APPENDICES

APPENDIX A

List of the chemicals and materials used in the study

Chemicals/Materials	Source
β- NADPH	Oriental Yeast, Japan
1-Chloro-2, 4-dinitrobenzene	Fluka A.G., Switzerland
2-aminophenol	Sigma-Aldrich, USA
4', 6-diamidino-2-phenylindole dihydrochloride	Invitrogen, USA
5, 5'-Dithio-bis (2-Nitrobenzoic acid)	Sigma-Aldrich, USA
Absolute ethanol	BDH, England
Acrylamide	Biorad, USA
Ammonium persulphate	Carlo-Erba, Italy
Ammonium sulfamate	Sigma-Aldrich, USA
2-Aniline	BDH, England
Ascorbic acid	Merck A.G.,Germany
Bis-acrylamide	Biorad, USA
Bovine serum albumin	Sigma-Aldrich, USA
Butanol	BDH, England
Calcium chloride dihydrate	Merck A.G.,Germany
Chloroform	LAB-SCAN, Thailand
Collagenase type IV	Invitrogen, USA
Copper sulphate	Merck A.G.,Germany
Cytochrome c Type VI	Sigma-Aldrich, USA
Diethyl ether	BDH, England
Disodium hydrogen orthophosphate	BDH, England
Ethylene diamine tetraacetic acid	Sigma-Aldrich, USA

Ethylene glycol tetraacetic acid	Sigma-Aldrich, USA
Folin & Ciocalteu's phenol reagent	Fluka A.G., Switzerland
Formalin	BDH, England
Glucose-6-phosphate	Sigma-Aldrich, USA
Glucose-6-phosphate dehydrogenase	Sigma-Aldrich, USA
Glutathione (Reduced form)	Wako, Japan
Glycerol	Sigma-Aldrich, USA
Glycine	Vivantis, Malaysia
Hemin	Sigma-Aldrich, USA
HEPES, free acid	Amresco, USA
Hydrochloric	BDH, England
Isopropanol	Sigma-Aldrich, USA
Magnesium chloride	APS Finechem, Australia
Malondialdehyde bis(dimethyl acetal)	Sigma-Aldrich, USA
Mercaptoethanol	Sigma-Aldrich, USA
Methanol	BDH, England
N-naphthlethylene diamine	BDH, England
Phenol red sodium salt	Amresco, USA
Phenylmethylsulphonyl fluoride	Sigma-Aldrich, USA
Potassium chloride	Carlo-Erba, Italy
Potassium cyanide	Merck A.G.,Germany
Potassium Dihydrogen Phosphate	May and Baker, England
Potassium sodium tatrate	Mallinckrodt chemical work, USA
Skimmed milk	Merck A.G.,Germany
Sodium carbonate	BDH, England
Sodium chloride	BDH, England
Sodium dihydrogenphosphate	BDH, England
Sodium dodecyl sulfate	Biorad, USA
Sodium hydrogen carbonate	BDH, England
Sodium hydroxide	BDH, England
Sodium nitrite	AJAX Chemicals, Australia

TEMED	USB, USA
Thiobarbituric acid	Sigma-Aldrich, USA
Trichloroacetic acid	BDH, England
Tris base	Vivantis, Malaysia
Triton x-100	Sigma-Aldrich, USA
Tween-20	USB, USA
Tween-80	BDH, England
UDP-glucuronic acid	Sigma-Aldrich, USA

APPENDIX B

List of the instruments used in the study

Instrument	Model	Source
Analytical balance	300A	Precisa, USA
Blotting apparatus	Trans-blot SD cell	Biorad, USA
Centrifugator	PMC-060	Tomy Seiko, Japan
	22R D-78532	Mikro, Germany
Electrophoretic apparatus	AE-6500	Atto corp., Japan
Film cassette	RPN 11649	Amersham, England
Fluorescent microscope	AX-70	Olympus, Japan
Freezer (-86^{o}C)	0838	Forma Scientific, USA
Homogenizer	HS-30E	Daihan, Korea
Hot plate/stirrer	HPMS	Whatman, USA
Light microscope		Olympus, Japan
Microplate reader	MCC/340	ICN, Flow, USA
Mini rocker		Biosan, Taiwan
pH meter	320	Mettler Toledo, USA
Peristaltic pump	MP-100	Tokyo Rikakikai, Japan

