APPENDIX

APPENDIX A

List of chemicals and materials used in the study

All chemicals and reagents used in this study are analytical grade and are listed as follows:

Chemicals/Materials	Source
Absolute ethanol	Merck, Darmstadt, Germany
Aflatoxin B_1	Sigma-Aldrich, St. Louis, MO, USA
Asiaticoside	Sigma Chemical Co., Dorset, UK
Agarose	Sigma-Aldrich, St. Louis, MO, USA
Bovine serum albumin	Sigma-Aldrich, St. Louis, MO, USA
Crystal violet	Sigma-Aldrich, St. Louis, MO, USA
Deoxynivalenol	Sigma Chemical Co., Dorset, UK
Deoxyribonuclease I	Invitrogen, USA
Dimethyl sulfoxide (DMSO)	Sigma Chemical Co., Dorset, UK
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco laboratories, Paisley, UK
DMEM without phenol red	Gibco laboratories, Paisley, UK
Ethidium bromide	Sigma Chemical Co., Dorset, UK
Fetal bovine serum	Sigma Chemical Co., Dorset, UK
Fumonisin B_1	Sigma Chemical Co., Dorset, UK
Mouse iNOS relative RT-PCR kit	Ambion, UK
Mouse TNF-α relative RT-PCR kit	Ambion, UK
Glass filter paper	Amersham, UK
Heparin	Sigma Chemical Co., Dorset, UK
HEPES	Sigma-Aldrich, St. Louis, MO, USA
Histopaque	Amersham, UK
HRP-conjugated rabbit anti-mouse IgG	Zymed Company, San Francisco

HRP-conjugated rabbit anti-mouse IgM	Zymed Company, San Francisco
HotStartTaq DNA polymerase	Qiagen, UK
Human IL-2 ELISA assay kit	eBioscience Co., Wembley, UK
Human TNF-α ELISA assay kit	eBioscience Co., Wembley, UK
Hydrochloric acid	Sigma Chemical Co., Dorset, UK
Hydrogen peroxide	Sigma Chemical Co., Dorset, UK
Isopropanol	Sigma Chemical Co., Dorset, UK
Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4	Sigma Chemical Co., Dorset, UK
Magnesium chloride	Sigma-Aldrich, St. Louis, MO, USA
[methyl-^{3}H] thymidine	Amersham, UK
Millipore filter membrane (0.22 μm)	Whatman, UK
Mouse TNF-α ELISA assay kit	eBioscience Co., Wembley, UK
MTT [3-(4,5-di-methylthizol-2-yl)-2,5-diphenyltetrazolium bromide]	Sigma Chemical Co., Dorset, UK
N-(1-naphthyl)-ethylenediamine dihydrochloride	Sigma Chemical Co., Dorset, UK
Ninety six-well flat bottom tissue culture plate	Nunc Inc., Hereford, UK
Ninety six-well ELISA plate	Nunc Inc., Hereford, UK
Penicillin-streptomycin	Gibco, USA
Phosphoric acid	Sigma-Aldrich, St. Louis, MO, USA
Phytohemagglutinin (PHA)	Sigma-Aldrich, St. Louis, MO, USA
Pokeweed mitogen (PWM)	Sigma-Aldrich, St. Louis, MO, USA
Polymyxin B mix	Sigma Chemical Co., Dorset, UK
Potassium chloride	Sigma-Aldrich, St. Louis, MO, USA
Potassium hydrogen phosphate	Sigma-Aldrich, St. Louis, MO, USA
RNAgent® Total RNA Isolation kit	Promega, USA
RPMI-1640	Gibco laboratories, Paisley, UK
Six-well flat bottom tissue culture plate	Nunc Inc., Hereford, UK
Sodium chloride	Sigma-Aldrich, St. Louis, MO, USA
Sodium nitrite	Sigma Chemical Co., Dorset, UK
Sodium bicarbonate	Sigma-Aldrich, St. Louis, MO, USA

