n=2. (Procedure was demonstrated in the Materials and Methods).

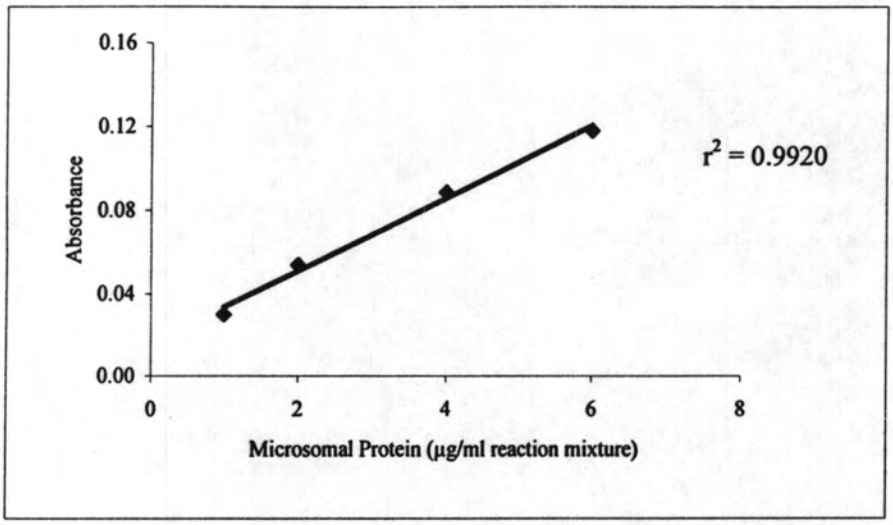

Figure A5 Linearity of the aniline 4- hydroxylation assay using for determination of CYP2E1 activity.

Correlation between amounts of microsomal protein used in the reaction and the corresponding fluorometric absorbances of the product was shown to possess a coefficient of determination (r^2) of 0.9920. Each point was mean of n=2. (Procedure was demonstrated in the Materials and Methods).

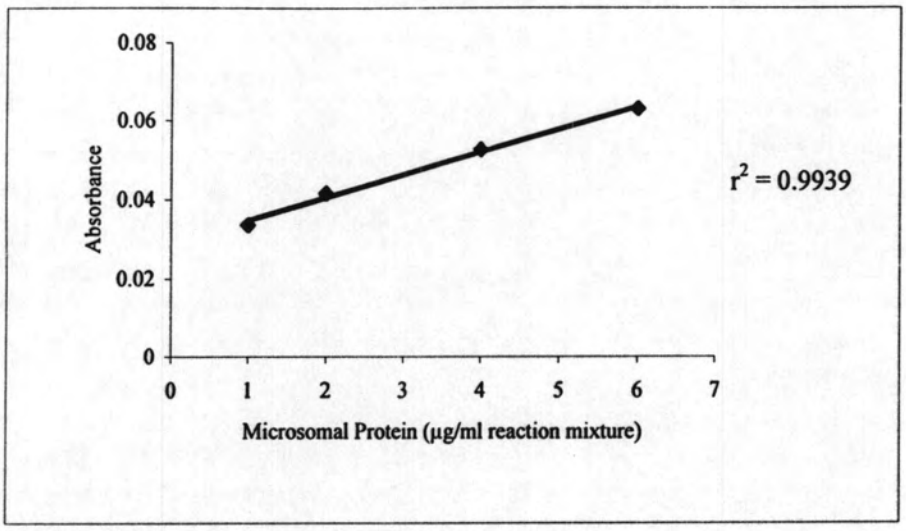

Figure A6 Linearity of the erythromycin N-demethylation assay using for determination of CYP3A activity.

Correlation between amounts of microsomal protein used in the reaction and the corresponding fluorometric absorbances of the product was shown to possess a coefficient of determination (r^2) of 0.9939. Each point was mean of n=2. (Procedure was demonstrated in the Materials and Methods).