Refrigerator	SR-F511	Sanyo, Thailand
Ultracentrifuge	L-100 XP	Beckman Coulter
UV-Spectrophotometer	UV-1700	Shimazu, Japan
Vortex	G-560E	Scientific industries, USA
Water bath	W-350	Mammert, Germany

APPENDIX C

Reagents and buffers preparation

1. Preparation of mediums for liver-cell suspension

1.1 Preperfusion medium

NaCl	8	g
KCl	0.4	g
KH_2PO_4	0.06	g
Na_2HPO_4	0.09	g
EGTA	0.195	g
HEPES	2.39	g
$NaHCO_3$	0.35	g

After dissolve in distilled water, adjust pH to 7.4 with 1 N NaOH and bring to 1L with distilled water.

1.2 Collagenase medium

NaCl	8	g
KCl	0.4	g
KH_2PO_4	0.06	g
Na_2HPO_4	0.09	g
Phenol red	0.01	g
$CaCl_2 \cdot 2H_2O$	0.56	g
HEPES	2.39	g
$NaHCO_3$	0.35	g
Collagenase type IV	0.5	g

After completely dissolve in 1 liter of distilled water, adjust pH to 7.4 with 1 N NaOH

1.3 Phosphate buffer saline ($Mg^{2+,}$ $Ca^{2+,}$ free)

NaCl	8	g
KCl	0.2	g
KH_2PO_4	0.2	g
Na_2HPO_4	1.15	g

After dissolve in distilled water, adjust pH to 7.4 with 1 N NaOH and fill to 1 liter.

2. Preparation of buffers for microsome fraction

2.1 Homogenizing buffer

KCl	11.5	g
EDTA	0.37	g

After completely dissolved in deionized water, add 1 ml of 0.25 M PMSF and fill to 1 liter

2.2 Microsome suspention buffer

KH_2PO_4	0.14	g
EDTA	0.004	g
DTT	0.002	g
Glycerol	3	ml

After completely dissolved in deionized water, adjust pH to 7.4 with conc. KOH and fill to 10 mL.

3. Preparation of SDS-PAGE reagent and buffer

3.1 30% Acrylamode

Acryamide	300	g
Bisacrylamide	8	g

After dissolve in deionized water and fill to 100 mL, store at 4 ^{o}C.

3.2 Separating gel buffer pH 8.8

Tris base	19.71	g
SDS	4	g

After completely dissolved in deionized water, adjust pH to 8.8 with conc. HCl and fill to 1 liter.

3.3 Stacking gel buffer pH 6.8

Tris base	60.57	g
SDS	8	g

After completely dissolved in deionized water, adjust pH to 6.8 with 1 N NaOH and fill to 1 liter.

3.4 10% Ammonium persulphate

Dissolve 10 g of ammonium persulphate in deionized water and fill to 100 mL.

3.5 Sample buffer pH 6.8

Tris base	0.15	g
SDS	0.40	g
Glycerol	2	ml
2-mercaptoethanol	1	ml
0.002% bromophenol blue	5	µl

Dissolve in deionized water, adjust pH to 6.8 with conc. HCl and fill to 1 liter.

3.6 Electrode buffer

Tris base	3.03	g
Glycine	4.41	g
SDS	1	g

Dissolve in deionized water and fill to 1 liter.

3.7 Blotting buffer

Tris Base	12.11	g
Glycine	14.4	g

After dissolve in deionized water, mix 200 mL of methanol and bring to 1liter.

4. Preparation of immunostaining buffer

4.1 Phophate buffer saline

$NaH_2PO_4 \cdot H_2O$	1.56	g
NaCl	9.00	g

Dissolve in deionized water, adjust pH to 7.5 with 1 N NaOH and fill to 1liter.