Sodium hydrogen phosphate	Sigma-Aldrich, St. Louis, MO, USA
Sulfanilamide	Sigma Chemical Co., Dorset, UK
Sulfuric acid	Sigma-Aldrich, St. Louis, MO, USA
Tissue culture flask	Nunc Inc., Hereford, UK
ThermoScript™ RT-PCR system	Invitrogen, USA
TMB substrate	Sigma-Aldrich, St. Louis, MO, USA
Trypan blue dye	Sigma Chemical Co., Dorset, UK
Tween-20	Sigma Chemical Co., Dorset, UK
Sample bag	Amersham, UK
Scintillation fluid	Amersham, UK
Skimmed milk	Difco Laboratory, USA
Sodium Chloride	Merck, Darmstadt, Germany
Sodium hydrogen carbonate	Merck, Darmstadt, Germany
Sodium lauryl sulfate	Sigma-Aldrich, St. Louis, MO, USA
Total RNA isolation kit	Promega, USA
Whatman filter paper	Whatman, UK

APPENDIX B

List of instrument used in the study

Instrument	Source
Analytical balance	Satorius
Autoclave	Tomy Seiko Co., Japan
Capillary electrophoresis	Beckman Instruments Inc., CA, USA
Cell harvester	Wallac
Centrifuge	Jouan, France
CO_2 incubator	Foma Scientific
Electrophoresis unit	Amersham
ELISA reader	ICN, Flow, USA
Freeze dryer	Martin Christ, Germany
Heat sealer	Wallac
High speed microcentrifuge	Tommy, USA
Inverted microscope	Olympus, USA
Laminar flow	HOLTEN Lamina Air
Light microscope	Olympus, USA
Liquid nitrogen tank	International Cryogenics Inc.
Liquid scintillation counter (β-counter)	Wallac
Refrigerator (-20 oC)	Sanyo, Thailand
Refrigerator (-80 oC)	Foma Scientific
Master Cycler Gradient (PCR amplifier)	Eppendorf
UV spectrophotometer	Shimadzu Co., Japan
Vacuum rotating evaporator	Tokyo Rikakikai Co., Ltd., Japan
Water bath	Yamaha, Japan

APPENDIX C

Reagents and buffers preparation

1. Reagents for cell culture

1.1 RPMI-1640 medium

RPMI-1640 powder	10.4	g
HEPES	0.357	g
$NaHCO_3$	0.2	g
Antibiotics (Penicillin-streptomycin)	1.0	ml

Add distilled water to 1 liter and adjusted pH to 7.4 and sterile by Millipore filter membrane (0.22 μm).

1.2 Complete RPMI-1640 medium

RPMI-1640	90	ml
Fetal bovine serum	10	ml

1.3 DMEM medium

DMEM	13.5	g
HEPES	3.57	g
$NaHCO_3$	2 .0	g
Antibiotics (Penicillin-streptomycin)	1.0	ml

Add distilled water to 1 liter and adjusted pH to 7.4 and sterile by Millipore filter membrane (0.22 μm).

1.4 Complete DMEM medium

DMEM	90	ml
Fetal bovine serum	10	ml

1.5 0.4% Trypan blue

Trypan blue dye	0.4	g
Add phosphate buffer saline to	100	ml

Filter through Whatman filter paper no. 2 and kept at room temperature.

2. Reagents for ELISA

2.1 Phosphate buffer saline (PBS)

KCl	0.4	g
KH_2PO_4	0.4	g
Na_2HPO_4	2.3	g
NaCl	16.0	g

Add distilled water 2 liters and adjusted pH to 7.4.

2.2 0.05%Tween-PBS (Washing buffer)

PBS	1000	ml
Tween 20	0.5	ml

2.3 0.5% skimmed milk (Blocking buffer)

Skimmed milk	5	g
PBS	100	ml

2.4 Citrate buffer, pH 5.0

Citric acid trisolium salt	7.35	g

Add distilled water to 500 ml and adjusted pH to 5.0.