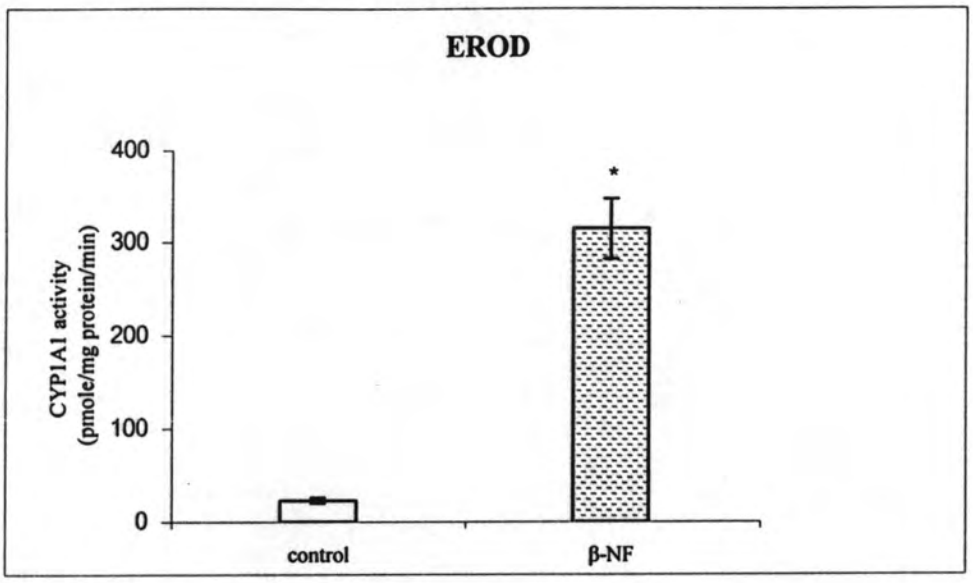

Figure A7 Effect of ß-NF on rat hepatic CYP1A1 activity. Rats were received ß-NF 80 mg/kg/day intraperitoneally for 3 days or corn oil for the treatment group and the control group, respectively. The individual bar graph represented mean of EROD activity with a standard error of the mean (n=4). * $p < 0.05$; ß-NF induced group vs. control group.

	CYP 1A1 activity	
Rat no.	Control group	β-NF induced group
1	15.83	301.68
2	24.29	230.12
3	30.25	381.89
4	21.68	343.27
Average	23.02	314.24
SEM	32.47	32.47

Unit express as pmol/mg protein/min

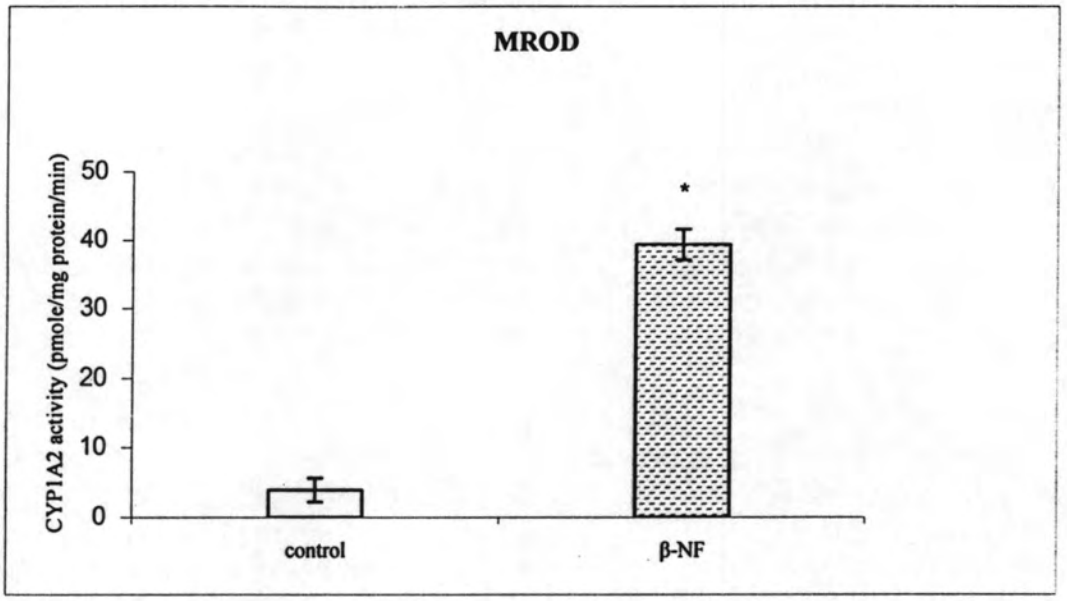

Figure A8 Effect of ß-NF on rat hepatic CYP1A2 activity. Rats were received ß-NF 80 mg/kg/day intraperitoneally for 3 days or corn oil for the treatment group and the control group, respectively. The individual bar graph represented mean of MROD activity with a standard error of the mean (n=4). * $p < 0.05$; ß-NF induced group vs. control group.