4.2 Tween-phophate buffer saline

$NaH_2PO_4 \cdot H_2O$	1.56	g
NaCl	9.00	g

Dissolve in deionized water, after completely dissolved, add 0.5 ml tween-20 and adjust pH to 7.5 with 1N NaOH and fill to 1liter.

4.3 5% skimmed milk

Dissolve 5 g of non-fat dried milk in TPBS and fill to 100 ml.

4.4 10% BSA

Dissolve 10 g of BSA TPBS and fill to 100 ml.

4.5 TPBS-0.2% BSA

Mix 100 ml of TPBS with 2 ml of 10% BSA.

5. Preparation of reagents and buffer for lipid peroxidation assay (Thiobarbituric acid method)

5.1 0.1 M Sodium phosphate buffer pH 7.0

Dissolve 6.2404 g of Na_2HPO_4 in 200 ml of distilled water.

Dissolve 14.3256 g of NaH_2PO_4 in 200 ml of distilled water.

Add NaH_2PO_4 solution into the Na_2HPO_4 solution until pH value reaches 7.0.

5.2 Malondialdehyde dimethyl acetal (MDA) stock solution

MDA	5	µl
Methanol	5	ml
0.2 M HCl	5	ml

All chemicals mix and store the reagent at room temperature (freshly prepare)

5.3 50% Trichloroacetic acid (TCA)

Dissolve 50 g of TCA in 70 ml of distilled water and made up to 100 ml.

5.4 0.67% Thiobarbituric acid (TBA)

Dissolve 0.67 g of TBA in 90 ml of distilled water and made up to 100 ml.

6. Preparation of reagents and buffer for NADPH-P450 reductase activity assay

6.1 0.3M potassium phosphate buffer (pH 7.5)

Dissolve 4.08 g of KH_2PO_4 in deionized water, adjust pH to 7.5 with conc. KOH and made up to 100 ml.

6.2 10 mM NADPH

Dissolve 9.1 mg of NADPH in 1 ml of deionized water.

6.3 1mM Cytochrome c Type VI (Horse Heart Type VI)

Dissolve 12.384 mg of Cytochrome *c* in 1 ml of deionized water.

6.4 50 mM KCN

Dissolve 32.56 mg of KCN in deionized water and made up to10 ml.

7. Preparation of reagents and buffer for heme oxygenase activity assay

7.1 0.1M potassium phosphate buffer containing $MgCl_2$, pH 7.4

Dissolve 13.61 g of KH_2PO_4 in deionized water, adjust pH to 7.4 with conc. KOH. Add 0.406 g of $MgCl_2$ and made up to 1 liter.

7.2 400 μM Hemin

Dissolve 2.6 mg of hemin in dichloromethane 10 ml

7.3 8mM Guclose-6-phosephate

Dissolve 22.57 mg of Guclose-6-phosephate in deionized water10 ml

7.4 6.4 mM β-NADPH

Dissolve 5.33 mg of *β-NADPH* in deionized water 1 ml

8. Preparation of reagents and buffer for NADPH quinone reductase activity assay

8.1 37.5mM Tris-Hcl buffer, pH 7.4

Dissolve 4.54 g of Tris in deionized water, adjust pH to 7.4 with conc. HCl and made up to 1 liter.

8.2 30 mg/ml BSA

Dissolve 30 mg of BSA in 1 ml of deionized water.

8.3 36 mM DCPIP

Dissolve 104.4 mg of DCPIP in 10 ml of deionized water.

8.4 300 mM β-NADPH

Dissolve 1 g of NADPH in 4 ml of deionized water.

8.5 4.5 mM FAD

Dissolve 37.32 m g of FAD in 10 ml of deionized water.

9. Preparation of reagents and buffer for UDP-glucuronyl transferase activity assay

9.1 0.1 M Tris buffer pH 8.0

Dissolve 12.1 g of Tris in deionized water, adjust pH to 8 with conc. HCl and made up to 100 ml.

9.2 1mM 2-aminophenol

Dissolve 11 mg of 2-aminophenol in 90 ml of distilled water and made up to 100 ml.

9.3 20%(w/v) TCA

Dissolve 200 g of TCA in distilled water and made up to 1 liter.

