

**PHYTOCHEMICAL STUDIES OF
LIPPIA NODIFLORA (L.) MICHX AND
ITS ANTI-HYPERURICEMIC ACTIVITY**

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NODIFLORA (L.) MICHX AND ITS
ANTI-HYPERURICEMIC
ACTIVITY**

by

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TABLE OF CONTENTS

Acknowledgement	ii
Table of Contents	iv
List of Tables	x
List of Figures	xiv
List of Abbreviations	xviii
List of Appendices	xxii
Abstrak	xxix
Abstract	xxxii
 CHAPTER 1 : INTRODUCTION	 1
1.1 Medicinal plants as potential source for drug discovery against hyperuricemia	1
1.2 <i>Lippia nodiflora</i>	3
1.2.1 Taxonomy classification of <i>Lippia nodiflora</i>	3
1.2.2 Morphology of <i>Lippia nodiflora</i>	4
1.2.3 Ethnobotanical uses of <i>Lippia nodiflora</i>	5
1.2.4 Phytochemicals of <i>Lippia nodiflora</i>	8
1.2.5 Pharmacological properties of <i>Lippia nodiflora</i>	11
1.3 Hyperuricemia	14
1.3.1 Nucleotides and purine nucleotides	14
1.3.2 Purine metabolism: <i>de novo</i> synthesis and salvage pathways of purine nucleotides	15
1.3.3 Xanthine oxidoreductase	16
1.3.4 Uric acid	17
1.3.5 Degradation of uric acid	17
1.3.6 Excretion of uric acid	19
1.4 The etiology of hyperuricemia	20

1.4.1	Uric acid overproduction	20
1.4.2	Uric acid underexcretion	21
1.5	Prevalence of hyperuricemia	22
1.6	Co-morbidities of hyperuricemia	24
1.7	Management of hyperuricemia	25
1.7.1	Uricosuric agents	26
1.7.2	Uricostatic agents or XOD inhibitors	27
1.7.3	Uricolytic agents	29
1.8	Problem statement	30
1.9	Objectives	31
1.10	Significance of the study	32
1.11	Research outline	34
CHAPTER 2: MATERIALS AND METHODS		35
2.1	Chemicals and reagents	35
2.2	Instruments	36
2.3	Animals	37
2.4	Bioactivity-guided isolation of <i>Lippia nodiflora</i>	37
2.4.1	Plant material	37
2.4.2	Preparation of <i>Lippia nodiflora</i> methanol extract	38
2.4.3	Bioactivity-guided fractionation and isolation of <i>Lippia nodiflora</i> methanol extract	38
2.5	Phytochemical analysis of bioactive compounds of <i>Lippia nodiflora</i>	41
2.5.1	HPLC method development and validation	41
2.5.1(a)	HPLC system and chromatographic conditions	41
2.5.1(b)	Purity check of the isolated bioactive compounds	41
2.5.1(c)	Preparation of the calibration standards	42

2.5.1(d)	Linearity, limit of detection (LOD) and limit of quantification (LOQ)	42
2.5.1(e)	Precision, accuracy and recovery	43
2.5.2	Preparation of <i>Lippia nodiflora</i> plant samples for phytochemical analysis	43
2.6	<i>In vitro</i> XOD inhibitory activity of <i>Lippia nodiflora</i>	45
2.6.1	<i>In vitro</i> XOD assay	45
2.6.2	Evaluation of the <i>in vitro</i> XOD inhibitory activities of <i>Lippia nodiflora</i> plant samples	45
2.7	<i>In vivo</i> antihyperuricemic activity of <i>Lippia nodiflora</i>	46
2.7.1	Establishment of chemically-induced hyperuricemic rat model	46
2.7.1(a)	Effect of potassium oxonate and hypoxanthine on the rat serum uric acid level	46
2.7.1(b)	Determination of the optimal hypoxanthine dose	47
2.7.1(c)	Chemically-induced hyperuricemic rat model	47
2.7.2	Evaluation of the rat serum uric acid lowering activities of <i>Lippia nodiflora</i> plant samples	49
2.7.2(a)	Single-dose experiment	49
2.7.2(b)	Repeated-dose experiment	49
2.7.3	Evaluation of the hypouricemic activities of <i>Lippia nodiflora</i> plant samples	50
2.7.4	<i>In vivo</i> rat liver XOD and XDH inhibitory studies of <i>Lippia nodiflora</i> plant samples	51
2.7.4(a)	Preparation of cytosolic fraction	51
2.7.4(b)	Determination of protein concentration of cytosolic fractions	52
2.7.4(c)	Determination of optimal protein concentration	52