	CYP 1A2 activity	
Rat no.	Control group	β-NF induced group
1	3.21	39.90
2	2.29	35.82
3	8.85	36.23
4	1.31	45.59
Average	3.91	39.38
SEM	1.69	2.26

Unit express as pmol/mg protein/min

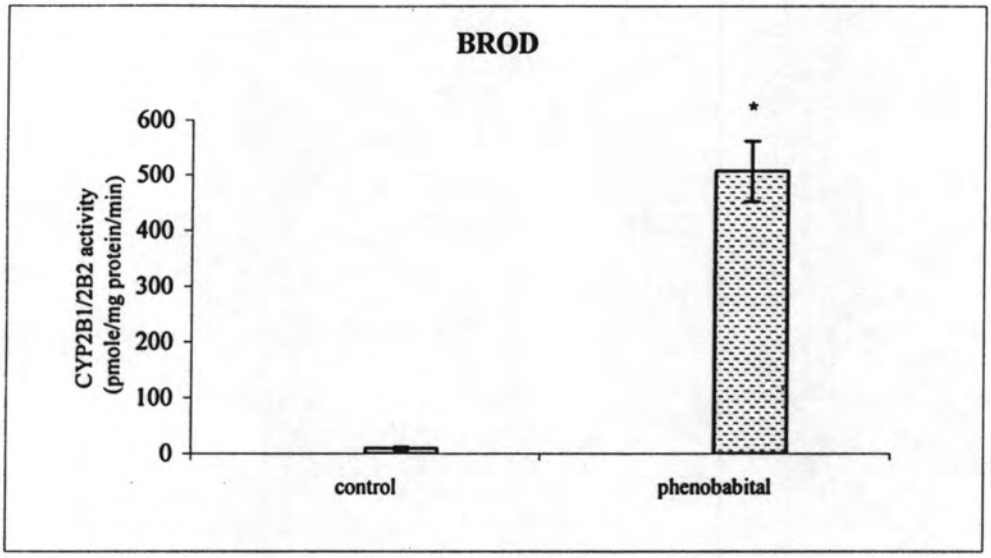

Figure A9 Effect of phenobabital on rat hepatic CYP1A1 activity. Rats were received phenobabiral 80 mg/kg/day intraperitoneally for 3 days or corn oil for the treatment group and the control group, respectively. The individual bar graph represented mean of BROD activity with a standard error of the mean (n=4). * $p < 0.05$; ß-NF induced group vs. control group.

	CYP 2B1/2B2 activity	
Rat no.	Control group	Phenobarbital induced group
1	16.44	549.50
2	10.44	397.29
3	5.14	440.04
4	5.57	641.36
Average	9.40	507.04
SEM	2.64	55.06