9.4 6%(w/v) TCA

Dissolve 60 g of TCA in distilled water and made up to 1 liter.

9.5 0.15 M $MgCl_2$

Dissolve 15.25 g of $MgCl_2$ in distilled water and made up to 500 ml.

9.6 *1% (w/v) Triton x-100*

Dissolve 100 mg of triton x-100 in distilled water and made up to 50 ml.

9.7 0.02 M ascorbic acid

Dissolve 180 mg of ascorbic acid in distilled water and made up to 50 ml.

9.8 20%(w/v) TCA in 0.1 M phosphate buffer pH 2.7

Dissolve 200 g of TCA in 0.1 M phosphate buffer pH 2.7 made up to 1 liter.

9.9 6%(w/v) TCA in 0.1 M phosphate buffer pH 2.7

Dissolve 60 g of TCA in 0.1 M phosphate buffer pH 2.7 made up to 1 liter.

9.10 0.1% (w/v) sodium nitrite

Dissolve 100 mg of sodium nitrite in distilled water and made up to 100 ml (fleshly prepare).

9.11 0.5 % (w/v) ammonium sulfamate

Dissolve 500 mg of ammonium sulfamate in distilled water and made up to 100 ml.

9.12 0.1% (w/v) N-naphthlethylene diamine

Dissolve 100 mg of N-naphthlethylene diamine in distilled water and made up to 100 ml.

9.13 0.1 mM aniline in 6%(w/v) TCA

Aniline (HCl salt)	13	mg
TCA	6	g

Dissolve in distilled water and fill to 100 ml.

10. Preparation of reagents and buffer for glutathione-*S*-transferase activity assay

10.1 0.2 M potassium phosphate buffer pH 6.5

Dissolve 2.7 g of KH_2PO_4 in 80 ml of deionized water, adjust pH to 6.5 with conc. KOH and made up to 100 ml.

10.2 10 mM 1-chloro-2,4-dinitrobenzene

Dissolve 102 mg of 1-chloro-2, 4-dinitrobenzene in 20 ml of absolute ethanol. After completely dissolved, total volume was adjusted to 50 ml by deionized water.

10.3 10 mM reduced glutathione

Dissolve 307 mg of reduced glutathione in 10 ml of deionized water.

11. Preparation of reagents and buffers for total protein assay by Lowry method

11.1 2% Na_2CO_3 in 0.1 N NaOH (Reagent A)

Dissolve 10 g of Na_2CO_3 in 400 ml of deionized water. After completely dissolved, add 2 g of NaOH and adjust volume to 500 ml.

11.2 0.5% $CuSO_4 \cdot 5H_2O$ (Reagent B)

Dissolve 1 g of $CuSO_4 \cdot 5H_2O$ in deionized water and made up to 200 ml.

11.3 1% potassium sodium tartrate (Reagent C)

Dissolve 1 g of potassium sodium tartrate in deionized water and made up to 100 ml.

11.4 Carbonate-copper solution (Reagent D)

Mix reagent A, B, and C at ratio 50:1:1.

11.5 Diluted Folin reagent

Dilute Folin-Ciocalteu's phenol reagent in deionized water at equal volume.

APPENDIX D

Extraction and isolation of pinocembrin from fingerroot

1. Extraction and isolation

Figure S-1 presents the procedure of pinocembrin isolated from *Boesenbergia pandurata.* Briefly, chopped-dried rhizomes (1 kg) of *Boesenbergia pandurata* were three times extracted with n-hexane, ethyl acetate at room temperature, followed by filtration. The solvent was evaporated under reduced pressure to afford the n-hexane (29.09 g), ethyl acetate (79.99 g), respectively.

A part of the ethyl acetate extract (150 g) was further subjected to column chromatography on silica gel (400 g) eluting with hexane/ethyl acetate/methanol to yield 6 fractions (F1–F6). Fraction F5 was chromatographed by on silica gel (400 g) eluting with hexane/ methanol to give 5 subfractions (F1–F5). Subfraction F5 (9g) was recrystallized from ethanol to give yellow crystal of pinocembrin (130 mg), Figure S-2.

