

**PHYTOCHEMICAL, PHARMACOLOGICAL AND  
PHARMACOKINETIC STUDIES OF  
*PHYLLANTHUS NIRURI* LINN. LIGNANS AS  
POTENTIAL ANTIHYPERURICEMIC AGENTS**

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**by**

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**Thesis submitted in fulfilment  
of the requirements for the degree of  
Doctor of Philosophy**

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*To **GOD** for giving me **LIFE**,  
my **parents** for showing me the **WAY**,  
and my **dear** for making my life **COMPLETE**.*

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## TABLE OF CONTENTS

	Page
<b>ACKNOWLEDGEMENT</b>	iii
<b>TABLE OF CONTENTS</b>	iv
<b>LIST OF TABLES</b>	ix
<b>LIST OF FIGURES</b>	xi
<b>LIST OF APPENDICES</b>	xvi
<b>LIST OF ABBREVIATIONS</b>	xxiii
<b>ABSTRAK</b>	xxv
<b>ABSTRACT</b>	xxviii
<b>CHAPTER ONE : INTRODUCTION</b>	
1.1 Hyperuricemia: A global scenario and its management	1
1.2 Literature review	6
1.2.1 Biochemistry and physiology of uric acid	6
1.2.1.1 Biosynthesis and regulation of uric acid formation	6
1.2.1.2 Degradation of uric acid	8
1.2.1.3 Uric acid disposal	9
1.2.2 Hyperuricemia	11
1.2.2.1 Experimental hyperuricemia in rodents	12
1.2.3 Role of medicinal plants and natural products in hyperuricemia	14
1.2.4 <i>Phyllanthus niruri</i> L.	16
1.2.4.1 Botanical aspects and geographical distributions	16
1.2.4.2 Chemical constituents of <i>Phyllanthus niruri</i> L.	17
1.2.4.3 Ethnobotanical uses of <i>Phyllanthus niruri</i> L.	27
1.2.4.4 Pharmacological properties of <i>Phyllanthus niruri</i> L.	28
1.2.4.5 Toxicological evaluation of <i>Phyllanthus niruri</i> L.	33
1.2.5 Lignans	34
1.2.5.1 Importance of lignans in biological system	34
1.2.5.2 Separation and analysis of plant lignans	39
1.2.5.3 Metabolism, bioavailability and pharmacokinetics of lignans	40
1.3 Objectives of the present study	42
1.4 Outline of the present study	43

## CHAPTER TWO : MATERIALS AND METHODS

### SECTION I CHEMISTRY

2A	Bioactivity-guided fractionation of <i>Phyllanthus niruri</i> L. and isolation of lignans	45
2.1	Plant materials	45
2.2	Instrumentations	45
2.3	Chemicals and reagents	46
2.4	Preparation of <i>P. niruri</i> methanol extract	46
2.5	Bioactivity-guided fractionation of the <i>P. niruri</i> methanol extract and isolation of lignans	47
2B	Analytical studies of lignans of <i>Phyllanthus niruri</i> L.	51
2.6	Standards, samples and chemicals	51
2.7	Instrumentations	51
2.8	Development and validation of a HPLC method for analysis of lignans from <i>P. niruri</i>	52
2.8.1	Development of a HPLC method	52
2.8.1.1	Peak purity	52
2.8.1.2	Chromatographic conditions	52
2.8.1.3	Limits of detection and limits of quantification	53
2.8.1.4	Calibration curve and linearity	53
2.8.2	HPLC-fluorescence detection method validation	53
2.8.2.1	Standards preparation	53
2.8.2.2	Precision and accuracy	54
2.8.2.3	Recovery	54
2.9	Quantification of lignans in <i>P. niruri</i> samples	54
2.10	Profiling and quantification of lignans in <i>P. niruri</i> methanol extract, fractions and sub-fractions	55

### SECTION II PHARMACOLOGY

2C	Preparation of chemically induced hyperuricemic rat model and studies on the antihyperuricemic effect of <i>Phyllanthus niruri</i> L. extracts and their lignans	56
2.11	Chemicals and reagents	56
2.12	Animals	56
2.13	Effect of different blood sampling technique on plasma uric acid	57

2.14	Optimization of multiple blood sampling interval by cardiac puncture technique	58
2.15	Effect of potassium oxonate and uric acid administration on plasma uric acid	58
2.16	Optimization of hyperuricemia in rat model	59
2.17	Effect of <i>P. niruri</i> methanol extract on plasma uric acid of normal rats	59
2.18	Effect of <i>P. niruri</i> methanol extract on plasma uric acid of hyperuricemic rats	60
2.19	Dose-response relationship of the antihyperuricemic effect of <i>P. niruri</i> methanolic extract	60
2.20	Effect of <i>P. niruri</i> methanol extract, fractions and sub-fractions on plasma uric acid of hyperuricemic rats	61
2.21	Effect of lignans on plasma uric acid of hyperuricemic rats	61
2.22	Dose-response relationship of the antihyperuricemic effect of phyllanthin	62
2.23	Statistical analysis	62
2D	Studies on <i>in vitro</i> and <i>in vivo</i> xanthine oxidase enzyme inhibitory effect of <i>Phyllanthus niruri</i> L. extracts and their lignans	63
2.24	Enzymes and chemicals	63
2.25	<i>In vitro</i> XO inhibitory study	63
2.25.1	<i>In vitro</i> XO assay	63
2.25.2	<i>In vitro</i> XO inhibitory effect of <i>P. niruri</i> methanol extract, fractions, sub-fractions and lignans	64
2.26	<i>In vivo</i> XO Inhibitory Study	65
2.26.1	Animals	65
2.26.2	Enzyme preparation	66
2.26.3	Protein content determination	66
2.26.4	<i>In vivo</i> XO assay optimization	67
2.26.4.1	Optimization of protein content	67
2.26.4.2	Optimization of incubation time	68
2.26.4.3	Optimization of xanthine concentration	68
2.26.5	Optimized <i>in vivo</i> XO assay	68
2.26.6	Effect of potassium oxonate- and uric acid-induced hyperuricemia on XO activity	69
2.26.7	Effect of <i>P. niruri</i> methanol extract on XO activity of hyperuricemic rats	70
2.26.8	Effect of <i>P. niruri</i> methanol extract, fractions and sub-fractions on XO activity of hyperuricemic rats	70

2.26.9	Effect of lignans on XO activity of hyperuricemic rats	71
2.27	Statistical analysis	71
2E	Studies on the effect of <i>Phyllanthus niruri</i> L. extracts and their lignans on urinary uric acid excretion and clearance of hyperuricemic rats	72
2.28	Chemicals and reagents	72
2.29	Animals	72
2.30	Effect of potassium oxonate- and uric acid–induced hyperuricemia on daily urinary uric acid excretion	72
2.31	Effect of <i>P. niruri</i> methanol extract on daily urinary uric acid excretion of hyperuricemic rats	73
2.32	Effect of <i>P. niruri</i> methanol extract, fractions and sub-fractions on urinary uric acid excretion and clearance of hyperuricemic rats	74
2.33	Effect of lignans on urinary uric acid excretion and clearance of hyperuricemic rats	75
2.34	Dose-response relationship of phyllanthin uricosuric effect	76
2.35	Pyrazinamide suppression test	76
2.36	Statistical analysis	76

### **SECTION III PHARMACOKINETIC**

2F	Pharmacokinetic and bioavailability study of lignans of <i>Phyllanthus niruri</i> L.	77
2.37	Chemicals and reagents	77
2.38	Animals	77
2.39	Instrumentations	77
2.40	Sample preparation	77
2.41	HPLC assay validation	78
2.41.1	Standards preparation	78
2.41.2	Chromatographic conditions	78
2.41.3	Limits of detection and limits of quantification	78
2.41.4	Calibration curve and linearity	79
2.41.5	Precision and recovery	79
2.41.6	Recovery	79
2.42	Pharmacokinetic and bioavailability study of lignans	79
2.43	Data analysis	80



## CHAPTER THREE : RESULTS

### SECTION I CHEMISTRY

3A	Bioactivity-guided fractionation of <i>Phyllanthus niruri</i> L. and isolation of lignans	82
	3.1 Characterization and structural elucidation of lignans from <i>P. niruri</i> L.	82
3B	Analytical studies of lignans of <i>Phyllanthus niruri</i> L.	108
	3.2 Development and validation of a HPLC method for analysis of lignans from <i>P. niruri</i>	108
	3.3 Quantification of lignans in <i>P. niruri</i> samples	112
	3.4 Profiling and quantification of lignans in methanol extract, fractions and sub-fractions of <i>P. niruri</i>	117

### SECTION II PHARMACOLOGY

3C	Preparation of chemically induced hyperuricemic rat model and studies on the antihyperuricemic effect of <i>Phyllanthus niruri</i> L. extracts and their lignans	120
	3.5 Preparation of chemically induced hyperuricemic rat model	120
	3.6 Studies on antihyperuricemic effect of <i>P. niruri</i> extracts and their lignans	129
3D	Studies on <i>in vitro</i> and <i>in vivo</i> xanthine oxidase enzyme inhibitory effect of <i>Phyllanthus niruri</i> L. extracts and their lignans	136
	3.5 <i>In vitro</i> XO inhibitory study	136
	3.6 <i>In vivo</i> XO inhibitory study	139
3E	Studies on the effect of <i>Phyllanthus niruri</i> L. extracts and their lignans on urinary uric acid excretion and clearance of hyperuricemic rats	145

### SECTION III PHARMACOKINETIC

3F	Pharmacokinetics and bioavailability study of lignans of <i>Phyllanthus niruri</i> L.	161
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<b>CHAPTER FOUR : DISCUSSION</b>	<b>168</b>
----------------------------------	------------

<b>CHAPTER FIVE : CONCLUSION</b>	<b>188</b>
----------------------------------	------------

<b>CHAPTER SIX : SUGGESTION FOR FURTHER WORK</b>	<b>191</b>
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<b>REFERENCES</b>	<b>193</b>
-------------------	------------

<b>APPENDICES</b>	<b>215</b>
-------------------	------------

<b>PUBLICATIONS</b>	<b>306</b>
---------------------	------------

## LIST OF TABLES

		Page
Table 1.1	Substances that alter the renal tubular handling of uric acid	10
Table 1.2	Classification of hyperuricemia	11
Table 1.3	The ethnobotanical uses of <i>Phyllanthus niruri</i> L.	28
Table 1.4	Pharmacological activities of plant lignans	35
Table 3.1a	<sup>1</sup> H-NMR assignments of phyllanthin ( <b>PN 1</b> )	86
Table 3.1b	<sup>13</sup> C-NMR assignments of phyllanthin ( <b>PN 1</b> )	87
Table 3.2	HSQC correlations of <b>PN 1</b>	87
Table 3.3a	<sup>1</sup> H-NMR assignments of hypophyllanthin ( <b>PN 2</b> )	91
Table 3.3b	<sup>13</sup> C-NMR assignments of hypophyllanthin ( <b>PN 2</b> )	92
Table 3.4	HSQC correlations of <b>PN 2</b>	93
Table 3.5a	<sup>1</sup> H-NMR assignments of phyltetralin ( <b>PN 3</b> )	98
Table 3.5b	<sup>13</sup> C-NMR assignments of phyltetralin ( <b>PN 3</b> )	99
Table 3.6	HSQC correlations of <b>PN 3</b>	100
Table 3.7a	<sup>1</sup> H-NMR assignments of niranthin ( <b>PN 4</b> )	105
Table 3.7b	<sup>13</sup> C-NMR assignments of niranthin ( <b>PN 4</b> )	106
Table 3.8	HSQC correlations of <b>PN 4</b>	107
Table 3.9	Calibration results, LOD and LOQ values of lignans of <i>P. niruri</i> analyzed by HPLC-UV and HPLC-fluorescence detection methods	108
Table 3.10	Recovery, within-day and between-day precision and accuracy values of <i>P. niruri</i> lignans analyzed by HPLC-fluorescence detection method	110
Table 3.11	Content of lignans of <i>P. niruri</i> in samples collected from various locations in Penang, Malaysia	116

Table 3.12	Content of lignans in methanol extract, fractions and sub-fractions of <i>P. niruri</i>	117
Table 3.13	Antihyperuricemic effect-lignans content relationship of <i>P. niruri</i> methanol extract, its fractions and sub-fractions	135
Table 3.14	Repeatability of the <i>in vitro</i> XO assay using allopurinol as standard	137
Table 3.15	<i>In vitro</i> XO inhibitory activity and mean IC <sub>50</sub> values of <i>P. niruri</i> methanol extract, fractions, sub-fractions and lignans	138
Table 3.16	Calibration results, LOD and LOQ values of lignans of <i>P. niruri</i> in rat plasma analyzed by HPLC-fluorescence detection method	162
Table 3.17	Recovery, within-day and between-day precision and accuracy values for <i>P. niruri</i> lignans in plasma analyzed by HPLC-fluorescence detection method	164
Table 3.18	Pharmacokinetic parameters of lignans in rat plasma after intravenous and oral administration of lignan rich <i>Phyllanthus niruri</i> L. extract (fraction 4)	167