2.5 TMB solution

3,3',5,5'-tetramethylbenzidine	5.0	g
Dimethylsulfoxide	500	µl

2.6 TMB substrate

Citrate buffer	10.0	ml
TMB solution	100	µl

3. Griess's reagent

Reagent A:

N-(1-naphthyl)-ethylenediamine dihydrochloride	0.1	g
Add distilled water to	100	ml

Reagent B:

Sulfanilamide	1.0	g
Add 2.5% H_3PO_4 to	100	ml

Mixed one part of Reagent A with one part of Reagent B to make working Griess's reagent. Stored at 4°C.

4. Reagents for RT-PCR

4.1 0.5 M EDTA

Disodium EDTA	18.6	g

Add distilled water to 100 ml and adjusted pH to 8.0.

4.2 5x TBE Buffer

Tris-base	54.0	g
Boric acid	27.5	g
0.5 M EDTA	20.0	ml
Distilled water to	1000	ml

4.3 1 mg/ml Ethidium bromide

Ethidium bromide	0.001	g
DEPC treated water	1	ml

Stored at 4 °C in dark.

4.4 2% Agarose

Agarose	2	g
0.5x TBE	100	ml
1 mg/ml Ethidium bromide	50	µl

4.5 1 M Tris-HCl, pH 8.0

Tris-base	121.14	g
Deionized water to	1000	ml

Sterile by autoclave

4.6 Gel loading solution

Glycerol	3.7	ml
Bromophenyl blue	2.5	mg
Xylene cyanol	2.5	mg
1 M Tris-HCl, pH 8.0	200	µl
500 mM EDTA	100	µl
Nuclease-free water to	10	ml

4.7 Component for cDNA synthesis (RT reaction)

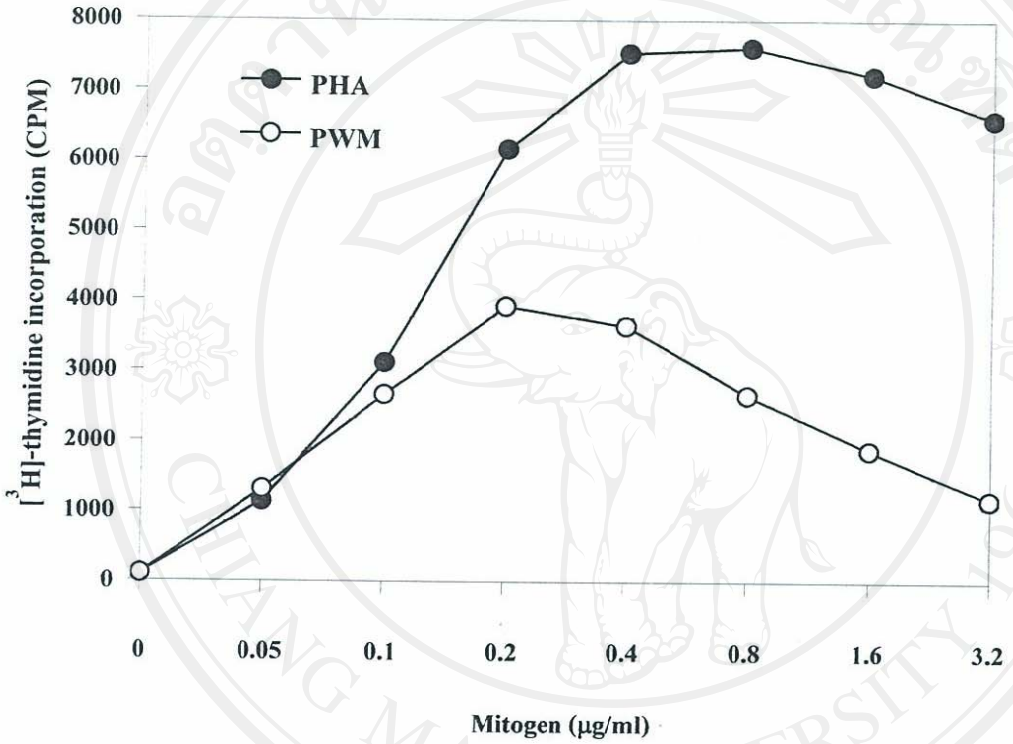
5x cDNA synthesis buffer	4	µl
0.1 M DTT	1	µl
RNaseOUT™ (40 U/µl)	1	µl
DEPC-treated water	1	µl
ThermoScript™ RT (15 U/µl)	1	µl
50 ng/ml Random hexamer	1	µl
RNA sample (10 pg-5 µg)	x	µl
10 mM dNTP mix	2	µl
DEPC-treated water to	20	µl