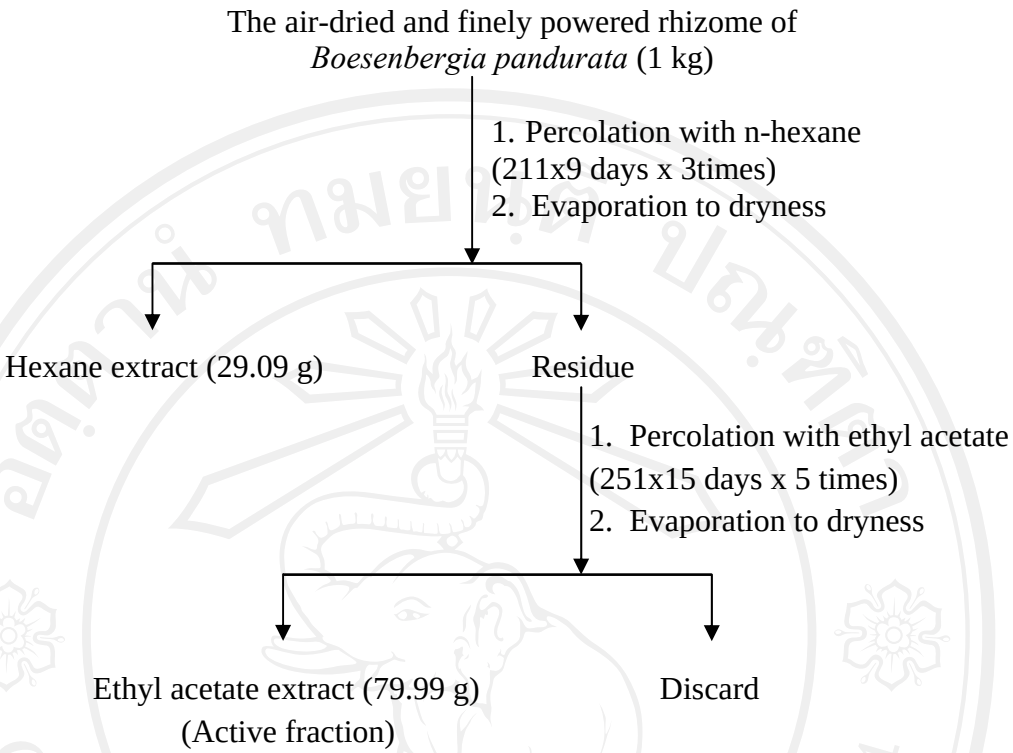

Figure S-1 Extraction scheme of *Boesenbergia pandurata*

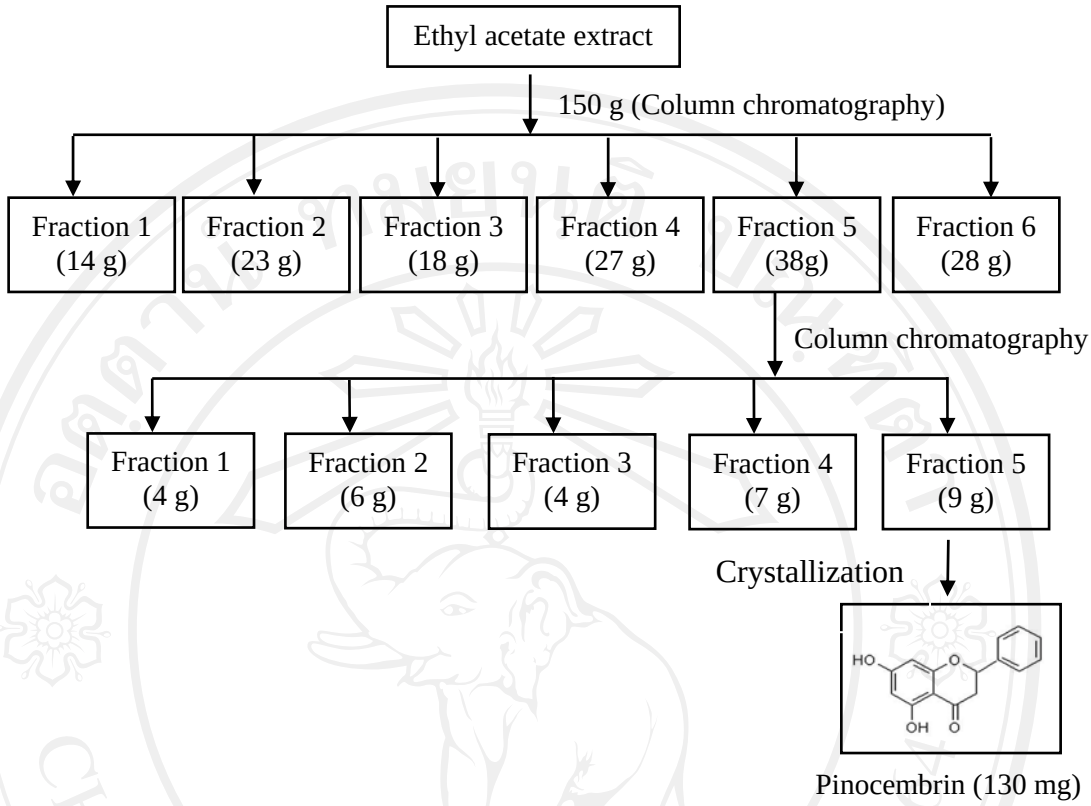

Figure S-2 Isolation scheme of pinocembrin

Structure elucidation of 5, 7 dihydroxyflavanone (Pinocembrin)

UV λ_{max}^{MeOH} nm (log ε): 288 (4.10), 323 (3.83).

FTIR υ_{max}^{KBr} cm^{-1}: 3415 (O-H stretching), 1630 (C=O stretching of ketone), 1583, 1487, 1467, 1357, 1302, 1089.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 12.13 (*s*, 1H, 5-OH), 10.85 (*br s*, 1H, 7-OH), 7.41 (1H, *m*, 2′,3′,4′,5′,6′), 5.93 (*d*, 1H, *J*= 2.1 Hz, H-8), 5.90 (*d*, 1H, *J*= 2.1 Hz, H-6) 5.58 (*dd*, 1H, *J*= 13, 3 Hz, H-2), 3.23(*dd*, 1H, *J*= 18, 13 Hz, H-3a), 2.72(*dd*, 1H, *J*= 18, 3 Hz, H-3a)

^{13}C-NMR (100 MHz, $CDCl_3$): δ 195.81 (C-4), 166.63 (C-7), 163.42 (C-5), 162.63 (C-9), 138.61 (C-1′), 128.44(C-3′-5′), 126.49 (C-2′,6′), 101.71 (C-10), 95.85 (C-6), 78.29 (C-2), 42.07(C-3).

EIMS m/z (% relative intensity): 256 (M^+, 64.73), 255 (100), 238 (29.91), 213 (4.60), 179 (73.29), 152 (16.92), 124 (25.01), 103(8.14), 96 (11.27), 78(13.08), 69(11.87), 50(8.83).

Crystals: yellow needles from EtOH m.p. 201.7-202.2 ^{o}C (Lit. m.p. 200-201^{o}C).