## LIST OF FIGURES

		Page
Figure 1.1	Pathways for biosynthesis of purines and the formation of uric acid	7
Figure 1.2	Enzymatic degradation of uric acid	8
Figure 1.3	<i>Phyllanthus niruri</i> L.; (A) whole plant (B) aerial part (C) leaves	16
Figure 1.4	Flow chart of the outline of the present study	44
Figure 2.1	Schematic diagram on the outline of bioactivity-guided fractionation of <i>Phyllanthus niruri</i> L.	48
Figure 2.2	Schematic diagram on the isolation of lignans from <i>Phyllanthus niruri</i> L.	49
Figure 3.1	HPLC chromatogram of mixed standard solution of <i>P. niruri</i> lignans ( <b>PN 1</b> , 1250 ng/ml; <b>PN 2</b> , 6250 ng/ml; <b>PN 3</b> , 1250 ng/ml; <b>PN 4</b> , 2500 ng/ml) obtained from HPLC-fluorescence detection method	109
Figure 3.2	HPLC chromatograms of methanol extract of <i>P. niruri</i> aerial parts collected from various geographical locations at 20 µg/ml. (A) Bukit Mertajam (B) Simpang Ampat (C) Air Itam (D) Sungai Dua (E) Balik Pulau (F) Seberang Jaya	114
Figure 3.3	HPLC chromatograms of methanol extract of different parts of <i>P. niruri</i> plant sample collected from Bukit Mertajam. (A) roots at 50 µg/ml (B) leaves at 20 µg/ml (C) branches at 50 µg/ml (D) stems at 50 µg/ml (E) fruits at 50 µg/ml	115
Figure 3.4	HPLC chromatograms of methanol extract, fractions and sub-fractions of <i>P. niruri</i> . (A) methanol extract at 50 µg/ml (B) fraction 2 at 100 µg/ml (C) fraction 3 at 100 µg/ml (D) fraction 4 at 20 µg/ml (E) <i>n</i> -hexane sub-fraction of fraction 4 at 5 µg/ml (F) chloroform sub-fraction of fraction 4 at 50 µg/ml (G) <i>n</i> -butanol sub-fraction of fraction 4 at 100 µg/ml	118
Figure 3.5	Comparison of plasma uric acid concentrations obtained by cardiac puncture and tail-cut method	121
Figure 3.6	Effect of multiple cardiac puncture blood sampling on red blood cells count	122
Figure 3.7	Effect of multiple cardiac puncture blood sampling on haemoglobin concentrations	122
Figure 3.8	Effect of multiple cardiac puncture blood sampling on haematocrit values	123

Figure 3.9	Effect of multiple cardiac puncture blood sampling on platelet count	123
Figure 3.10	Effect of multiple cardiac puncture blood sampling on white blood cells count	124
Figure 3.11	Effect of multiple cardiac puncture blood sampling on plasma uric acid concentrations	124
Figure 3.12	Effect of single intraperitoneal administration of potassium oxonate (200 mg/kg) on plasma uric acid concentrations	127
Figure 3.13	Effect of single intraperitoneal administration of uric acid (2g/kg) on plasma uric acid concentrations	127
Figure 3.14	Effect of intraperitoneal potassium oxonate (200 mg/kg) alone and intraperitoneal potassium oxonate (200 mg/kg) with oral uric acid (2 g/kg) on plasma uric acid concentrations	128
Figure 3.15	Effect of intraperitoneal potassium oxonate (200 mg/kg) with oral uric acid (0.5, 1 or 2 g/kg) on plasma uric acid concentrations	128
Figure 3.16	Effect of oral <i>P. niruri</i> methanol extract (PN MeOH, 200 mg/kg once daily) on plasma uric acid concentrations of normal rats	130
Figure 3.17	Effect of oral <i>P. niruri</i> methanol extract (PN MeOH, 200 mg/kg once daily) on plasma uric acid concentrations of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	130
Figure 3.18	Effect of oral methanol extracts of <i>P. niruri</i> (PN MeOH, 100 – 1000 mg/kg) and standard drugs given once daily on plasma uric acid concentrations of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	131
Figure 3.19	Effect of single intraperitoneal administration of <i>P. niruri</i> methanol extract (PN MeOH) and fractions (Fr 1, 2, 3 and 4) each at 50 mg/kg on plasma uric acid concentrations of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	133
Figure 3.20	Effect of single intraperitoneal administration of sub-fractions of fraction 4 (50 mg/kg) on plasma uric acid concentrations of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	133
Figure 3.21	Effect of single intraperitoneal administration of <i>P. niruri</i> lignans and clinically used drugs on plasma uric acid concentrations of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	134
Figure 3.22	Dose-response relationship of phyllanthin antihyperuricemic effect in hyperuricemic rats induced by oral uric acid (1 g/kg)	134

	and intraperitoneal potassium oxonate (200 mg/kg)	
Figure 3.23	Effect of protein concentration of cytosolic fractions in the final assay mixture on uric acid concentrations	140
Figure 3.24	Effect of incubation period of the final assay mixture on uric acid concentrations	140
Figure 3.25	Effect of xanthine concentration in the final assay mixture on uric acid concentrations	141
Figure 3.26	Effect of hyperuricemia induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg) on liver XO activity	141
Figure 3.27	Effect of oral methanol extracts of <i>P. niruri</i> (PN MeOH, 100 – 1000 mg/kg) and allopurinol (50 mg/kg) given once daily on liver XO activity of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	143
Figure 3.28	Effect of single intraperitoneal administration of methanol extract of <i>P. niruri</i> (PN MeOH) and its fractions (Fr 1, 2, 3 and 4) each at 50 mg/kg on liver XO activity of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	143
Figure 3.29	Effect of single intraperitoneal administration of sub-fractions of fraction 4 (50 mg/kg) on liver XO activity of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	144
Figure 3.30	Effect of single intraperitoneal administration of lignans of <i>P. niruri</i> and allopurinol on liver XO activity of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	144
Figure 3.31	Effect of hyperuricemia induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg) on daily water intake	146
Figure 3.32	Effect of hyperuricemia induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg) on daily urine output	146
Figure 3.33	Effect of oral methanol extracts of <i>P. niruri</i> (PN MeOH, 100 – 1000 mg/kg) and clinically used drugs given once daily on daily water intake of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	147
Figure 3.34	Effect of oral methanol extracts of <i>P. niruri</i> (PN MeOH, 100 – 1000 mg/kg) and clinically used drugs given once daily on daily urine output of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	148
Figure 3.35	Effect of single intraperitoneal administration of methanol	150

	extract of <i>P. niruri</i> (PN MeOH) and its fractions (Fr 1, 2, 3 and 4) each at 50 mg/kg on water intake and urine output of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	
Figure 3.36	Effect of single intraperitoneal administration of sub-fractions of fraction 4 (50 mg/kg) on water intake and urine output of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	150
Figure 3.37	Effect of single intraperitoneal administration of lignans of <i>P. niruri</i> and clinically used drugs on water intake and urine output of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	151
Figure 3.38	Effect of single intraperitoneal administration of phyllanthin on water intake and urine output of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	151
Figure 3.39	Effect of hyperuricemia induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg) on daily urinary excretion of uric acid	153
Figure 3.40	Effect of oral methanol extracts of <i>P. niruri</i> (PN MeOH, 100 - 1000 mg/kg) and clinically used drugs given once daily on daily urinary excretion of uric acid of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	154
Figure 3.41	Effect of oral methanol extracts of <i>P. niruri</i> (PN MeOH, 100 -1000 mg/kg) and clinically used drugs given once daily on cumulative urinary excretion of uric acid of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	155
Figure 3.42	Effect of single intraperitoneal administration of methanol extract of <i>P. niruri</i> (PN MeOH) and its fractions (Fr 1, 2, 3 and 4) each at 50 mg/kg on urinary excretion and clearance of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	158
Figure 3.43	Effect of single intraperitoneal administration of sub-fractions of fraction 4 (50 mg/kg) on urinary excretion and clearance of uric acid of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	158
Figure 3.44	Effect of single intraperitoneal administration of lignans of <i>P. niruri</i> and clinically used drugs on urinary excretion and clearance of uric acid of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	159
Figure 3.45	Effect of single intraperitoneal administration of phyllanthin on urinary excretion and clearance of uric acid of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal	159

potassium oxonate (200 mg/kg)

- Figure 3.46 Effect of single intraperitoneal administration of clinically used drugs and phyllanthin on urinary excretion of uric acid in the pyrazinamide administered hyperuricemic rats 160
- Figure 3.47 HPLC chromatograms of *P. niruri* lignans in rat plasma. (A) Blank rat plasma; (B) Rat plasma spiked with 625.5, 3125, 625.5 and 1250 ng/ml of **PN 1**, **PN 2**, **PN 3** and **PN 4**, respectively; (C) Lignan rich *P. niruri* extract (fraction 4) at 20 µg/ml (D) Rat plasma at 1 hr after intravenous administration of 5 mg/kg lignan rich *P. niruri* L. extract (fraction 4 containing 0.89, 0.20, 0.08 and 0.40 mg/kg of **PN 1**, **PN 2**, **PN 3** and **PN 4**, respectively) 163
- Figure 3.48 Mean plasma concentration-time profiles of lignans after intravenous administration of 5 mg/kg of lignan rich *P. niruri* extract (fraction 4) 165
- Figure 3.49 Mean plasma concentration-time profiles of lignans after oral administration of 50 mg/kg of lignan rich *P. niruri* extract (fraction 4) 165



## LIST OF APPENDICES

		Page
Appendix 1a	Animal Ethics Committee approval letter for animal studies (Part 1)	215
Appendix 1b	Animal Ethics Committee approval letter for animal studies (Part 2)	216
Appendix 1c	Animal Ethics Committee approval letter for animal studies (Part 3)	217
Appendix 2.1a	UV spectrum of phyllanthin ( <b>PN 1</b> )	218
Appendix 2.1b (i)	IR spectrum of phyllanthin ( <b>PN 1</b> )	219
Appendix 2.1b(ii)	IR spectrum of phyllanthin ( <b>PN 1</b> ) after drying	220
Appendix 2.1c	MS spectrum of phyllanthin ( <b>PN 1</b> )	221
Appendix 2.1d	<sup>1</sup> H NMR spectrum of phyllanthin ( <b>PN 1</b> )	222
Appendix 2.1e	<sup>13</sup> C NMR spectrum of phyllanthin ( <b>PN 1</b> )	223
Appendix 2.1f	DEPT spectrum of phyllanthin ( <b>PN 1</b> )	224
Appendix 2.1g	COSY spectrum of phyllanthin ( <b>PN 1</b> )	225
Appendix 2.1h	HSQC spectrum of phyllanthin ( <b>PN 1</b> )	226
Appendix 2.1i	HMBC spectrum of phyllanthin ( <b>PN 1</b> )	227
Appendix 2.1j	ROESY spectrum of phyllanthin ( <b>PN 1</b> )	228
Appendix 2.2a	UV spectrum of hypophyllanthin ( <b>PN 2</b> )	229
Appendix 2.2b	IR spectrum of hypophyllanthin ( <b>PN 2</b> )	230
Appendix 2.2c	MS spectrum of hypophyllanthin ( <b>PN 2</b> )	231
Appendix 2.2d	<sup>1</sup> H NMR spectrum of hypophyllanthin ( <b>PN 2</b> )	232
Appendix 2.2e	<sup>13</sup> C NMR spectrum of hypophyllanthin ( <b>PN 2</b> )	233
Appendix 2.2f	DEPT spectrum of hypophyllanthin ( <b>PN 2</b> )	234
Appendix 2.2g	COSY spectrum of hypophyllanthin ( <b>PN 2</b> )	235
Appendix 2.2h	HSQC spectrum of hypophyllanthin ( <b>PN 2</b> )	236
Appendix 2.2i	HMBC spectrum of hypophyllanthin ( <b>PN 2</b> )	237
Appendix 2.2j	ROESY spectrum of hypophyllanthin ( <b>PN 2</b> )	238
Appendix 2.3a	UV spectrum of phyltetralin ( <b>PN 3</b> )	239
Appendix 2.3b	IR spectrum of phyltetralin ( <b>PN 3</b> )	240
Appendix 2.3c	MS spectrum of phyltetralin ( <b>PN 3</b> )	241
Appendix 2.3d	<sup>1</sup> H NMR spectrum of phyltetralin ( <b>PN 3</b> )	242
Appendix 2.3e	<sup>13</sup> C NMR spectrum of phyltetralin ( <b>PN 3</b> )	243