4.8 Component for PCR reaction

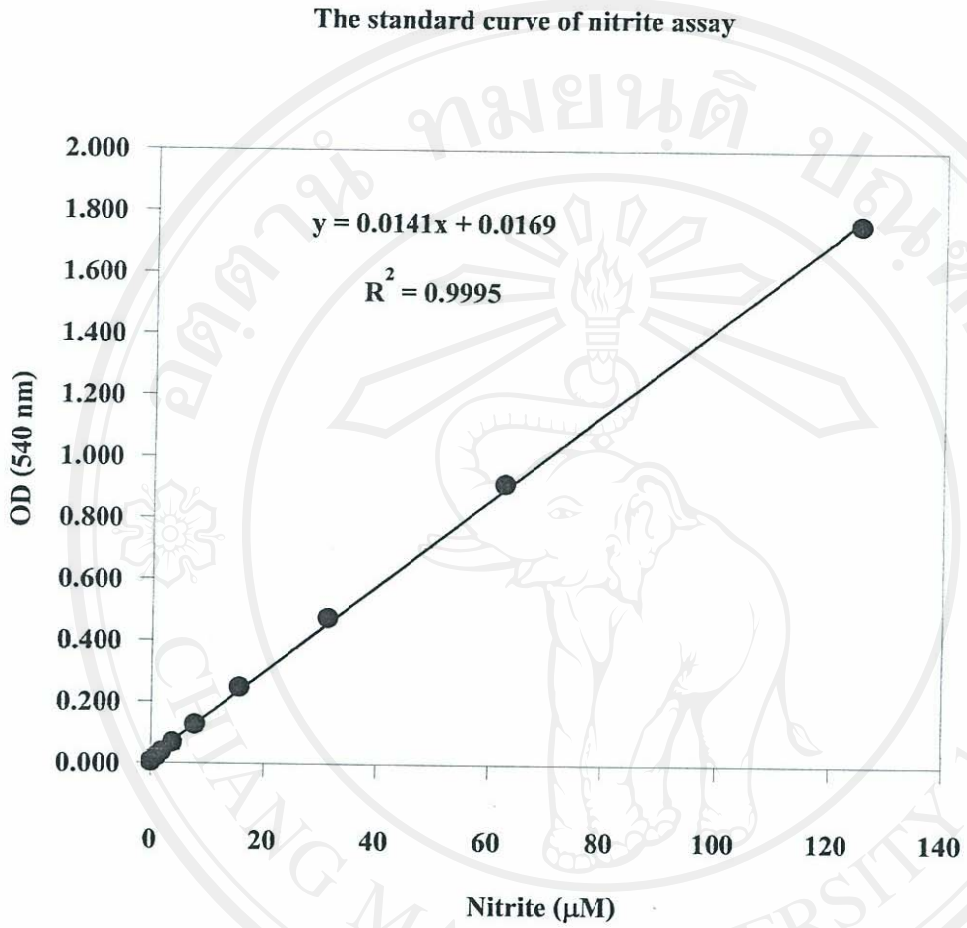
10x PCR buffer	2.5	µl
2 mM dNTP mix	2.5	µl
Gene specific primer	2.0	µl
18S primer: competimer (2:8)	2.0	µl
HotStartTaq DNA polymerase	2.5	µl
cDNA sample	1.0	µl
DEPC-treated water	12.5	µl

APPENDIX D

The titration of mitogens used in lymphocyte activation assay



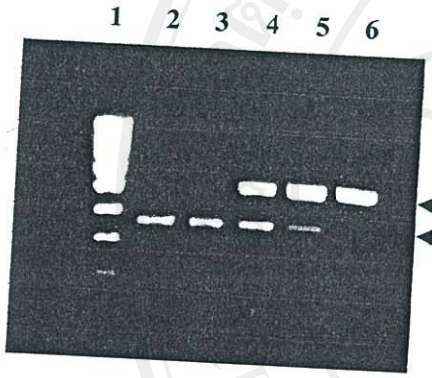
APPENDIX E



APPENDIX F

Determination of the optimal ratio of 18S primer: competimer for RT-PCR

This experiment examined what ratios of primers to competimers are needed to have both the target-of-interest, and the 18S control target amplify to give similar yields of product.