CURRICULUM VITAE

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Publications

1. Charoensin S, Punvittayagul C, Pompimon W, Mevatee U and Wongpoomchai R. Toxicological and clastogenic evaluation of pinocembrin and pinostrobin isolated from *Boesenbergia pandurata* in Wistar rats. *Thai J Toxicol*. (inpress).
2. Punvittayagul C., Charoensin S., Taya S., Pompimon W., Wongpoomchai R. Effect of pinocembrin on xenobiotic-metabolizing enzymes in rat liver. Poster presentation at the 3rd Asian Pacific Regional ISSX Meeting, Bangkok, Thailand. May 10-12, 2009. Drug Met. Rev. 2009, 41(2): 72.
3. Taya S., Charoensin S., Punvittayagul C., and Wongpoomchai R. Effect of *Cleistocalyx nervosum* on antioxidant and detoxifying enzymes in Wistar rat. Poster presentation at the 3rd Asian Pacific Regional ISSX Meeting, Bangkok, Thailand. May 10-12, 2009. Drug Met. Rev. 2009, 41(2): 88-89.
4. Taya S., Punvittayagul C., Chewonarin T., and Wongpoomchai R. Effect of aqueous extract from *Cleistocalyx nervosum* on oxidative status in rat liver. Proceeding at the 2nd National Conference in Toxicology, Bangkok, Thailand. December 17-18, 2009. *Thai J Toxicol.* 24(2) 101-105.