Appendix 2.3f	DEPT spectrum of phyltetralin ( <b>PN 3</b> )	244
Appendix 2.3g	COSY spectrum of phyltetralin ( <b>PN 3</b> )	245
Appendix 2.3h	HSQC spectrum of phyltetralin ( <b>PN 3</b> )	246
Appendix 2.3i	HMBC spectrum of phyltetralin ( <b>PN 3</b> )	247
Appendix 2.3j	ROESY spectrum of phyltetralin ( <b>PN 3</b> )	248
Appendix 2.4a	UV spectrum of niranthin ( <b>PN 4</b> )	249
Appendix 2.4b	IR spectrum of niranthin ( <b>PN 4</b> )	250
Appendix 2.4c	MS spectrum of niranthin ( <b>PN 4</b> )	251
Appendix 2.4d	<sup>1</sup> H NMR spectrum of niranthin ( <b>PN 4</b> )	252
Appendix 2.4e	<sup>13</sup> C NMR spectrum of niranthin ( <b>PN 4</b> )	253
Appendix 2.4f	DEPT spectrum of niranthin ( <b>PN 4</b> )	254
Appendix 2.4g	COSY spectrum of niranthin ( <b>PN 4</b> )	255
Appendix 2.4h	HSQC spectrum of niranthin ( <b>PN 4</b> )	256
Appendix 2.4i	HMBC spectrum of niranthin ( <b>PN 4</b> )	257
Appendix 2.4j	NOESY spectrum of niranthin ( <b>PN 4</b> )	258
Appendix 2.5	Fluorescence spectrum of mixed standard solution of <i>P. niruri</i> lignans ( <b>PN 1</b> , 1250 ng/ml; <b>PN 2</b> , 6250 ng/ml; <b>PN 3</b> , 1250 ng/ml; <b>PN 4</b> , 2500 ng/ml)	259
Appendix 2.6	HPLC chromatogram of mixed standard solution of <i>P. niruri</i> lignans ( <b>PN 1</b> , 2500 ng/ml; <b>PN 2</b> , 12 500 ng/ml; <b>PN 3</b> , 2500 ng/ml; <b>PN 4</b> , 5000 ng/ml) obtained from HPLC-UV detection method (a) using acetonitrile-deionized water system (55:45) (b) using methanol-deionized water system (55:45)	260
Appendix 3.1	Comparison of plasma uric acid obtained by cardiac puncture and tail-cut method	261
Appendix 3.2	Effect of multiple cardiac puncture blood sampling on red blood cells count	261
Appendix 3.3	Effect of multiple cardiac puncture blood sampling on haemoglobin concentrations	262
Appendix 3.4	Effect of multiple cardiac puncture blood sampling on haematocrit values	262
Appendix 3.5	Effect of multiple cardiac puncture blood sampling on platelet count	263
Appendix 3.6	Effect of multiple cardiac puncture blood sampling on white blood cells count	264
Appendix 3.7	Effect of multiple cardiac puncture blood sampling on plasma uric acid concentrations	264

Appendix 3.8	Effect of single intraperitoneal administration of potassium oxonate (200 mg/kg) and uric acid (2gm/kg) on plasma uric acid concentrations	265
Appendix 3.9	Effect of intraperitoneal potassium oxonate (200 mg/kg) alone and intraperitoneal potassium oxonate (200 mg/kg) with oral uric acid (2 g/kg) on plasma uric acid concentrations	266
Appendix 3.10	Effect of intraperitoneal potassium oxonate (200 mg/kg) with oral uric acid (0.5, 1 or 2 g/kg) on plasma uric acid concentrations	267
Appendix 3.11	Effect of oral <i>P. niruri</i> methanol extract (PN MeOH, 200 mg/kg once daily) on plasma uric acid concentrations of normal rats	268
Appendix 3.12	Effect of oral <i>P. niruri</i> methanol extract (PN MeOH, 200 mg/kg once daily) on plasma uric acid concentrations of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	268
Appendix 3.13	Effect of oral methanol extracts of <i>P. niruri</i> (PN MeOH, 100 – 1000 mg/kg) and standard drugs given once daily on plasma uric acid concentrations of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	269
Appendix 3.14	Effect of single intraperitoneal administration of <i>P. niruri</i> methanol extract (PN MeOH) and fractions (Fr 1, 2, 3 and 4) each at 50 mg/kg on plasma uric acid concentrations of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	270
Appendix 3.15	Effect of single intraperitoneal administration of sub-fractions of fraction 4 (50 mg/kg) on plasma uric acid concentrations of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	270
Appendix 3.16	Effect of single intraperitoneal administration of <i>P. niruri</i> lignans and clinically used drugs on plasma uric acid concentrations of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	271
Appendix 3.17	Dose-response relationship of phyllanthin antihyperuricemic effect in hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	271
Appendix 4.1	Repeatability of the <i>in vitro</i> XO assay using allopurinol as standard	272
Appendix 4.2	<i>In vitro</i> XO inhibitory activity of <i>P. niruri</i> methanol extract, fractions, sub-fractions and lignans	273
Appendix 4.3	Mean IC <sub>50</sub> values of <i>in vitro</i> xanthine oxidase inhibition of <i>P. niruri</i> methanol extract, fractions, sub-fractions and lignans	274
Appendix 4.4	Effects of protein concentration of cytosolic fraction, incubation period and xanthine concentration in the assay mixture on uric acid concentrations	276
Appendix 4.5	Liver weight and protein content of cytosolic fraction of the liver homogenates of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	277

	treated with oral methanol extracts of <i>P. niruri</i> (PN MeOH, 100 – 1000 mg/kg) and allopurinol (50 mg/kg) once daily	
Appendix 4.6	Effect of oral methanol extracts of <i>P. niruri</i> (PN MeOH, 100 – 1000 mg/kg) and allopurinol (50 mg/kg) given once daily on liver XO activity of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	278
Appendix 4.7	Liver weight and protein content of cytosolic fraction of the liver homogenates of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg) treated with single intraperitoneal administration of methanol extract of <i>P. niruri</i> (PN MeOH) and its fractions (Fr 1, 2, 3 and 4) and sub-fractions of fraction 4 each at 50 mg/kg	279
Appendix 4.8	Effect of single intraperitoneal administration of methanol extract of <i>P. niruri</i> (PN MeOH) and its fractions (Fr 1, 2, 3 and 4) and sub-fractions of fraction 4 each at 50 mg/kg on liver XO activity of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	280
Appendix 4.9	Liver weight and protein content of cytosolic fraction of the liver homogenates of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg) treated with single intraperitoneal administration of lignans of <i>P. niruri</i> and allopurinol	281
Appendix 4.10	Effect of single intraperitoneal administration of lignans of <i>P. niruri</i> and allopurinol on liver XO activity of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	281
Appendix 5.1	Effect of hyperuricemia induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg) on daily water intake	282
Appendix 5.2	Effect of hyperuricemia induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg) on daily urine output	282
Appendix 5.3	Effect of oral methanol extracts of <i>P. niruri</i> (PN MeOH, 100 – 1000 mg/kg) and clinically used drugs given once daily on daily water intake of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	283
Appendix 5.4	Effect of oral methanol extracts of <i>P. niruri</i> (PN MeOH, 100 – 1000 mg/kg) and clinically used drugs given once daily on daily urine output of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	284
Appendix 5.5	Effect of single intraperitoneal administration of methanol extract of <i>P. niruri</i> (PN MeOH) and its fractions (Fr 1, 2, 3 and 4) each at 50 mg/kg on water intake of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	285
Appendix 5.6	Effect of single intraperitoneal administration of sub-fractions of fraction 4 (50 mg/kg) on water intake of hyperuricemic rats	285

	induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	
Appendix 5.7	Effect of single intraperitoneal administration of lignans of <i>P. niruri</i> and clinically used drugs on water intake of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	286
Appendix 5.8	Effect of single intraperitoneal administration of phyllanthin on water intake of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	286
Appendix 5.9	Effect of single intraperitoneal administration of methanol extract of <i>P. niruri</i> (PN MeOH) and its fractions (Fr 1, 2, 3 and 4) each at 50 mg/kg on urine output of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	287
Appendix 5.10	Effect of single intraperitoneal administration of sub-fractions of fraction 4 (50 mg/kg) on urine output of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	287
Appendix 5.11	Effect of single intraperitoneal administration of lignans of <i>P. niruri</i> and clinically used drugs on urine output of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	288
Appendix 5.12	Effect of single intraperitoneal administration of phyllanthin on urine output of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	288
Appendix 5.13	Effect of hyperuricemia induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg) on daily urinary excretion of uric acid	289
Appendix 5.14	Effect of oral methanol extracts of <i>P. niruri</i> (PN MeOH, 100 – 1000 mg/kg) and clinically used drugs given once daily on daily urinary excretion of uric acid of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	290
Appendix 5.14a	Effect of oral methanol extracts of <i>P. niruri</i> (PN MeOH, 100 – 1000 mg/kg) and clinically used drugs given once daily on fold increase in urinary excretion of uric acid compared to normal or hyperuricemic control rats	291
Appendix 5.15	Effect of oral methanol extracts of <i>P. niruri</i> (PN MeOH, 100 -1000 mg/kg) and clinically used drugs given once daily on cumulative urinary excretion of uric acid of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	292
Appendix 5.16	Effect of single intraperitoneal administration of methanol extract of <i>P. niruri</i> (PN MeOH) and its fractions (Fr 1, 2, 3 and 4) each at 50 mg/kg on urinary excretion of uric acid of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	293

Appendix 5.17	Effect of single intraperitoneal administration of sub-fractions of fraction 4 (50 mg/kg) on urinary excretion of uric acid of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	293
Appendix 5.18	Effect of single intraperitoneal administration of lignans of <i>P. niruri</i> and clinically used drugs on urinary excretion of uric acid of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	294
Appendix 5.19	Effect of single intraperitoneal administration of phyllanthin on urinary excretion of uric acid of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	294
Appendix 5.20	Effect of single intraperitoneal administration of methanol extract of <i>P. niruri</i> (PN MeOH) and its fractions (Fr 1, 2, 3 and 4) each at 50 mg/kg on urinary uric acid clearance of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	295
Appendix 5.21	Effect of single intraperitoneal administration of sub-fractions of fraction 4 (50 mg/kg) on urinary uric acid clearance of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	295
Appendix 5.22	Effect of single intraperitoneal administration of lignans of <i>P. niruri</i> and clinically used drugs on urinary uric acid clearance of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	296
Appendix 5.23	Effect of single intraperitoneal administration of phyllanthin on urinary uric acid clearance of hyperuricemic rats induced by oral uric acid (1 g/kg) and intraperitoneal potassium oxonate (200 mg/kg)	296
Appendix 5.24	Effect of single intraperitoneal administration of clinically used drugs and phyllanthin on urinary excretion of uric acid in the pyrazinamide administered hyperuricemic rats	297
Appendix 6.1	Plasma concentration-time profile of phyllanthin ( <b>PN 1</b> ) after intravenous administration of 5 mg/kg of lignan rich <i>P. niruri</i> extract	298
Appendix 6.2	Plasma concentration-time profile of hypophyllanthin ( <b>PN 2</b> ) after intravenous administration of 5 mg/kg of lignan rich <i>P. niruri</i> extract	299
Appendix 6.3	Plasma concentration-time profile of phyltetralin ( <b>PN 3</b> ) after intravenous administration of 5 mg/kg of lignan rich <i>P. niruri</i> extract	300
Appendix 6.4	Plasma concentration-time profile of niranthin ( <b>PN 4</b> ) after intravenous administration of 5 mg/kg of lignan rich <i>P. niruri</i> extract	301
Appendix 6.5	Plasma concentration-time profile of phyllanthin ( <b>PN 1</b> ) after oral administration of 50 mg/kg of lignan rich <i>P. niruri</i> extract	302

Appendix 6.6	Plasma concentration-time profile of hypophyllanthin ( <b>PN 2</b> ) after oral administration of 50 mg/kg of lignan rich <i>P. niruri</i> extract	303
Appendix 6.7	Plasma concentration-time profile of phyltetralin ( <b>PN 3</b> ) after oral administration of 50 mg/kg of lignan rich <i>P. niruri</i> extract	304
Appendix 6.8	Plasma concentration-time profile of niranthin ( <b>PN 4</b> ) after oral administration of 50 mg/kg of lignan rich <i>P. niruri</i> extract	305

## LIST OF ABBREVIATIONS

Abs	absorbance
ANOVA	analysis of variance
AUC <sub>0→∞</sub>	area under plasma concentration-time curve
BBM	brush border membrane
<sup>13</sup> C	carbon
C <sub>max</sub>	peak concentration
CL	clearance
COSY	correlation spectroscopy
CP-1	cardiac puncture - sampling interval 1 day
CP-3	cardiac puncture - sampling interval 3 day
CP-5	cardiac puncture - sampling interval 5 day
CP-7	cardiac puncture - sampling interval 7 day
CV	coefficient of variation
D.B.E	double bond equivalents
DEPT	distortionless enhancement by polarization transfer
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ED <sub>50</sub>	50 % effective dose
ECF	extracellular fluid
EDTA	ethylenediamine tetraacetic acid
F	absolute oral bioavailability
FAD	flavin adenine dinucleotide
Fe/S	iron-sulphur
FTIR	fourier transformed infra red
GC	gas chromatography
GCMS	gas chromatography-mass spectrophotometer
<sup>1</sup> H	proton
HBeAg	hepatitis B virus envelope antigen
HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
HMBC	heteronuclear multiple-bond correlation
HPLC	high-performance liquid chromatography
HPLC-CEA	high-performance liquid chromatography-coulometric electrode array
HPLC-DAD	high-performance liquid chromatography-diode array detector
HPLC-EC	high-performance liquid chromatography-electrochemical
HPLC-MS	high-performance liquid chromatography-mass spectrophotometer
HPLC-UV	high-performance liquid chromatography-ultra violet
HPTLC	high-performance thin layer chromatography
hr	hour
HSQC	heteronuclear single-quantum correlation
Hz	hertz
IC <sub>50</sub>	50 % inhibitory concentration
i.d.	internal diameter
IR	infra red
<i>J</i>	coupling constant
KBr	potassium bromide
k <sub>e</sub>	elimination rate constant