Lane 1: molecular weight marker
Lane 2: iNOS primer only
Lane 3: 1:9 (primer: competimer)
Lane 4: 2:8 (primer: competimer)
Lane 5: 3:7 (primer: competimer)
Lane 6: 18S primer only

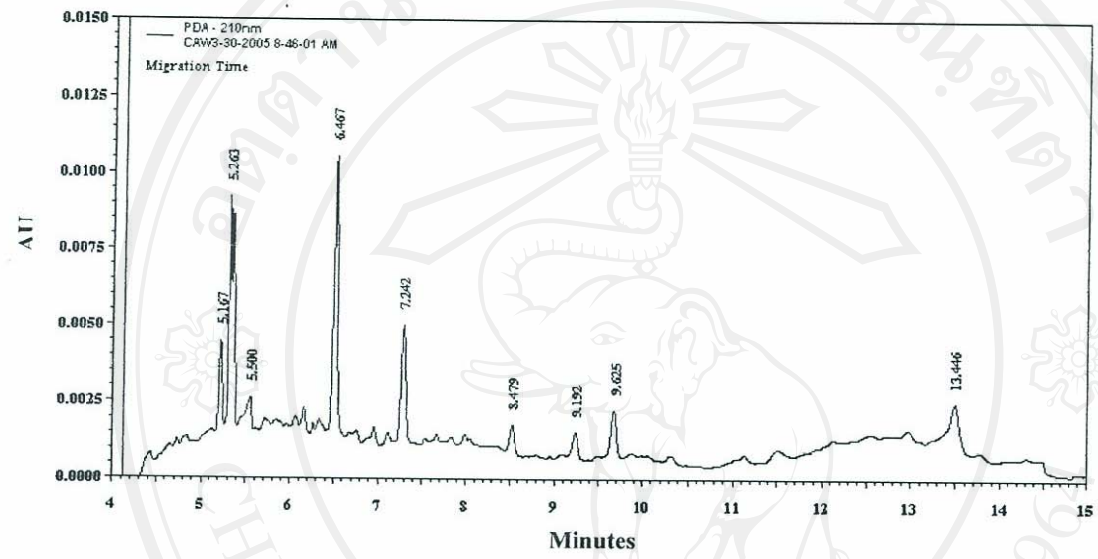
Lane 1: molecular weight marker
Lane 2: TNF-α primer only
Lane 3: 1:9 (primer: competimer)
Lane 4: 2:8 (primer: competimer)
Lane 5: 3:7 (primer: competimer)
Lane 6: 18S primer only

The correct ratio of 18S primer to competimer for use in RT-PCR was 2:8 (lane 4), which the level of 18S product was most similar to the level of both gene specific products.