Unit express as pmol/mg protein/min

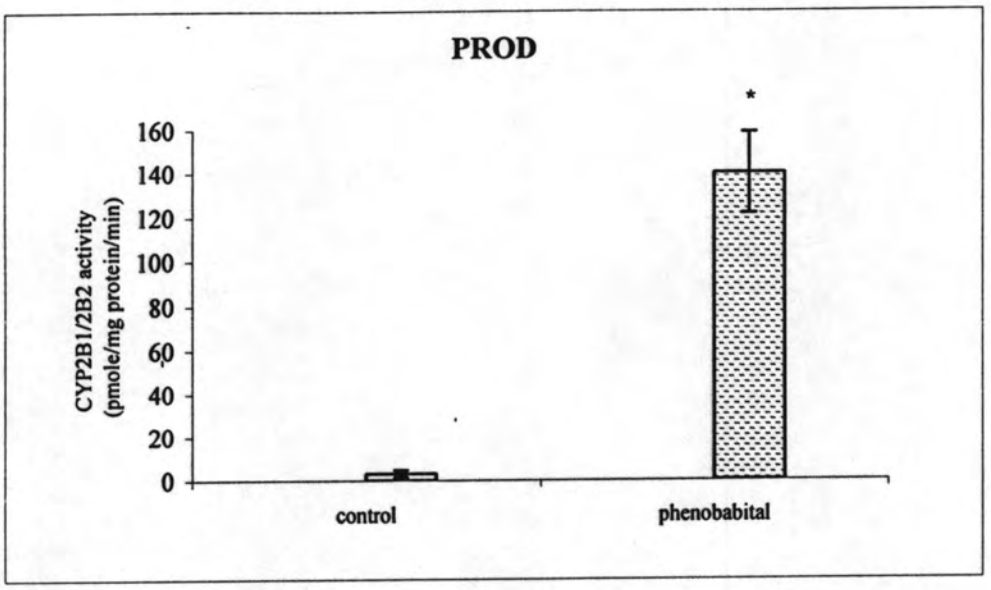

Figure A10 Effect of phenobabital on rat hepatic CYP1A1 activity. Rats were received phenobabiral 80 mg/kg/day intraperitoneally for 3 days or corn oil for the treatment group and the control group, respectively. The individual bar graph represented mean of PROD activity with a standard error of the mean (n=4). * p< 0.05; ß-NF induced group vs. control group.

	CYP 2B1/2B2 activity	
Rat no.	Control group	Phenobarbital induced group
1	9.42	164.76
2	1.76	121.49
3	3.02	97.01
4	3.07	176.62
Average	4.32	139.97
SEM	1.73	18.58

Unit express as pmol/mg protein/min

Appendix B

An *in vivo* enzyme activity study

Table B1 Microsomal protein concentration of individual male rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	57.18	92.14	70.14	107.14
2	99.00	89.94	86.29	58.29
3	66.23	86.94	42.11	83.23
4	94.37	44.67	100.23	70.09
5	65.56	56.44	90.94	47.00
6	65.22	55.62	91.94	50.11
7	72.67	76.94	56.23	82.66
8	51.89	76.22	61.17	65.07
9	34.09	64.56	71.33	56.89
10	79.94	55.07	60.18	66.00

Unit expressed as mg/ml

Table B2 Microsomal protein concentration of individual female rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	23.43	27.43	40.00	74.86
2	33.43	43.29	58.00	48.80
3	45.37	28.80	68.43	39.80
4	43.23	33.94	41.37	27.80
5	28.09	28.09	51.23	23.94
6	38.51	36.94	41.44	46.89
7	43.66	33.67	33.44	31.67
8	37.66	34.29	33.78	38.67
9	38.56	28.33	40.78	38.44
10	21.56	32.44	29.11	31.07

Unit expressed as mg/ml

Table B3 Hepatic microsomal total CYP content of individual male rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	0.53	0.37	0.40	0.16
2	0.26	0.23	0.26	0.40
3	0.33	0.26	0.55	0.21
4	0.27	0.23	0.27	0.25
5	0.30	0.51	0.22	0.21
6	0.26	0.67	0.36	0.57
7	0.35	0.26	0.40	0.26
8	0.30	0.29	0.31	0.42
9	0.21	0.29	0.36	0.35
10	0.41	0.27	0.37	0.42
Average	0.32	0.34	0.35	0.32
SEM	0.03	0.04	0.03	0.04

Unit expressed as nmol/mg protein

Table B4 Hepatic microsomal total CYP content of individual female rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	0.25	0.30	0.40	0.23
2	0.25	0.30	0.20	0.24
3	0.31	0.27	0.12	0.25
4	0.27	0.36	0.50	0.34
5	0.34	0.34	0.29	0.34
6	0.21	0.30	0.22	0.28
7	0.14	0.23	0.27	0.16
8	0.22	0.32	0.26	0.42
9	0.41	0.30	0.42	0.47
10	0.25	0.29	0.35	0.25
Average	0.26	0.30	0.30	0.29
SEM	0.02	0.01	0.04	0.03

Unit expressed as nmol/mg protein

Table B5 Hepatic microsomal EROD activity of individual male rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	57.49	33.87	42.90	81.87
2	30.44	63.98	69.70	42.75
3	36.02	36.24	77.10	24.44
4	25.66	62.29	85.30	25.61
5	64.16	54.36	31.50	55.26
6	49.07	78.28	95.00	96.33
7	31.61	49.51	102.10	59.86
8	25.37	33.24	32.90	61.13
9	16.24	52.80	31.70	40.70
10	67.48	33.99	53.70	70.74
Average	41.08	49.85	62.19	55.86
SEM	5.64	4.90	8.50	7.36