LD <sub>50</sub>	50 % lethal dose
LOD	limits of detection
LOQ	limits of quantification
MS	mass spectrometer
NAD	nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
PN	<i>Phyllanthus niruri</i>
PN MeOH	<i>Phyllanthus niruri</i> methanol extract
<b>PN 1</b>	phyllanthin
<b>PN 2</b>	hypophyllanthin
<b>PN 3</b>	phyltetralin
<b>PN 4</b>	niranthin
PRPP	5-phosphoribosyl-1-pyrophosphate
PTFE	polytetrafluoroethylene
RBC	red blood cells
$r^2$	coefficient of determination
R <sub>f</sub>	retention factor
ROESY	rotating frame Overhauser effect spectroscopy
S1	first segment
S2	second segment
S3	third segment
SEM	standard error of mean
S/N	signal to noise
t <sub>1/2</sub>	biological half-life
T <sub>max</sub>	time to reach peak concentration
TLC	thin layer chromatography
TMS	tetramethylsilane
UV	ultraviolet
V <sub>d</sub>	volume of distribution
v/v	volume over volume
w/w	weight over weight
XD	xanthine dehydrogenase
XO	xanthine oxidase
XOR	xanthine oxidoreductase

**KAJIAN FITOKIMIA, FARMAKOLOGI DAN FARMAKOKINETIK LIGNAN  
*PHYLLANTHUS NIRURI* LINN. SEBAGAI AGEN ANTIHIPERURISEMIK BERPOTENSI**

**ABSTRAK**

Ekstrak metanol dari daun *Phyllanthus niruri* L. menunjukkan aktiviti antihiperurisemik oral yang bergantung dos di dalam tikus hiperurisemia yang diaruh dengan kalium oksonat dan asid urik. Fraksinasi ekstrak tersebut melalui kromatografi resin memberi fraksi kurang polar yang menunjukkan penurunan tertinggi dalam asid urik plasma. Penulenan seterusnya fraksi itu berdasarkan aktiviti antihiperurisemik menghasilkan empat lignan, filantin, hipofilantin, filtetralin dan nirantin. Struktur kimia sebatian-sebatian ini dielusidasi dan dikenalpasti melalui perbandingan takat lebur, spektra resonans magnetik nukleus, ultraungu, inframerah dan jisim mereka dengan nilai yang dilaporkan sebelumnya. Filantin menunjukkan kesan antihiperurisemik yang tertinggi bila dibanding dengan lignan yang lain. Pada 20 mg/kg, filantin menurunkan asid urik plasma ke tahap yang serupa dengan 10 mg/kg benzbromaron dan allopurinol. Akan tetapi, filantin tidak berupaya untuk menurunkan secara signifikan asid urik plasma ke tahap lebih rendah daripada tahap tikus normourisemik walaupun pada dos tertinggi, 20mg/kg.

Mekanisme bagi aktiviti antihiperurisemik *P. niruri* dan lignannya telah dikaji menggunakan esei enzim xantina oksidase dan kajian urikosurik. Ekstrak metanol *P. niruri* menunjukkan aktiviti perencatan xantina oksidase *in vitro* dan *in vivo* yang sederhana dengan masing-masing IC<sub>50</sub> sebanyak 39.39 µg/ml dan ED<sub>50</sub> sebanyak 157.91 mg/kg. Akan tetapi, lignan tidak menunjukkan perencatan xantina oksidase *in vitro* dan menunjukkan aktiviti perencatan *in vivo* yang agak lemah pada 10 mg/kg. Sebaliknya, rawatan oral ekstrak metanol *P. niruri* (100 – 1000 mg/kg) menunjukkan aktiviti urikosurik dengan peningkatan sebanyak 1.10 hingga 7.14 ganda dalam ekskresi asid urik urin berbanding tikus

hiperurisemik yang tidak menerima sebarang rawatan. Lignan, filantin, hipofilantin dan filtetralin pada 10 mg/kg turut menunjukkan ekskresi dan klearans asid urik lebih tinggi sehingga 2.51 dan 11.0 ganda, masing-masing lebih tinggi berbanding tikus hiperurisemik kawalan. Filantin menunjukkan potensi yang serupa dengan benzbromaron dan probenesid pada dos 10 mg/kg dan peningkatan dalam ekskresi serta klearans asid urik urin bergantung pada dos. Berdasarkan penemuan kajian ini, kesan antihiperurisemik ekstrak metanol *P. niruri* mungkin disebabkan terutamanya oleh kesan urikosurik dan sebahagian kecil melalui perencatan xantina oksidase, manakala kesan antihiperurisemik lignan diakibatkan oleh kesan urikosuriknya. Pemberian bersama pirazinamida dan benzbromaron atau filantin kepada tikus hiperurisemik menunjukkan penekanan signifikan dalam aktiviti urikosurik mereka tidak seperti tikus yang diberi pirazinamida bersama probenesid. Filantin menunjukkan aktiviti urikosurik menyerupai benzbromaron, mungkin melalui perencatan penyerapan semula pada tapak post-perembesan tubul berlingkar proksimal.

Kaedah analisis baru yang mudah dan sensitif menggunakan kromatografi cecair prestasi tinggi dengan pengesanan pendarfluor telah dibangunkan untuk penentuan empat lignan yang telah dipencilkan. Kaedah ini mempunyai had pengesanan untuk filantin, hipofilantin, filtetralin dan nirantin sebanyak 80, 8, 80 dan 40 kali, masing-masing lebih sensitif berbanding nilai yang diperolehi dengan kaedah pengesanan ultraungu. Kaedah tersebut telah berjaya diaplikasi bagi kuantifikasi lignan dalam sampel pokok *P. niruri* serta dalam kajian farmakokinetik dan biokeperolehan lignan dalam tikus. Kandungan lignan tertinggi didapati pada daun, diikuti buah, dahan dan batang manakala bahagian akar mempunyai kandungan lignan paling rendah. Selepas pemberian intravena kepada tikus, lignan dikeluarkan secara perlahan dari badan dengan nilai klearans min yang kecil serta nilai separuh hayat min antara 3.35 hingga 4.40 jam. Kepekatan plasma puncak berikutan

pemberian oral dicapai selepas 1 jam. Akan tetapi, penyerapan lignan tersebut tidak lengkap dengan nilai kiraan bagi biokeperolehan oral mutlak sebanyak 0.62, 1.52, 4.01 dan 2.66 % masing-masing untuk filantin, hipofilantin, filtetralin dan nirantin.

**PHYTOCHEMICAL, PHARMACOLOGICAL AND PHARMACOKINETIC STUDIES OF  
*PHYLLANTHUS NIRURI* LINN. LIGNANS AS POTENTIAL ANTIHYPERURICEMIC AGENTS**

**ABSTRACT**

The methanol extract from the leaves of *Phyllanthus niruri* L. showed dose-dependent oral antihyperuricemic activity in potassium oxonate- and uric acid-induced hyperuricemic rats. Fractionation of the extract by resin chromatography gave a less polar fraction which exhibited the highest reduction of plasma uric acid. Further antihyperuricemic-guided purification of the fraction afforded four lignans, phyllanthin, hypophyllanthin, phylltetralin and niranthin. Their structures were elucidated and confirmed by comparison of their physico-chemical properties, nuclear magnetic resonance, ultraviolet, infrared and mass spectra with those reported previously. Phyllanthin showed the highest dose-dependent antihyperuricemic effect when compared with that of the other lignans. At 20 mg/kg, phyllanthin decreased the plasma uric acid to the same extent as 10 mg/kg of benzbromarone and allopurinol. However, phyllanthin was not able to significantly reduce the plasma uric acid level below that of normouricemic rats even at the highest dose of 20 mg/kg.

The mechanisms of antihyperuricemic activity of *P. niruri* and its lignan constituents were investigated using the xanthine oxidase enzyme assay and uricosuric studies. *P. niruri* methanol extract exhibited moderate *in vitro* and *in vivo* xanthine oxidase inhibitory activity with an IC<sub>50</sub> of 39.39 µg/ml and an ED<sub>50</sub> of 157.91 mg/kg, respectively. However, the lignans did not display xanthine oxidase inhibition *in vitro* and showed a relatively weak *in vivo* inhibitory activity at 10 mg/kg. On the other hand, oral treatment with *P. niruri* methanol extracts (100 - 1000 mg/kg) showed uricosuric activity of 1.10 to 7.14 folds increase in urinary uric acid excretion when compared to the non-treated hyperuricemic rats. Likewise, the lignans, phyllanthin, hypophyllanthin and phylltetralin at 10 mg/kg

exhibited up to 2.51 and 11.0 fold higher in urinary uric acid excretion and clearance, respectively compared to the hyperuricemic control rats. Phyllanthin at 10 mg/kg increased the urinary uric acid excretion and clearance in a dose-dependent manner and exhibited similar potency with those of benzbromarone and probenecid. Based on the findings of the present study, it seems very likely that the antihyperuricemic effect of *P. niruri* methanol extract may be attributable mainly to its uricosuric action and partly through xanthine oxidase inhibition, while the antihyperuricemic effect of the lignans was attributable to their uricosuric action. The co-administration of pyrazinamide with benzbromarone or phyllanthin to the hyperuricemic rats exhibited a significant depression of their uricosuric activity unlike those rats given pyrazinamide and probenecid. Phyllanthin showed uricosuric activity resembling that of benzbromarone, probably by the inhibition of reabsorption at the post-secretory site of the proximal convoluted tubule.

A new, simple and sensitive analytical method using HPLC with fluorescence detection was developed for the simultaneous determination of the four isolated lignans. The method recorded limits of detection for phyllanthin, hypophyllanthin, phyltetralin and niranthin of 80, 8, 80 and 40 times, respectively more sensitive than those derived from the HPLC-UV detection method. The method was successfully applied for quantification of the lignans in *P. niruri* plant samples and pharmacokinetic and bioavailability studies of the lignans in rats. The highest amount of lignans was found in the leaves followed by the fruits, branches and stem whilst the roots have the least amount of lignans. Following intravenous administration to the rats, the lignans were eliminated slowly from the body with a small mean clearance value and a mean half-life of 3.35 to 4.40 hr. Their peak plasma concentration upon oral administration was achieved after 1 hr. However, their absorption was incomplete with a calculated absolute oral bioavailability of 0.62, 1.52, 4.01 and 2.66 % for phyllanthin, hypophyllanthin, phyltetralin and niranthin, respectively.

## CHAPTER ONE

### INTRODUCTION

#### 1.1 Hyperuricemia: A global scenario and its management

Hyperuricemia or high level of blood uric acid is a common biochemical abnormality encountered in clinical practice. About 10 % of adults are documented to have hyperuricemia at least once in their lifetime (Dincer *et al.*, 2002). The prevalence of hyperuricemia in the general population has been reported to be from 5 to 30 %, although it is higher in some ethnic groups (Vazquez-Mellado *et al.*, 2004). For instance, Klemp *et al.* (1997) reported that hyperuricemia was more commonly found in Maori men (27.1 %) than in European men (9.4 %) while Chou and Lai (1998) reported that the prevalence of hyperuricemia was 41.4 % among Taiwan aborigines. Meanwhile, Li *et al.* (1997) found that the prevalence of hyperuricemia were higher in urban than rural population of Beijing. Hyperuricemia seems to be more prevalent worldwide, probably due to improvements in standard of living, increasing longevity and the usage of certain drugs such as salicylate and pyrazinamide. This has resulted in significant morbidity and increase in costs of the health care system (Vazquez-Mellado *et al.*, 2004; Kim *et al.*, 2003; Klemp *et al.*, 1997).

Hyperuricemia is often associated with a number of human diseases (Ruilope and Garcia-Puig, 2001). Classically, hyperuricemia is a major risk factor for gout, urolithiasis and uric acid nephropathy. In addition, it has also been linked with other diseases such as diabetes mellitus, preeclampsia, hypertension, vascular diseases and stroke or clinical symptoms such as lipid abnormalities, insulin resistance and obesity (Kim *et al.*, 2003; Dincer *et al.*, 2002; Ghei *et al.*, 2002; Li *et al.*, 1997; Campion *et al.*, 1987). These complications develop depending on both the level and duration of hyperuricemia.

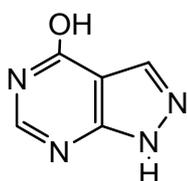
Primary intervention in patients with symptomatic hyperuricemia or its associated gout include patient education, lifestyle changes and pharmacological therapy. Lifestyle modifications such as weight reduction, decreased alcohol consumption and dietary purine intake may help to decrease blood uric acid. However, many patients will still need medication to control their hyperuricemia (Kong *et al.*, 2004; Wright and Pinto, 2003; Liote, 2003; Wood, 1999). Despite a long history of hyperuricemia and gout, there are only a limited number of drugs currently used in clinical practice and they belong to two classes, the xanthine oxidase (XO) inhibitors and the uricosuric agents. An example of clinically used XO inhibitor is allopurinol, while uricosuric agents include probenecid and benzbromarone.

XO inhibitors reduce the blood uric acid level by inhibition of XO enzyme that is responsible for the formation of uric acid from purines. Consequence to the inhibition, the blood and urinary concentrations of uric acid are reduced and there is a simultaneous increase in the excretion of the more soluble uric acid precursors, xanthine and hypoxanthine. Patients who are categorized as overproducers of uric acid or those with renal insufficiency are best treated with XO inhibitors (Wright and Pinto, 2003; Wood, 1999).