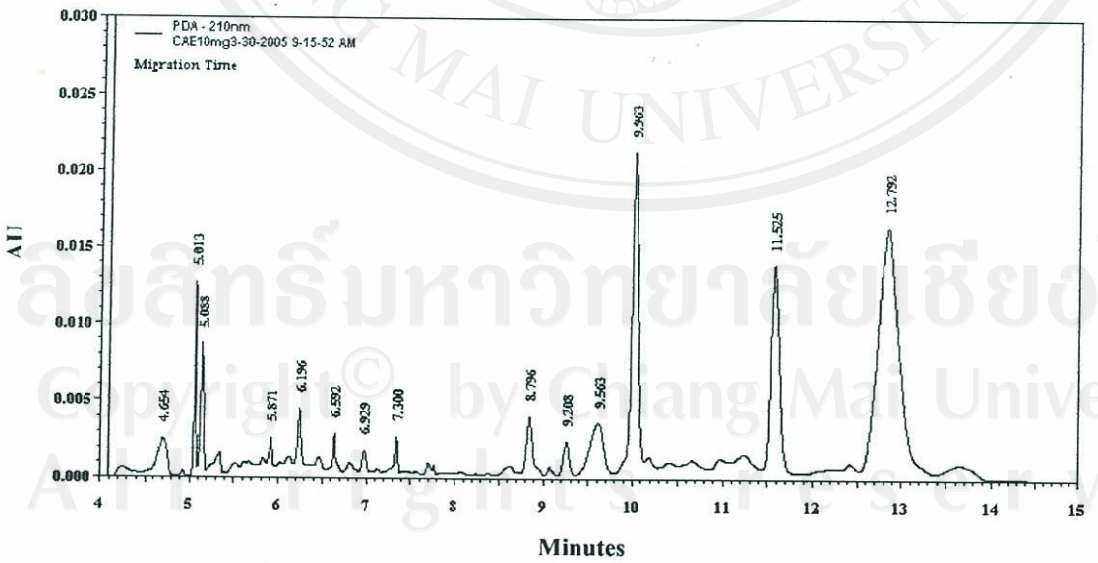
APPENDIX G

Fingerprints of *Centella asiatica* and *Rhinacanthus nasutus* extracts

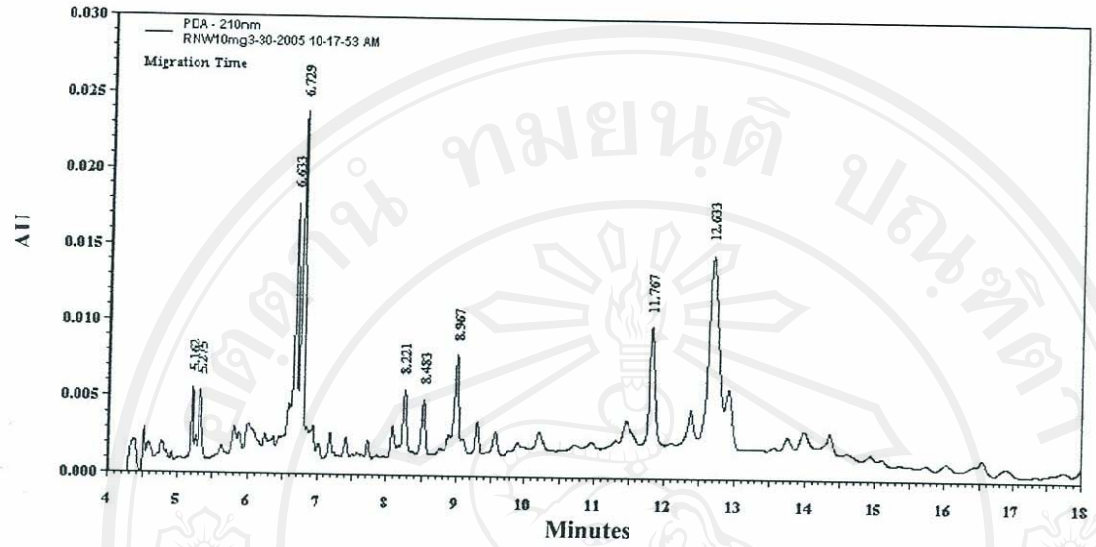
Water extract of *C. asiatica*



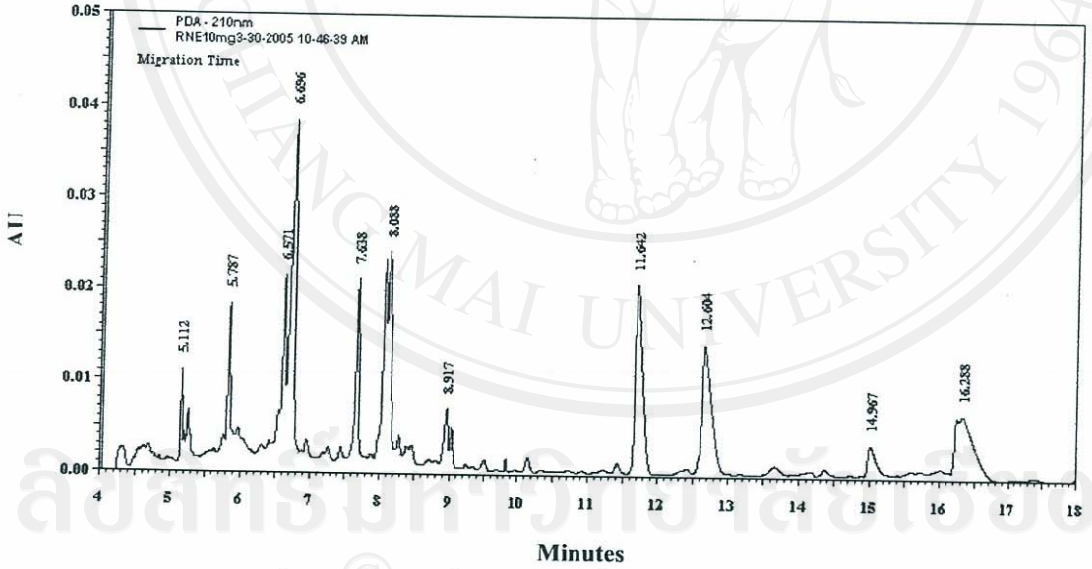
Ethanol extract of *C. asiatica*



Water extract of *R. nasutus*



Ethanol extract of *R. nasutus*



The fingerprints of both plant extracts were determined by capillary electrophoresis using the following condition:

Capillary:	Uncoated fused silica (diameter 75 μM, Length 30 cm)
Capillary temperature:	25 ^{o}C
Separation buffer:	30mM borate buffer, pH 9.0
Separation voltage:	15kV
Detection:	PDA 210 nm

PUBLICATIONS FOR THESIS

1. **Punturee K**, Wild CP, Vinitketkumneun U. Thai medicinal plants modulate nitric oxide and tumor necrosis factor-alpha in J774.2 mouse macrophages. *J Ethnopharmacol*. 2004: 95; 183-9.

2. **Punturee K**, Kasinrerk W, Wild CP, Vinitketkumnuen U. Immunomodulatory effects of Thai medicinal plants on the mitogen stimulated proliferation of human peripheral blood mononuclear cells *in vitro*. *Chiangmai Med Bull*. 2005: 44; 1-12.

3. **Punturee K**, Wild CP, Kasinrerk W, Vinitketkumnuen U. Immunomodulatory activities of *Centella asiatica* and *Rhinacanthus nasutus* extracts. (Submitted).