Unit expressed as pmol/mg protein/min

Table B6 Hepatic microsomal EROD activity of individual female rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	55.73	35.06	56.69	51.50
2	51.69	22.51	23.45	19.54
3	52.48	35.42	29.95	23.17
4	46.45	45.73	26.13	25.27
5	21.65	62.33	17.45	87.71
6	28.19	75.96	19.86	89.10
7	40.29	16.45	21.89	50.84
8	46.02	73.74	61.51	42.01
9	18.30	80.79	39.20	44.41
10	41.44	55.08	46.92	48.14
Average	40.22	50.30	34.30	48.16
SEM	4.17	7.22	5.02	7.60

Unit expressed as pmol/mg protein/min

Table B7 Hepatic microsomal MROD activity of individual male rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	13.43	11.29	9.60	19.16
2	8.25	8.31	5.30	7.01
3	7.60	8.02	22.80	9.73
4	7.69	10.95	24.60	9.43
5	5.33	10.60	7.10	13.28
6	18.25	18.30	17.20	4.90
7	8.38	10.21	20.10	10.56
8	2.80	12.87	10.10	17.45
9	16.86	3.78	3.10	10.44
10	14.67	17.20	6.10	8.37
Average	9.82	11.15	12.60	11.03
SEM	1.79	2.49	1.35	1.40

Unit expressed as pmol/mg protein/min

Table B8 Hepatic microsomal MROD activity of individual female rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	13.01	23.09	16.23	6.49
2	17.65	5.85	5.46	8.23
3	9.74	7.42	8.61	15.02
4	22.02	11.39	9.64	16.13
5	9.81	9.18	8.92	13.69
6	17.38	12.97	14.70	15.91
7	7.22	15.91	18.58	14.70
8	17.57	13.20	8.91	7.91
9	5.16	4.54	5.30	5.14
10	14.83	15.03	28.87	12.51
Average	13.43	11.85	12.52	11.57
SEM	1.70	1.17	2.29	1.30

Unit expressed as pmol/mg protein/min

Table B9 Hepatic microsomal BROD activity of individual male rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	25.51	18.02	33.10	39.34
2	19.33	23.43	44.90	34.71
3	26.54	16.42	63.80	24.96
4	14.95	37.82	31.10	24.96
5	45.08	34.77	16.60	35.05
6	30.13	22.65	30.70	33.42
7	17.24	18.19	65.70	14.65
8	23.67	23.52	7.70	39.34
9	16.95	25.75	16.40	19.47
10	26.33	2.07	27.50	45.19
Average	23.16	22.26	33.75	31.10
SEM	3.07	6.12	3.14	3.07

Unit expressed as pmol/mg protein/min

Table B10 Hepatic microsomal BROD activity of individual female rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	4.67	7.08	26.39	12.91
2	3.47	4.65	8.17	3.72
3	9.72	10.75	4.50	5.96
4	11.87	18.39	3.75	5.00
5	8.01	9.78	7.35	6.77
6	14.11	7.30	5.67	7.34
7	4.79	9.37	3.20	14.22
8	14.89	14.03	7.13	6.76
9	2.65	8.33	5.97	3.21
10	20.62	13.01	5.48	5.58
Average	9.48	10.26	7.76	7.14
SEM	1.89	1.26	2.12	1.15

Unit expressed as pmol/mg protein/min

Table B11 Hepatic microsomal PROD activity of individual male rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	7.63	11.03	10.46	6.88
2	5.48	9.86	13.15	5.55
3	3.58	11.97	17.07	3.77
4	4.03	2.37	12.12	3.77
5	3.84	14.66	4.87	35.86
6	5.78	14.01	13.01	16.82
7	11.37	0.00	16.76	4.67
8	8.79	11.84	7.34	9.41
9	6.09	5.67	6.71	10.38
10	8.68	3.10	9.73	5.22
Average	6.28	8.45	11.13	10.23
SEM	0.92	1.65	1.30	3.11

Unit expressed as pmol/mg protein/min

Table B12 Hepatic microsomal PROD activity of individual female rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	2.88	1.92	10.47	2.95
2	4.55	5.57	7.24	1.68
3	1.51	3.75	1.28	4.02
4	7.60	7.60	1.29	5.12
5	3.50	2.42	5.24	2.24
6	6.00	1.64	1.05	3.74
7	6.24	5.90	3.41	7.32
8	1.29	6.36	1.93	4.36
9	2.82	9.95	3.76	1.57
10	5.22	4.17	3.73	4.91
Average	4.16	4.92	3.94	3.79
SEM	0.67	0.84	0.95	0.56