Allopurinol was developed in 1956 for use as an adjuvant in chemotherapy, however, it was found to possess the ability to lower serum uric acid level (Khoo and Leow, 2000). Allopurinol (**1**) (4-hydroxypyrazolo [3,4-d] pyrimidine) is a potent inhibitor and substrate for XO. It is the only clinically available drug belonging to the XO inhibitor group that is most widely prescribed for the management of hyperuricemia and gout (Dincer *et al.*, 2002; Khoo and Leow, 2000). It has been used for the therapy of both primary hyperuricemia and gout or secondary hyperuricemia that is due to haematological disorders or antineoplastic therapy. A response to allopurinol is seen about two days after initiation of therapy and is maximal after about seven to ten days (Wood, 1999).



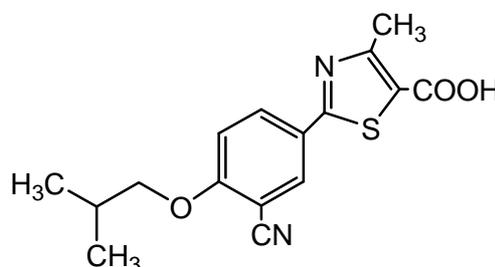
Allopurinol itself is metabolized by the XO enzyme, to its active metabolite oxypurinol (2). Although the half-life of allopurinol is 1 to 3 hours, its metabolite has a longer half-life ranging from 18 to 33 hours, thus prolonging the therapeutic effectiveness of allopurinol administered as a single dose (Spector, 1977). Common adverse effects associated with allopurinol administration include a variety of skin rashes, hypersensitivity, gastrointestinal upset, hepatotoxicity, hepatitis and fever. Approximately 2 to 10 % of patients, especially the elderly with renal impairment, have developed a pruritic erythematous rash, which prevented further administration of allopurinol (Fam, 2001; Khoo and Leow, 2000). A more severe and life threatening hypersensitivity syndrome in which patients develop toxic epidermal necrolysis, fever, hepatitis, eosinophilia and deterioration of renal function has also been reported in approximately 0.4 % of patients (Zhu *et al.*, 2004; Dincer *et al.*, 2002; Kong *et al.*, 2002; Fam, 2001; Khoo and Leow, 2000; Wood, 1999; Osada *et al.*, 1993). The use of allopurinol has also led to the appearance of allopurinol allergic-patients throughout the world (Fam, 2001).



1 allopurinol



2 oxypurinol

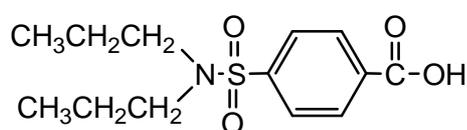


3 febuxostat

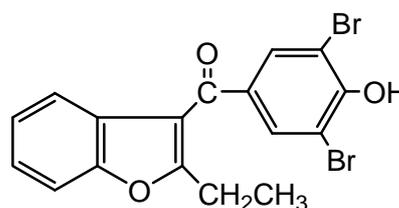
Recently, febuxostat (3) [2-(3-cyano-4-isobutoxyphenyl)-4-methylthiazole-5-carboxylic acid], a selective inhibitor of XO was developed in Japan. Febuxostat may become an alternative effective drug to allopurinol for use in the treatment of hyperuricemia and gout (Takano *et al.*, 2005; Komoriya *et al.*, 1993; Osada *et al.*, 1993). Clinical trials on the efficacy and tolerability of febuxostat in normal subjects and patients with hyperuricemia or gout, have found that the drug significantly reduced the serum uric acid level in a dose-dependent manner at a lower dose than allopurinol (Bruce, 2006;

Becker *et al.*, 2005a; Becker *et al.*, 2005b). It was generally well tolerated; the most common adverse effects were liver function abnormalities, diarrhea, headache, nausea, vomiting, abdominal pain, arthralgias and musculoskeletal symptoms (Bruce, 2006; Pohar and Murphy, 2006).

In contrast to the XO inhibitors, drugs belonging to the uricosuric group reduce the blood uric acid level by increasing its excretion. This agent competes with uric acid for the transport sites at the proximal tubules. Patients who are categorized as underexcretors of uric acid are the best candidates for uricosuric therapy (Perez-Ruiz *et al.*, 1998). Uricosuric drugs may also be used in patients who are intolerant of allopurinol but they are relatively ineffective in patients with poor renal function. The greatest potential risk of therapy with uricosuric drugs is the deposition of uric acid in the collecting tubules (Wright and Pinto, 2003; Wood, 1999).



**4** probenecid



**5** benzbromarone

Probenecid (**4**) [*p*-(dipropylsulfamoyl) benzoic acid] was initially developed in search for a drug to sustain blood level of penicillin by interfering with its renal excretion. In addition, it also inhibits the reabsorption of uric acid at the proximal tubule, thereby causing an increase in uric acid excretion. However, probenecid has a so-called “paradoxical effect”, whereby at therapeutic doses, it increases uric acid excretion while at much lower doses it decreases uric acid excretion (Dan and Koga, 1990; Frankfurt and Weinman, 1977).

Another uricosuric agent, benzbromarone (**5**) [3-(3,5-dibromo-4-hydroxybenzoyl)-2 ethylbenzofuran] also causes an increase in excretion of uric acid. However, the

paradoxical effect observed with probenecid is absent with benzbromarone. XO inhibition by benzbromarone was shown in some animal studies; however it does not inhibit XO in humans (Heel *et al.*, 1977). Benzbromarone is conjugated in the liver and excreted to the bile. Although it is effective in patients with renal insufficiency, it possesses a risk of severe hepatotoxicity (Dincer *et al.*, 2002; Fam, 2001; Perez-Ruiz *et al.*, 1998).

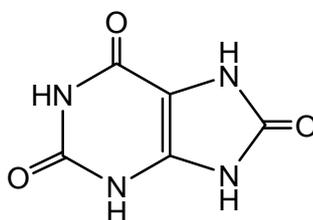
Besides the two classes of drugs, other pharmacological agents may also be used for the treatment of hyperuricemia and gout. Losartan and fenofibrate, in addition to their principal pharmacological activities, have blood uric acid lowering effect. Both of them diminish uric acid reabsorption at the proximal tubule and increase its excretion (Vazquez-Mellado *et al.*, 2004).

Generally in most patients, allopurinol or any of the uricosuric drugs will allow the achievement and maintenance of normouricemia. However in patients with comorbidities such as renal insufficiency, renal calculi, transplantation or allopurinol allergy, treatment options are narrow and could complicate the management of symptomatic hyperuricemia or gout (Kim *et al.*, 2003). Thus, a continuous development of novel antihyperuricemic drugs would be of great interest.

## 1.2 Literature review

### 1.2.1 Biochemistry and physiology of uric acid

Uric acid (2,6,8-trioxypurine) is a weak organic acid, due to the ionisable hydrogen at position 3 with an ionization constant of 5.75. This physicochemical property is an important determinant of the concentration and form of uric acid in the circulation or tissues. At pH 7.4 such as in blood or synovial fluids, about 98 % of uric acid is ionized as monosodium urate whereas at lower pH such in the urine, it exists mostly in free form (Ruilope and Garcia-puig, 2001).



6 Uric acid

#### 1.2.1.1 Biosynthesis and regulation of uric acid formation

The pool of uric acid in human is a balance between endogenous or exogenous sources of uric acid and degradation or elimination of uric acid. The exogenous sources for uric acid are the purine and purine precursors in the diets. Two endogenous sources contributing to the miscible pool of uric acid, firstly is the tissue catabolism via the breakdown of nucleic acids and nucleotides, and secondly is the *de novo* purine biosynthetic pathway (Newcombe, 1975).

The pathways for biosynthesis of purines and formation of uric acid are shown in Figure 1.1. 5-Phosphoribosyl-1-pyrophosphate (PRPP) is the starting compound for purine biosynthesis, which can also react with the preformed purine bases to form purine ribonucleotides directly by the so-called “salvage pathways” (Newcombe, 1975).

The first purine formed is inosinic acid that will be converted to free purine bases by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Part of the bases is reutilized through the salvage reaction with PRPP, and the remainder is degraded to free bases xanthine and hypoxanthine. Xanthine oxidoreductase (XOR) enzymes, convert both of these bases to uric acid (Seegmiller, 1976). They are known as the rate-limiting enzymes in purine catabolism.

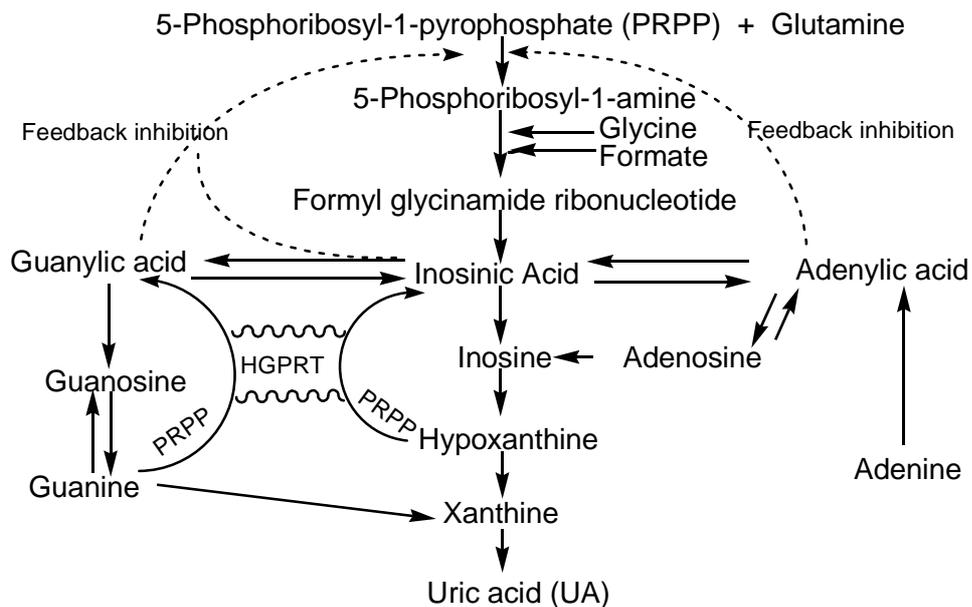


Figure 1.1 Pathways for biosynthesis of purines and the formation of uric acid (Seegmiller, 1976).

Xanthine oxidase (XO; EC 1.1.3.22) and xanthine dehydrogenase (XD; EC 1.1.1.204) are both members of the molybdenum hydroxylase flavoprotein family and often referred to as XOR (Pritsos, 2000). Structurally, XOR is a homodimer of 150-kDa subunits, with a N-terminal domain containing two iron-sulphur centres (Fe/S I and Fe/S II), a middle domain containing flavin adenine dinucleotide (FAD) site and a C-terminal domain containing a molybdenum cofactor and substrate binding site. Conversion of XD into XO form can be achieved by a variety of treatment, such as storage at -20 °C, adding proteolytic enzymes, organic solvents or thiol reagents and preincubation under anaerobic conditions (Delle-Corte and Stripe, 1972).

In mammals, the liver and intestine have the highest XOR activity (Pritsos, 2000; Krenitsky *et al.*, 1986). The primary structure, catalytic properties and cofactor requirements of XOR are highly conserved with a 90 % homology among rat, mouse and human XOR enzymes (Pritsos, 2000). The XOR enzymes catalyze the oxidation of hypoxanthine to xanthine and xanthine to uric acid. However, their mechanisms of action are different in that the XD reduces  $\text{NAD}^+$  (nicotinamide adenine dinucleotide) by a direct two-electron reduction whereas XO reduces molecular oxygen by a single electron. During the process, the substrates hypoxanthine and xanthine bind to the molybdenum site, and the electron acceptors  $\text{NAD}^+$  and  $\text{O}_2$  interact with the FAD cofactor (Pritsos, 2000; Fujimoto *et al.*, 2000, Mondal *et al.*, 2000).

### 1.2.1.2 Degradation of uric acid

Uric acid is formed mainly in the liver and only a small percentage (less than 5%) is bound to plasma proteins. Significant differences exist among the animals in the degradation of uric acid, whereby the lower forms of animal life possess a full complement of enzymes necessary for degrading uric acid completely into allantoin (**7**), allantoic acid (**8**) and urea (**9**) as shown in Figure 1.2 (Hitchings, 1978).

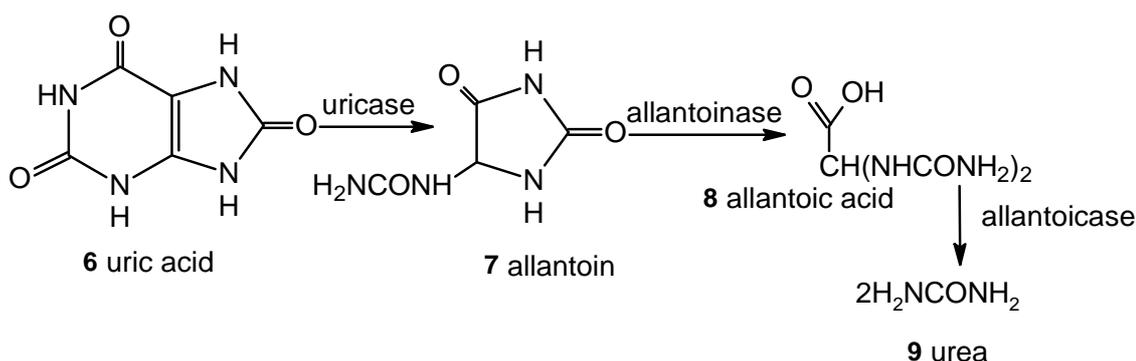


Figure 1.2 Enzymatic degradation of uric acid (Hitchings, 1978).