4. **Punturee K**, Wild CP, Vinitketkumnuen U. Effects of mycotoxin mixtures on lymphocytes and macrophages and its modulation by *Centella asiatica* extract. (Submitted).

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Publications:

1. **Punturee K**, Wild CP, Vinitketkumneun U. Thai medicinal plants modulate nitric oxide and tumor necrosis factor-alpha in J774.2 mouse macrophages. *J Ethnopharmacol.* 2004: 95; 183-9.
2. **Punturee K**, Kasinrerk W, Wild CP, Vinitketkumnuen U. Immunomodulatory effects of Thai medicinal plants on the mitogen stimulated proliferation of human peripheral blood mononuclear cells *in vitro*. *Chiangmai Med Bull.* 2005: 44; 1-12.
3. **Punturee K**, Wild CP, Kasinrerk W, Vinitketkumnuen U. Immunomodulatory activities of *Centella asiatica* and *Rhinacanthus nasutus* extracts. (Submitted).
4. **Punturee K**, Wild CP, Vinitketkumnuen U. Effects of mycotoxin mixtures on lymphocytes and macrophages and its modulation by *Centella asiatica* extract. (Submitted).
5. Pornprasert S, **Punturee K** and Vinitketkumneun U. Anti-proliferative and cytotoxic effects of *Murdannia loriformis* on leukemic cell lines. *Chiangmai Med Bull.* 2001: 40; 195-203.