Unit expressed as pmol/mg protein/min

Table B13 Hepatic microsomal aniline 4-hydroxylase activity of individual male rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	0.061	0.097	0.040	0.125
2	0.081	0.170	0.076	0.077
3	0.276	0.094	0.439	0.173
4	0.071	0.444	0.162	0.070
5	0.281	0.375	0.125	0.301
6	0.406	0.202	0.342	0.263
7	0.159	0.113	0.444	0.198
8	0.247	0.169	0.144	0.446
9	0.165	0.334	0.357	0.248
10	0.352	0.304	0.454	0.500
Average	0.210	0.230	0.258	0.240
SEM	0.038	0.040	0.052	0.046

Unit expressed as nmol/mg protein/min

Table B14 Hepatic microsomal aniline 4-hydroxylase activity of individual female rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	0.066	0.035	0.041	0.094
2	0.068	0.048	0.066	0.093
3	0.059	0.045	0.058	0.078
4	0.047	0.035	0.061	0.013
5	0.106	0.146	0.066	0.158
6	0.058	0.126	0.076	0.120
7	0.070	0.110	0.141	0.136
8	0.099	0.069	0.179	0.136
9	0.061	0.130	0.080	0.143
10	0.133	0.173	0.225	0.169
Average	0.077	0.092	0.099	0.114
SEM	0.008	0.016	0.019	0.014

Unit expressed as nmol/mg protein/min

Table B15 Hepatic microsomal erythromycin N-demethylase activity of individual male rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	0.232	0.107	0.226	0.209
2	0.226	0.247	0.369	0.185
3	0.214	0.043	0.297	0.223
4	0.307	0.272	0.21	0.241
5	0.272	0.272	0.204	0.340
6	0.330	0.325	0.348	0.278
7	0.209	0.139	0.313	0.249
8	0.229	0.253	0.151	0.309
9	0.303	0.439	0.359	0.229
10	0.284	0.346	0.396	0.309
Average	0.26	0.244	0.287	0.257
SEM	0.014	0.037	0.026	0.016

Unit expressed as nmol/mg protein/min

Table B16 Hepatic microsomal erythromycin N-demethylase activity of individual female rat

Rat No.	Group			
	Control	*Centella asiatica* group 1	*Centella asiatica* group 2	*Centella asiatica* group 3
1	0.220	0.077	0.137	0.298
2	0.316	0.191	0.256	0.310
3	0.197	0.284	0.286	0.209
4	0.307	0.394	0.099	0.354
5	0.284	0.197	0.388	0.342
6	0.093	0.278	0.198	0.272
7	0.389	0.266	0.334	0.235
8	0.253	0.152	0.271	0.278
9	0.099	0.271	0.331	0.205
10	0.155	0.331	0.166	0.305
Average	0.231	0.244	0.246	0.280
SEM	0.031	0.029	0.026	0.016

Unit expressed as nmol/mg protein/min

Appendix C

- An *in vitro* enzyme activity study

- Study protocol approval by Ethic Committee of Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

Table C1 Effect of the standardized extract of *C. asiatica* on CYP1A1 in an *in vitro* study.

Concentrations of the standardized extract of *C. asiatica* in the reaction mixture (μg/ml)	CYP1A1 activities (pmol/mg protein/min)			
	1	2	3	4
0	730.29	786.03	573.87	652.50
100	834.34	740.02	670.59	748.51
250	818.04	756.47	693.78	732.78
500	786.83	827.88	730.17	833.06
1000	850.96	868.20	770.96	853.40
2000	811.28	903.22	877.93	873.77

Various concentrations of the standardized extract of *C. asiatica* were co-incubated simultaneously with ER under the condition described in the Materials and Methods. The concentrations of the standardized extract of *C. asiatica* in the reaction mixture were 0,100, 250, 500, 1000 and 2000 μg/ml.

Table C2 Effect of the standardized extract of *C. asiatica* on CYP1A2 in an *in vitro* study.

Concentrations of the standardized extract of *C. asiatica* in the reaction mixture (μg/ml)	CYP1A2 activities (pmol/mg protein/min)			
	1	2	3	4
0	75.21	65.43	52.01	32.76
100	65.39	64.73	51.70	36.11
250	56.11	56.51	47.93	30.01
500	45.69	53.00	35.21	27.73
1000	49.50	48.10	38.30	18.00
2000	45.64	42.35	37.74	20.89

Various concentrations of the standardized extract of *C. asiatica* were co-incubated simultaneously with MR under the condition described in the Materials and Methods. The concentrations of the standardized extract of *C. asiatica* in the reaction mixture were 0,100, 250, 500, 1000 and 2000 μg/ml.