Most mammals possess the enzyme, uricase that catalyzes the degradation of uric acid to a more soluble allantoin (Ghei *et al.*, 2002; Ruilope and Garcia-Puig, 2001).

Only in man and great apes, uric acid remains as the end product of purine catabolism. Thus, humans have relatively higher level of blood uric acid (Wu *et al.*, 1992).

#### 1.2.1.3 *Uric acid disposal*

The kidney and gut are the main routes for the disposal of uric acid. Approximately 70 % of the uric acid is eliminated via the kidney while the remaining 30 % via the biliary and gastrointestinal system, where it undergoes degradation to allantoin by colonic bacteria. In individuals with renal insufficiency, the gastrointestinal track may be the major route of uric acid disposal. Mammals can be divided into two groups based on the net bidirectional transport of uric acid in the renal. Net reabsorption occurs in humans and other species such as cebus monkey, rats, mice and dogs whereas net secretion occurs in pigs and rabbits (Yamada *et al.*, 1999a; Roch-Ramel and Peters, 1978).

The renal mechanisms involved in the handling of uric acid are complex. Proximal convoluted tubules are the main sites of transtubular uric acid transport in humans as well as most animals. Renal handling of uric acid is considered to involve a four compartment model. Firstly, blood uric acid undergoes glomerular filtration followed by the second step, pre-secretory reabsorption at the first segment (S1) of the proximal tubule. The capacity of reabsorption at initial proximal tubule is large, where more than 95 % of the filtered uric acid will be reabsorbed, even in the presence of hyperuricemia (Liote 2003, Roch-Ramel and Peters, 1978). The third step involves uric acid secretion at the second segment (S2) followed by the fourth step, post-secretory reabsorption at the last segment (S3) of the proximal tubule (Itagaki *et al.*, 2005; Ghei *et al.*, 2002; Yamada *et al.*, 1999b; Steele, 1999). Based on the model, it has been reported that benzbromarone inhibits post-secretory reabsorption, while probenecid mainly inhibits post-secretory and partly inhibits pre-secretory reabsorption (Yamada *et al.*, 1999b; Dan *et al.*, 1990; Levinson and Sorenson, 1980; Heel *et al.*, 1977).

Following glomerulus filtration, uric acid enters the proximal tubule in its anionic form and due to its hydrophilic nature it hardly permeates the proximal tubular cells. At brush border membrane (BBM) of the proximal tubular cells, uric acid is transported by two distinct mechanisms, an anion exchanger and a voltage-dependent mechanism. Anion exchangers allow bidirectional transport and have been suggested to play a major role in uric acid reabsorption (Itagaki *et al.*, 2005; Enomoto *et al.*, 2002; Roch-Ramel and Guisan, 1999; Roch-Ramel *et al.*, 1994; Guggino *et al.*, 1983). The anion exchangers accept multiple monovalent organic anion, aliphatic or aromatic as well as chloride, bicarbonate and hydroxyl ions (Guggino *et al.*, 1983; Kahn *et al.*, 1983). Some of the endogenous compounds and drugs that may interfere with tubular transport of uric acid are listed in Table 1.1 (Roch-Ramel and Guisan, 1999).

Table 1.1: Substances that alter the renal tubular handling of uric acid (Roch-Ramel and Guisan, 1999)

<i>Substances that decrease uric acid excretion</i>	<i>Substances that increase uric acid excretion</i>
Lactate	Probenecid
Acetoacetate	Sulfinpyrazone
$\beta$ -Hydroxybutyrate	Benzbromarone
Nicotinate	Losartan (antihypertensive drug)
Pyrazinamide/pyrazinoate	Tienilic acid (diuretic)

Potential-sensitive transport system plays an important role in the efflux of organic anions including uric acid across BBM in rats, because the intracellular compartment has a more negative electrical potential than that of the luminal fluid in the proximal tubules (Itagaki *et al.*, 2005; Roch-Ramel *et al.*, 1994). Extracellular fluid volume (ECF) is another factor that influences the excretion of uric acid. Expansion of ECF will reduce the tubular reabsorption of uric acid. However the changes in the urine flow or pH have no effect in the excretion of uric acid (Steele, 1999).



### 1.2.2 Hyperuricemia

Hyperuricemia is defined as blood uric acid level of more than 7 mg/dl (420  $\mu$ mol/L) in men or more than 6 mg/dl (360  $\mu$ mol/L) in women (Vazquez-Mellado *et al.*, 2004; Kim *et al.*, 2003; Ruilope and Garcia-Puig, 2001). Ruilope and Garcia-Puig (2001) defined a blood uric acid level of more than 9 mg/dL as a severely hyperuricemic condition. Hyperuricemia results from overproduction or underexcretion of uric acid. About 80 to 90 % of the patients with hyperuricemia or gout are underexcretors of uric acid (Vazquez-Mellado *et al.*, 2004).

Table 1.2: Classification of hyperuricemia

1) Increased formation of uric acid	
Inherited enzyme defects	Hyperactivity of PRPP synthetase Decreased activity or deficiency of HGPRT
Disease states leading to purine overproduction	Myeloproliferative disorders Malignancies Hemolytic anaemia
Increased catabolism or decreased synthesis of adenosine triphosphate	Alcohol consumption Tissue hypoxia Excessive muscular exercise
Associated with drugs or dietary habits	Cytotoxic agents Fructose Excessive purine intake
2) Decreased renal clearance of uric acid	
Inherited defects of tubular function	-
Disease states leading to reduced uric acid clearance	Renal insufficiency Dehydration Acidosis (tissue hypoxia) Hyperparathyroidism Hypothyroidism
Associated with drugs	Diuretics (thiazide and loop) Ethanol Pyrazinamide Salicylates Cyclosporin

Genetic factors could be the major contributor to the high prevalence of hyperuricemia in some ethnic groups (Vazquez-Mellado *et al.*, 2004). Other factors which may

influence the blood uric acid concentration are age, sex, body weight, body surface area, body mass and socioeconomic status of an individual (Garcia-Puig *et al.*, 1986). Hyperuricemia can be classified as primary or secondary based on the underlying causative factors. Table 1.2 summarizes the pathophysiologic classification of hyperuricemic disorders and their respective underlying causes (Kim *et al.*, 2003; Ruilope and Garcia-puig, 2001; Nakanishi *et al.*, 1999; Li *et al.*, 1997).

#### 1.2.2.1 *Experimental hyperuricemia in rodents*

The presence of the enzyme uricase, is responsible for the lower plasma uric acid concentration observed in rodents. For example, the plasma uric acid concentration of normal rats ranges from 0.4 to 1.5 mg/dl (20 to 90  $\mu$ g/ml) (Roch-Ramel and Peters, 1978). Thus, to make the rodents more similar to man for studying hyperuricemia, the activity of uricase has to be reduced or eliminated. Experimentally, uricase activity in the liver can be suppressed either by uricase inhibitors, destroying a large part of the liver or by reducing the blood flow through the liver. By far, the most common method employed is by using uricase inhibitors such as salts of oxonic acid or analogs of xanthine and hypoxanthine such as 2,8-diazahypoxanthine, 2-azahypoxanthine, 8-azaxanthine and 8-azahypoxanthine (Newburger *et al.*, 1979; Roch-Ramel and Peters, 1978; Iwata *et al.*, 1973). Recently, transgenic hyperuricemic mice have been developed by removal of the uricase gene (Wu *et al.*, 1994; Bradely and Caskey, 1984).

Potassium oxonate is commonly used for induction of hyperuricemia in experimental animals, given either as injections or added to the diet. It has potent inhibitory effect on uricase enzyme but has comparatively insignificant effect on XO or on the transport of uric acid along the nephron (Mazzali *et al.*, 2002; De Rougement *et al.*, 1976; Iwata *et al.*, 1972; Johnson *et al.*, 1969; Fridovich, 1965). Potassium oxonate given as a single injection or as an injection followed by intravenous infusion causes

hyperuricemia that peaks at 1.5 to 2 hours and lasts for at least 5 hours (Kang *et al.*, 2002; Yonetani and Iwaki, 1983; Roch-Ramel and Peters, 1978). However, potassium oxonate is apparently metabolized or excreted rapidly, thus frequent injections are required to sustain uricase inhibitory activity. When potassium oxonate was given in the diet, blood uric acid level peaked at two weeks in the rats, then gradually decreased over the following 4 weeks, which may reflect enhanced extrarenal excretion and depressed production of uric acid (Kang *et al.*, 2002).

Most studies on animal hyperuricemia have employed simultaneous feeding of potassium oxonate (2 - 5 %) with other agents such as uric acid (1 - 3 %) or fructose to produce a higher and sustained level of plasma uric acid (Nakagawa *et al.*, 2003; Habu *et al.*, 2003; Mazzali *et al.*, 2001; Newburger *et al.*, 1979; Roch-Ramel and Peters, 1978; Starvic *et al.*, 1976; Johnson *et al.*, 1969). Fructose intake results in excess production of uric acid due to an increased degradation of nucleotides (Fields *et al.*, 1996; Fox and Kelley, 1972). However, addition of uric acid or fructose alone to the normal diet, produced no appreciable effect on plasma uric acid (Johnson *et al.*, 1969).

In oxonate- and uric acid-induced hyperuricemic animals, marked uricosuria was observed and the uric acid concentration in the renal tissue was considerably high causing intrarenal crystal deposition, interstitial nephritis and obstructive renal disease, as well as other impaired renal functions such as sodium, calcium and phosphate reabsorption and glomerular filtration (Habu *et al.*, 2003; Kang *et al.*, 2002; Mazzali *et al.*, 2002; Brown *et al.*, 1980; Roch-Ramel and Peters, 1978).

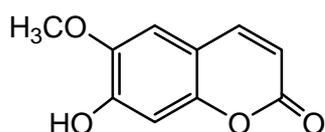
### 1.2.3 Role of medicinal plants and natural products in hyperuricemia

Traditional medicines are used in primary health care by about 75 to 80 % of the world population, especially in developing countries. The use of herbal medicine is also popular in some developed countries such as Germany, France and United States of America. The herbs and herbal extract sales in European Union and United States of America are estimated to be over US \$ 20 billion and \$ 8 billion annually, respectively, while the worldwide herbal medicine market is estimated to be \$ 30 - 60 billion (Kamboj, 2000). Hitherto, medicinal plants have been the source for a number of clinically important drugs such as morphine, atropine and digoxin and are excellent sources of lead compounds in the search for new drugs.

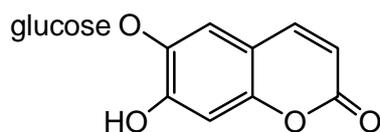
Diverse medicinal plants and natural products have been investigated as inhibitors of XO enzyme. Natural XO inhibitors from *in vitro* studies were reported from a variety of plants used as traditional herbal medicines such as *Coccinia grandis* and *Vitex negundo* in India (Umamaheswari *et al.*, 2007), *Chrysanthemum sinense* and *Tetracera scandens* in Vietnam (Nguyen *et al.*, 2004), *Cleodendrum floribundum*, *Eremophila maculata* and *Stemodia grossa* in Australia (Sweeney *et al.*, 2001), *Cinnamomum cassia*, *Chrysanthemum indicum* and *Lycopus europaeus* in China (Kong *et al.*, 2000a), *Larix laricina* in North America (Owen and Johns, 1999), *Hyptis obtusiflora* and *Hyptis lantanaefolia* in Panama (Gonzalez *et al.*, 1995) and *Hexachlamys edulis* and *Eugenia punicifolia* in Paraguay (Theduloz *et al.*, 1988). In general, the methanol extracts were found to be more active than the methanol-water or water extracts (Nguyen *et al.*, 2004; Kong *et al.*, 2000a). Chemical constituents from the flavonoids, polyphenols, tannins, xanthonenes, coumarins,  $\beta$ -carbolines and hydroxychalcones groups have been found to be potent inhibitors of XO (Owen and Johns, 1999; Gonzalez *et al.*, 1995; Hatano *et al.*, 1990; Hayashi *et al.*, 1988; Noro *et al.*, 1983).

Despite these findings, only a few of the natural products were evaluated for their antihyperuricemic activity *in vivo* using hyperuricemic animal models. Kong *et al.* (2004) reported that the extracts of a herbal mixture, Ermiao wan, containing phellodendri cortex and atractylodis rhizome, showed potent hypouricemic effect both in hyperuricemic and normal mice, whereas Zhu *et al.* (2004), showed that orally administered *Biota orientalis* extract reduced serum uric acid level of hyperuricemic mice. Similarly, Zhao *et al.* (2006) found that cassia oil extracted from *Cinnamomum cassia* reduced serum and hepatic uric acid level of hyperuricemic mice in a time- and dose-dependent manner partly by the inhibition of XO.

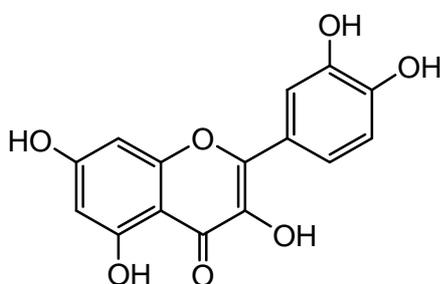
Scopoletin (**10**) isolated from *Erycibe obtusifolia* (Ding *et al.*, 2005), aesculin (**11**) from *Fraxinus rhynchophylla* (Kong *et al.*, 2002), quercetin (**12**) and rutin (**13**) from *Biota orientalis* (Zhu *et al.*, 2004) exhibited a potent antihyperuricemic effect after administration in hyperuricemic mice or rats. The effect of quercetin and rutin was mediated by inhibition of XO activity whereas, the effect of scopoletin was by both inhibition of XO activity and uricosuric pathway.