Presentations

1. Punvittayagul C., Pompimon W., Wongpoomchai R. Rat liver mutagenicity and antimutagenicity of pinocembrin extracted from fingerroot rhizome (*Boesenbergia pandurata*). Poster presentation at the 8th Annual Biochemical Research Meeting. October 13-14, 2008, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, page 43.
2. Charoensin S., Punvitayagul C., Taya S., Pompimon W., Mevatee U., and Wongpoomchai R. The inhibition of micronucleus formation of pinostrobin from fingerroot *(Boesenbergia pandurata*) in Wistar rat liver induced by diethylnitrosamine and its possibly inhibiting mechanism. Oral presentation at the Second International Conference on Natural Products for Health and Beauty (NATPRO). December 17-19, 2008, Naresuan University at Phayao, Phayao Province, Thailand, page 126.
3. Punvittayagul C., Pompimon W., Wongpoomchai R. Mutagenicity and antimutagenicity of pinocembrin extracted from fingerroot rhizome (*Boesenbergia pandurata*) in rat liver. Poster presentation at the 2nd International Conference on Natural Products for Health and Beauty (NATPRO). December 17-19, 2008, Naresuan University at Phayao, Phayao Province, Thailand, page 185.
4. Taya S., Charoensin S., Punvittayagul C., and Wongpoomchai R. Antioxidative properties of various extracts of ma-kiang (*Cleistocalyx nervosum* var. *paniala*). Poster presentation at the 2nd International Conference on Natural Products for Health and Beauty (NATPRO). December 17-19, 2008, Naresuan University at Phayao, Phayao Province, Thailand, page 223.
5. Taya S., Charoensin S., Punvittayagul C., Wongpornchai S, and Wongpoomchai R. Toxicological study of antioxidative substances extracted from Ma-kiang (*Cleistocalyx nervosum* var. *paniala*) in rat. Poster presentation at the International Congress for Innovation in Chemistry (PERCH-CIC Congress VI). May 3-6, 2009, Jomtien Plam Beach Hotel & Resort, Pattaya, Thailand, page 273.

6. Punvittayagul C., Charoensin S., Taya S., Pompimon W., Wongpoomchai R. Mutagenicity and antimutagenicity of pinocembrin isolated from *Boesenbergia pandurata* rhizome in rat liver. Poster presentation at the International Congress for Innovation in Chemistry (PERCH-CIC Congress VI). May 3-6, 2009, Jomtien Plam Beach Hotel & Resort, Pattaya, Thailand, page 299.
7. Punvittayagul C., Taya S., Charoensin S., Pompimon W., Wongpoomchai R. Evaluation of mutagenicity and antimutagenicity of pinocembrin by rat liver micronucleus test. Oral presentation at the 9th Annual Biochemical Research Meeting. October 8-9, 2009, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, page 9.
8. Taya S., Punvittayagul C., and Wongpoomchai R. Effect of *Cleistocalyx nervosum* extract on oxidative stress in early stages of chemicals induced hepatocarcinogenesis. Oral presentation at the 9th Annual Biochemical Research Meeting. October 8-9, 2009, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, page 10.
9. Punvittayagul C., Taya S., Pompimon W., Wongpoomchai R. Effect of pinocembrin on promotion stage in diethylnitrosamine-induced rat hepatocarcinogenesis. Poster presentation at the 2nd National Conference in Toxicology. December 17-18, 2009, Miracle Grand Convention Hotel, Bangkok, Thailand, page 170.

Scholarship

1. Center of excellence for innovation in chemistry (PERCH-CIC), year 2008-2009.