Table C3 Effect of the standardized extract of *C. asiatica* on CYP2B1/2B2 in an *in vitro* study.

Concentrations of the standardized extract of *C. asiatica* in the reaction mixture (μg/ml)	CYP2B1/2B2 activities (pmol/mg protein/min)			
	1	2	3	4
0	674.68	624.44	130.00	164.72
100	725.35	720.18	105.59	141.27
250	443.75	552.20	94.16	125.10
500	384.95	380.36	67.18	85.18
1000	197.79	137.54	55.46	52.42
2000	64.53	47.62	26.30	17.63

Various concentrations of the standardized extract of *C. asiatica* were co-incubated simultaneously with BR under the condition described in the Materials and Methods. The concentrations of the standardized extract of *C. asiatica* in the reaction mixture were 0,100, 250, 500, 1000 and 2000 μg/ml.

Table C4 Effect of the standardized extract of *C. asiatica* on CYP2B1/2B2 in an *in vitro* study.

Concentrations of the standardized extract of *C. asiatica* in the reaction mixture (μg/ml)	CYP2B1/2B2 activities (pmol/mg protein/min)			
	1	2	3	4
0	174.15	198.61	32.18	43.48
100	162.38	171.83	28.97	32.38
250	156.52	160.87	26.88	25.96
500	65.71	120.05	12.44	21.88
1000	60.46	64.89	15.19	8.88
2000	29.54	20.19	6.74	7.59

Various concentrations of the standardized extract of *C. asiatica* were co-incubated simultaneously with PR under the condition described in the Materials and Methods. The concentrations of the standardized extract of *C. asiatica* in the reaction mixture were 0,100, 250, 500, 1000 and 2000 μg/ml.

Table C5 Effect of the standardized extract of *C. asiatica* on CYP2E1 in an *in vitro* study.

Concentrations of the standardized extract of *C. asiatica* in the reaction mixture (μg/ml)	CYP2E1 activities (nmol/mg protein/min)			
	1	2	3	4
0	0.331	0.332	0.249	0.276
100	0.306	0.322	0.252	0.246
250	0.303	0.298	0.222	0.281
500	0.290	0.301	0.255	0.268
1000	0.237	0.218	0.240	0.240
2000	0.242	0.241	0.238	0.228

Various concentrations of the standardized extract of *C. asiatica* were co-incubated simultaneously with aniline 4-hydroxylase under the condition described in the Materials and Methods. The concentrations of the standardized extract of *C. asiatica* in the reaction mixture were 0,100, 250, 500, 1000 and 2000 μg/ml.

Table C6 Effect of the standardized extract of *C. asiatica* on CYP3A in an *in vitro* study.

Concentrations of the standardized extract of *C. asiatica* in the reaction mixture (μg/ml)	CYP3A activities (nmol/mg protein/min)			
	1	2	3	4
0	2.986	2.998	2.154	1.688
100	2.583	3.036	2.305	1.625
250	2.721	2.809	1.789	1.764
500	2.998	3.036	1.499	1.688
1000	3.036	2.809	1.839	1.764
2000	2.784	2.608	1.852	1.512

Various concentrations of the standardized extract of *C. asiatica* were co-incubated simultaneously with erythromycin N-demethylase under the condition described in the Materials and Methods. The concentrations of the standardized extract of *C. asiatica* in the reaction mixture were 0,100, 250, 500, 1000 and 2000 μg/ml.

CHULALONGKORN UNIVERSITY ANIMAL CARE AND USE COMMITTEE

Certificate of Project Approval	☑ Original ☐ Renew
Animal Use Protocol No. 07-33-003	**Approval No. 07-33-003**
Protocol Title Subchronic effects of the standardized extract of *Centella asiatica* on rat hepatic cytochrome P450	
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 November 12, 2007	**Date of Expiration** November 12, 2008
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.*	

VITAE

Miss Kornphimol Kulthong was born in June 22, 1978 in Chainat, Thailand. She graduated with a Bachelor Degree of Science in Chemistry in 2003 from the Faculty of Science, Srinakharinwirot University. Bangkok, Thailand.