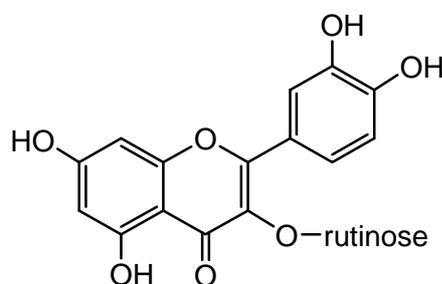
**10** scopoletin



**11** aesculin



**12** quercetin



**13** rutin

The search for new antihyperuricemic agents from medicinal plants and natural products is ongoing. Presently, the largest underexplored rainforest for the discovery of new drugs lies in tropical and subtropical regions of the world (Nguyen *et al.*, 2004). Malaysia being in this region is well known for its diverse nature and forest. Malaysians also use traditional and herbal remedies as an alternative choice for the prevention and treatment of diseases including gout and rheumatism. However, the validity of these claims has not been scientifically proven and therefore, is of interest to evaluate the antihyperuricemic effect of local Malaysian plants.

#### 1.2.4 *Phyllanthus niruri* L.

##### 1.2.4.1 *Botanical aspects and geographical distributions*

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Euphorbiales
Family	:	Euphorbiaceae
Genus	:	<i>Phyllanthus</i>
Species	:	<i>niruri</i>



Figure 1.3 *Phyllanthus niruri* L.; (A) whole plant (B) aerial part (C) leaves.

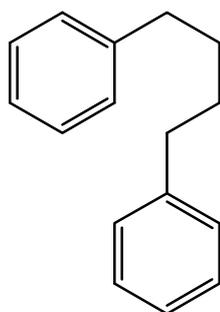
*Phyllanthus niruri* L., known locally as “dukong anak”, is found in most tropical and subtropical regions, commonly in fields, grasslands and forests. It is a small herb that grows up to 60 cm in height and can easily be differentiated from shrub species such as *P. pulcher* or *P. reticulatus*. The plant is quite herbaceous unlike *P. urinaria*, *P. simplex* or *P. maderaspatensis* which are woody at base (Unader *et al.*, 1995; Calixto *et al.*, 1998; Ridley, 1967). Its leaves are small and appear oblong with very short or absent petiole. The flowers are numerous, white to greenish in colour and minute, grouping at the axillary with a pedicel longer than *P. urinaria*. The fruit is a smooth surface and glabose capsule, in contrast to *P. urinaria* that has a echinate or warty capsule (Bee, 1964; Wiart, 2002).

#### 1.2.4.2 Chemical constituents of *Phyllanthus niruri* L.

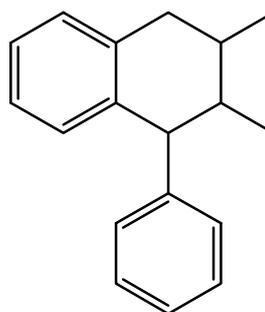
*P. niruri* has been the subject of much phytochemical studies since the mid 1960s. Different classes of organic compounds with various medical interest have been reported, the major being the lignans, tannins, polyphenols, alkaloids, flavonoids, terpenoids and steroids (Calixto *et al.*, 1998). The following chemical constituents have been isolated from *P. niruri*.

#### Lignans

Lignans isolated from *P. niruri* mostly belongs to two groups, the 1,4-diarylbutane and 1-aryltetralin though neolignans and lignans with other skeleton were also reported from this plant. The following lignans have been isolated from *P. niruri*:



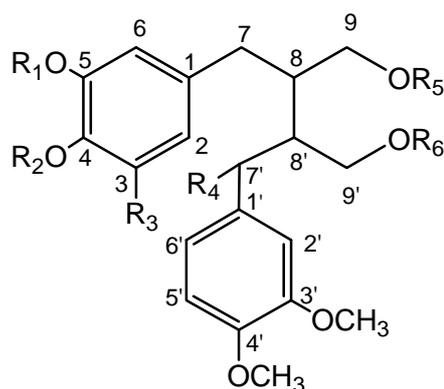
1,4-diarylbutane skeleton



1-aryltetralin skeleton

▪ *Diarylbutane lignans*

Phyllanthin (**14**) (Row and Srinivasalu, 1964), niranthin (**15**) (Anjaneyulu *et al.*, 1973), seco-isolariciresinol trimethyl ether (**16**), hydroxyniranthin (**17**) (Satyanarayana *et al.*, 1988), nirphyllin (**18**) (Singh *et al.*, 1989a), 2,3-desmethoxy seco-isolintetralin (**19**), 2,3-desmethoxy seco-isolintetralin diacetate (**20**), linnanthin (**21**), demethylenedioxy-niranthin (**22**) (Satyanarayana and Venkateswarlu, 1991).

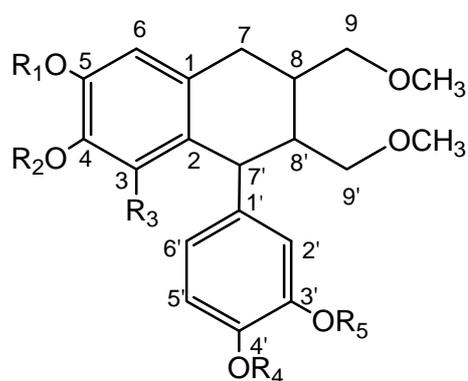
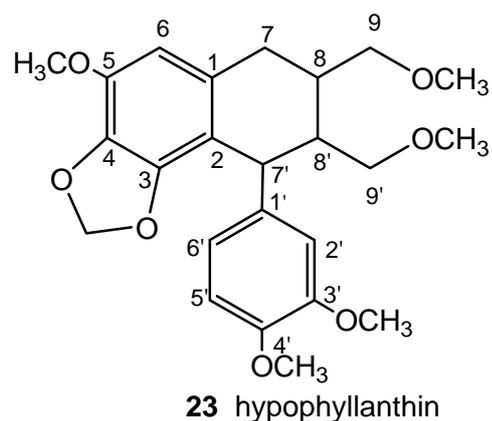
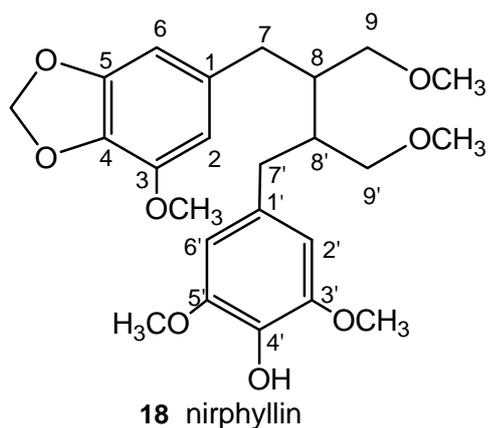


<b>14</b> phyllanthin	$R_1 = R_2 = \text{CH}_3$ $R_3 = R_4 = \text{H}$ $R_5 = R_6 = \text{CH}_3$
<b>15</b> niranthin	$R_1 + R_2 = \text{CH}_2$ $R_3 = \text{CH}_3$ $R_4 = \text{H}$ $R_5 = R_6 = \text{CH}_3$
<b>16</b> seco-isolariciresinol trimethyl ether	$R_1 = R_2 = \text{CH}_3$ $R_3 = R_4 = R_5 = \text{H}$ $R_6 = \text{CH}_3$
<b>17</b> hydroxyniranthin	$R_1 + R_2 = \text{CH}_2$ $R_3 = \text{CH}_3$ $R_4 = \text{OH}$ $R_5 = R_6 = \text{CH}_3$
<b>19</b> 2,3-desmethoxyseco-isolintetralin	$R_1 + R_2 = \text{CH}_2$ $R_3 = R_4 = R_5 = R_6 = \text{H}$
<b>20</b> 2,3-desmethoxyseco-isolintetralin diacetate	$R_1 + R_2 = \text{CH}_2$ $R_3 = R_4 = \text{H}$ $R_5 = R_6 = \text{COCH}_3$
<b>21</b> linnanthin	$R_1 = R_2 = R_3 = \text{CH}_3$ $R_4 = \text{H}$ $R_5 = R_6 = \text{CH}_3$
<b>22</b> demethylenedioxy-niranthin	$R_1 = R_2 = \text{H}$ $R_3 = \text{CH}_3$ $R_4 = \text{H}$ $R_5 = R_6 = \text{CH}_3$

▪ *Aryltetralin lignans*

Hypophyllanthin (**23**) (Row and Srinivasulu, 1964), nirtetralin (**24**), phyltetralin (**25**) (Anjaneyulu *et al.*, 1973), lintetralin (**26**) (Ward *et al.*, 1979), isolintetralin (**27**) (Huang *et al.*, 1992), neonirtetralin (**28**) (Wei *et al.*, 2002).





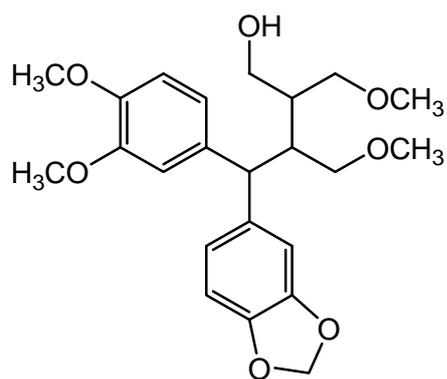
- 24** nirtetralin  $R_1 + R_2 = \text{CH}_2$   $R_3 = \text{OCH}_3$   $R_4 = R_5 = \text{CH}_3$
- 25** phlytetralin  $R_1 = R_2 = \text{CH}_3$   $R_3 = \text{H}$   $R_4 = R_5 = \text{CH}_3$
- 26** lintetralin  $R_1 = R_2 = \text{CH}_3$   $R_3 = \text{H}$   $R_4 + R_5 = \text{CH}_2$
- 27** isolintetralin  $R_1 + R_2 = \text{CH}_2$   $R_3 = \text{H}$   $R_4 = R_5 = \text{CH}_3$
- 28** neonirtetralin  $R_1 + R_2 = \text{CH}_2$   $R_3 = \text{OCH}_3$   $R_4 = R_5 = \text{CH}_3$

- *Other lignans*

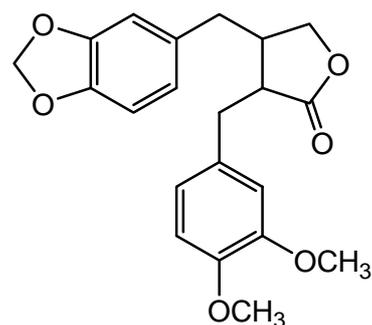
Seco-4-hydroxylintetralin (**29**), dibenzylbutyrolactone (**30**) (Satyanarayana *et al.*, 1988), hinokinin (**31**) (Huang *et al.*, 1992).

- *Neolignan*

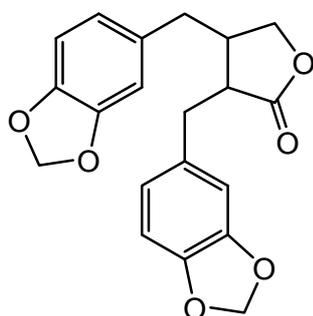
Phyllnirurin (**32**) (Singh *et al.*, 1989a).



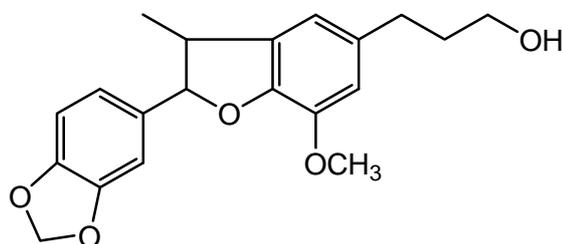
**29** seco-4-hydroxylintetralin



**30** dibenzylbutyrolactone



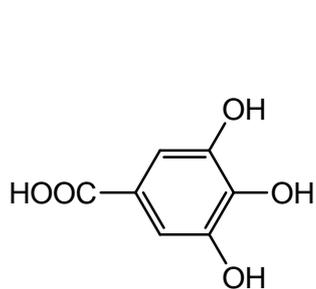
**31** hinokinin



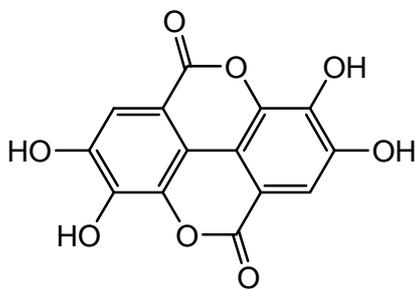
**32** phyllinurin

### Coumarins, tannins and related polyphenols

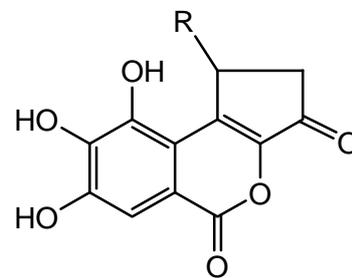
The following coumarins, tannins and polyphenols have been isolated from *P. niruri*: gallic acid (**33**), ellagic acid (**34**), brevifolin carboxylic acid (**35**), ethyl brevifolin carboxylate (**36**) (Shmizu *et al.*, 1989), methyl brevifolin carboxylate (**37**) (Iizuka *et al.*, 2006), geraniin (**38**) (Ueno *et al.*, 1988), corilagin (**39**) (Shmizu *et al.*, 1989), phyllanthusiin D (**40**) (Foo and Wong, 1992), amariin (**41**), amariinic acid (**42**), elaeocarpusin (**43**), geraniinic acid B (**44**), repandusinic acid (**45**), amarulone (**46**), furosin (**47**) (Foo, 1995), 1,6-digalloyl glucopyranoside (**48**) (Foo, 1993), catechin (**49**), epicatechin (**50**), galocatechin (**51**), epigallocatechin (**52**), epicatechin 3-O-gallate (**53**), epigallocatechin 3-O-gallate (**54**) (Ishimaru *et al.*, 1992).