Papers Presented at Conferences:

1. **Punturee K**, Vinitketkumneun U, Wild CP and Kasinrerk W. Immunomodulatory effect of Thai medicinal plants on the mitogen stimulated proliferation of human peripheral blood mononuclear cells *in vitro*. First national Symposium on Graduate Research, Chiang Mai University, Chiang Mai, Thailand, June 10-11, 2000.
2. **Punturee K**, Vinitketkumneun U, Wild CP and Kasinrerk W. Immunomodulatory effect of Thai medicinal plants on the mitogen stimulated proliferation of human peripheral blood mononuclear cells *in vitro*. The second national Seminar on Pharmaceutical Biotechnology, Holiday Garden Hotel, Chiang Mai, Thailand, June 21-23, 2000.
3. **Punturee K**, Kasinrerk W, Wild CP and Vinitketkumneun U. Immunomodulatory effect of Thai medicinal plants on the mitogen stimulated proliferation of human peripheral blood mononuclear cells *in vitro*. Takeo Wada Cancer Research Symposium, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, November 30-December 1, 2000.
4. **Punturee K**, Kasinrerk W, Wild CP and Vinitketkumneun U. Immunomodulatory effect of Thai medicinal plants on the mitogen stimulated proliferation of human peripheral blood mononuclear cells *in vitro*. The 8th World Congress on Clinical Nutrition, Phitsanuloke, Thailand, December 17-20, 2000.
5. **Punturee K**, Kasinrerk W, Wild CP and Vinitketkumneun U. *In vitro* and *in vivo* studies on the immunostimulating effect of *Centella asiatica*. RGJ-Ph.D. Congress II, Garden Beach Resort Hotel, Chonburi, Thailand, April 20-22, 2001.
6. **Punturee K**, Kasinrerk W, Wild CP and Vinitketkumneun U. *In vitro* and *in vivo* immunomodulating effects of Thai medicinal plants. The third National Seminar on Pharmaceutical Biotechnology, Chiang Mai, Thailand, June 27-29, 2001.
7. **Punturee K**, Kasinrerk W, Vinitketkumneun U and Wild CP. The effects of Thai medicinal plants on nitric oxide production in J774.2 mouse macrophage cell line. The 25th Anniversary Conference of United Kingdom Environmental Mutagen Society, University of Plymouth, Plymouth, UK, June 30-July 3, 2002.
8. **Punturee K**, Kasinrerk W, Wild CP and Vinitketkumneun U. The effects of *Centella asiatica* on mycotoxins-modulated nitric oxide and tumor necrosis factor-α production in

J774.2 mouse macrophages. The 2nd Annual Biochemical Research Meeting, Chiang Mai, Thailand, October 17-18, 2002.

9. **Punturee K**, Kasinrerk W, Wild CP and Vinitketkumneun U. Combined effects of mycotoxins on the immune system. The 3rd Annual Biochemical Research Meeting, Chiang Mai, Thailand, October 8-9, 2003.
10. **Punturee K**, Kasinrerk W, Wild CP and Vinitketkumneun U. Effects of *Centella asiatica* on mycotoxin-induced immunotoxicity. RGJ-Ph.D. Congress V, Jomtien Palm Beach Resort, Pattaya, Thailand, April 23-25, 2004.
11. **Punturee K**, Kasinrerk W, Wild CP and Vinitketkumneun U. Immunostimulating activity of *Centella asiatica* and *Rhinacanthus nasutus* extracts. The 4th International Symposium on Graduate Research, The Lotus Pang Suan Kaew Hotel, Chiang Mai, Thailand, August 10-11, 2004.
12. **Punturee K**, Kasinrerk W, Wild CP and Vinitketkumneun U. Immunomodulatory activity of *Centella asiatica* extracts. The 3rd Takeo Wada Cancer Research Symposium, Khon Kaen University, Khon Kaen, Thailand, February 24-25, 2005.