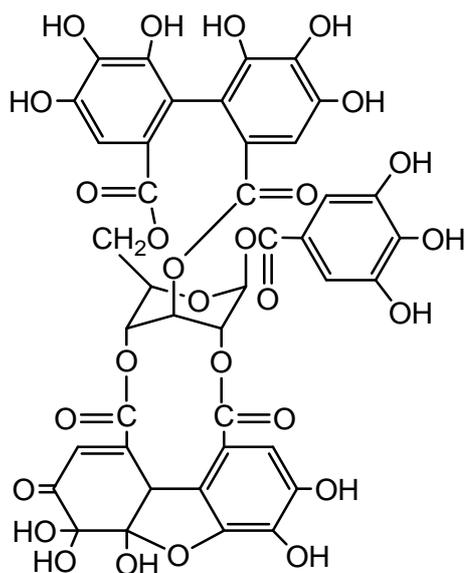
**33** gallic acid



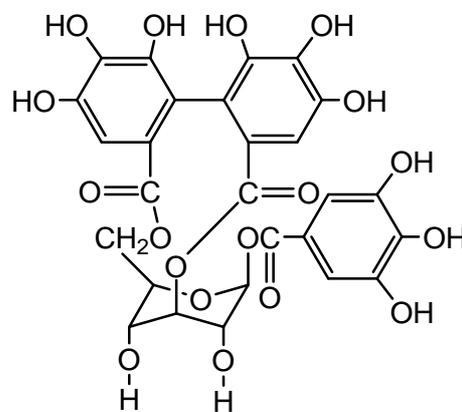
**34** ellagic acid



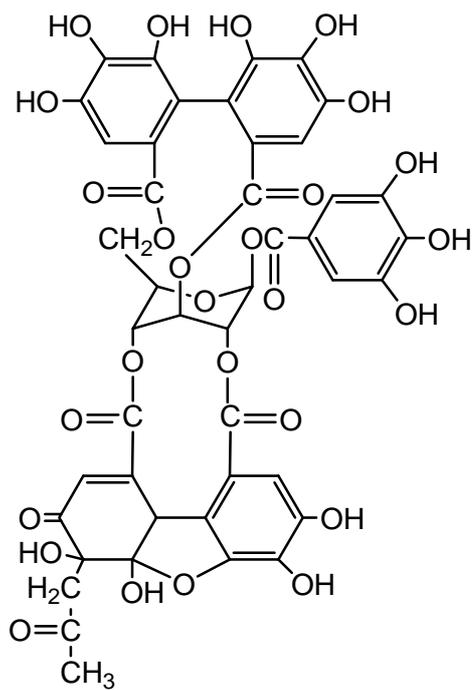
- R
- 35** brevifolin carboxylic acid    COOH  
**36** ethyl brevifolin carboxylate    COOCH<sub>2</sub>CH<sub>3</sub>  
**37** methyl brevifolin carboxylate    COOCH<sub>3</sub>



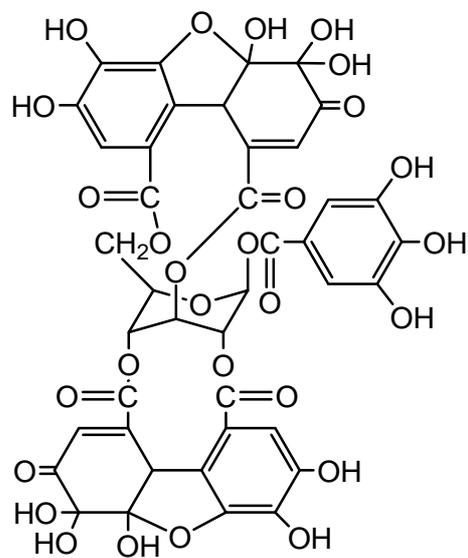
**38** geraniin



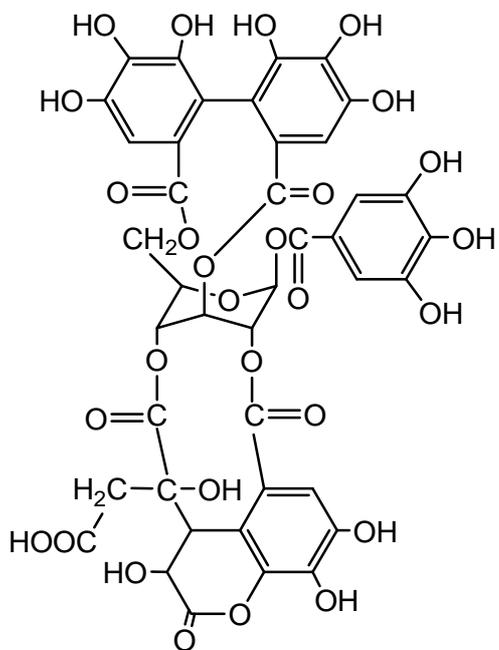
**39** corilagin



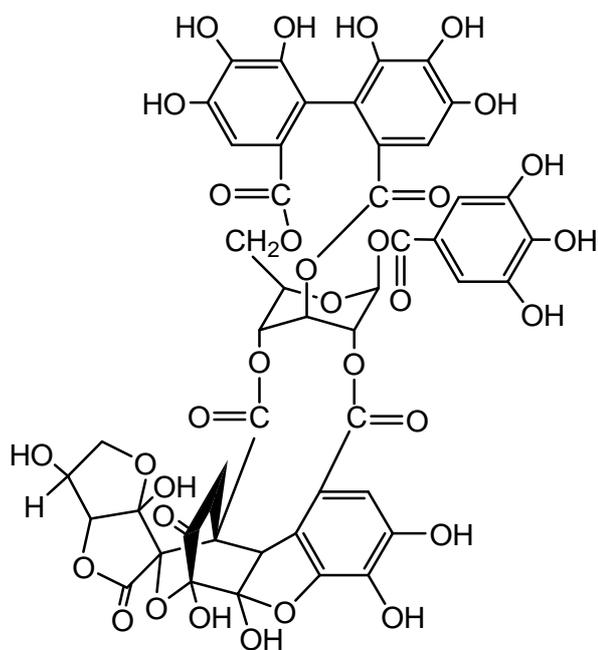
**40** phyllanthusiin D



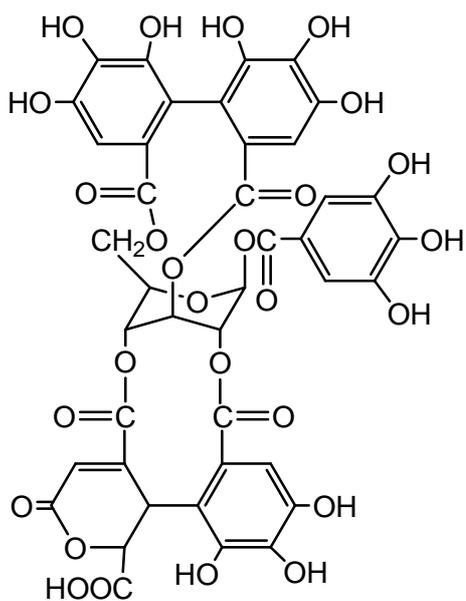
**41** amariin



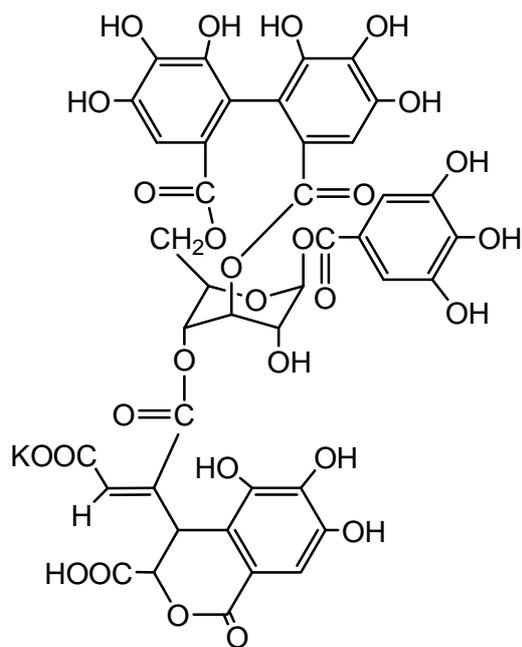
**42** amariinic acid



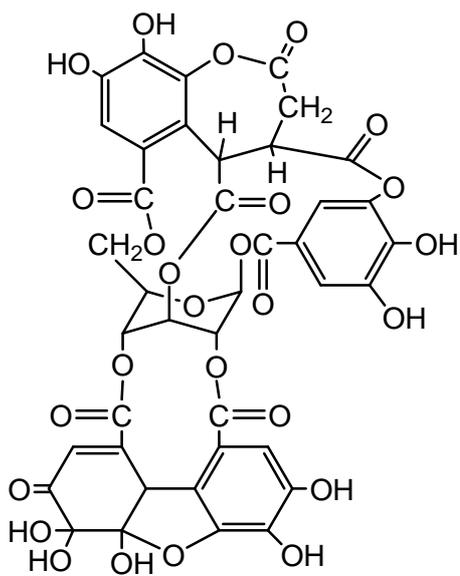
**43** elaeocarpusin



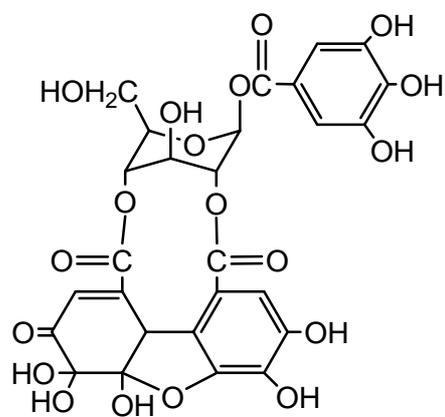
**44** geraniinic acid



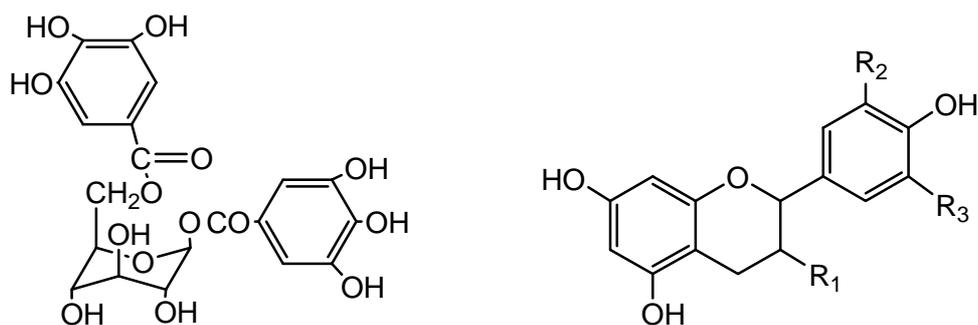
**45** repandusinic acid



**46** amarulone



**47** furosin



#### 48 1,6-digalloyl glucopyranoside

		R1	R2	R3
<b>49</b>	catechin	OH ( $\beta$ )	OH	H
<b>50</b>	epicatechin	OH ( $\alpha$ )	OH	H
<b>51</b>	gallocatechin	OH ( $\beta$ )	OH	OH
<b>52</b>	epigallocatechin	OH ( $\alpha$ )	OH	OH
<b>53</b>	epicatechin 3-O-gallate	O-gallate ( $\alpha$ )	OH	H
<b>54</b>	epigallocatechin 3-O-gallate	O-gallate( $\alpha$ )	OH	OH

### Flavonoids

Flavonoids reported from *P. niruri* plant belongs to the flavonols and flavanone subclasses and their respective glycosides. The following flavonoids have been isolated from *P. niruri*: quercetin (**12**), rutin (**13**), astragalin (**55**), quercitrin (**56**), isoquercitrin (**57**) (Nara *et al.*, 1977), kaempferol-4'-rhamnopyranoside (**58**), eridictyol-7-rhamno pyranoside (**59**) (Chauhan *et al.*, 1977), fisetin-4'-O-glucoside (**60**) (Gupta and Ahmed, 1984), quercetin-3-O-glucopyranoside (**61**) (Foo, 1993), kaempferol-3-O-rutinoside (**62**) (Qian-Cutrone *et al.*, 1996).

<b>55</b>	astragalin	R1 = OH	R2 = glucose	R3 = H
<b>56</b>	quercitrin	R1 = OH	R2 = rhamnose	R3 = OH
<b>57</b>	isoquercetin	R1 = OH	R2 = glucose	R3 = OH