2.7.4(d)	Determination of optimal incubation time	53
2.7.4(e)	Determination of optimal xanthine concentration	53
2.7.4(f)	Determination of optimal NAD ⁺ concentration	54
2.7.5	Evaluation of <i>in vivo</i> rat liver XOD and XDH activities of <i>Lippia nodiflora</i> plant samples	54
2.7.6	Molecular docking study	55
2.7.6(a)	Molecular docking of 6-hydroxyluteolin on xanthine oxidase	55
2.8	<i>In vivo</i> uricosuric activity of <i>Lippia nodiflora</i>	56
2.8.1	Evaluation of the <i>in vivo</i> uricosuric activities of <i>Lippia nodiflora</i> plant samples	56
2.9	Acute toxicity study	58
2.9.1	Evaluation of the acute oral toxicity of <i>Lippia nodiflora</i> extract and bioactive fraction	58
2.10	Pharmacokinetic and bioavailability studies of <i>Lippia nodiflora</i> bioactive chemical constituents	59
2.10.1	HPLC method development and validation	59
2.10.1(a)	HPLC system and chromatographic conditions	59
2.10.1(b)	Preparation of standards	59
2.10.1(c)	Linearity, limit of detection (LOD) and limit of quantification (LOQ)	59
2.10.1(d)	Precision, accuracy and recovery	60
2.10.2	Pharmacokinetic and bioavailability studies of arenarioside, verbascoside, 6-hydroxyluteolin, 6-hydroxyluteolin-7- <i>O</i> -glycoside, and nodifloretin	61
2.10.3	Preparation of plasma sample	62
2.10.4	Data analysis	62
2.11	Statistical analysis	63

CHAPTER 3: RESULTS	64
3.1 Structure elucidation and identification of bioactive compounds	64
3.1.1 Arenarioside (1)	64
3.1.2 Verbascoside (2)	75
3.1.3 6-Hydroxyluteolin (3)	84
3.1.4 6-Hydroxyluteolin-7- <i>O</i> -glycoside (4)	90
3.1.5 Nodifloretin (5)	97
3.2 Phytochemical analysis of bioactive compounds of <i>Lippia nodiflora</i>	103
3.2.1 HPLC method development and validation	103
3.2.2 Quantification of bioactive markers of <i>Lippia nodiflora</i> in plant samples	107
3.3 <i>In vitro</i> XOD inhibitory activities of <i>Lippia nodiflora</i> plant samples	114
3.4 Establishment of chemically-induced hyperuricemic rat model	118
3.4.1 Effect of potassium oxonate and hypoxanthine on the rat serum uric acid level	118
3.4.2 Determination of the optimal hypoxanthine dose	119
3.5 Evaluation of the rat serum uric acid lowering activities of <i>Lippia nodiflora</i> plant samples	120
3.5.1 Single-dose experiment	120
3.5.2 Repeated-dose experiment	122
3.5.3 Evaluation of the hypouricemic activities of <i>Lippia nodiflora</i> plant samples	124
3.5.4 Evaluation of the <i>in vivo</i> rat liver XOD and XDH activities of <i>Lippia nodiflora</i> plant samples	124
3.6 Molecular docking of 6-hydroxyluteolin on xanthine oxidase	130
3.7 Evaluation of the <i>in vivo</i> uricosuric activities of <i>Lippia nodiflora</i> plant samples	133

3.8	Evaluation of the acute oral toxicity of <i>Lippia nodiflora</i> extract and bioactive fraction	134
3.9	HPLC method development and validation for analysis of <i>Lippia nodiflora</i> bioactive chemical constituents	136
3.10	Application of HPLC method for pharmacokinetic and bioavailability studies of <i>Lippia nodiflora</i> bioactive chemical constituents	137
CHAPTER 4: DISCUSSION		146
CHAPTER 5: CONCLUSION AND SUGGESTIONS FOR FURTHER STUDY		163
5.1	Conclusion	163
5.2	Suggestions for further study	167
REFERENCES		169
APPENDICES		198
PUBLICATIONS AND CONFERENCE		312

LIST OF TABLES

		Page
Table 1.1	Ethnobotanical uses of <i>Lippia nodiflora</i> .	5
Table 1.2	Chemical constituents isolated from <i>Lippia nodiflora</i> .	8
Table 1.3	Reported pharmacological effects of <i>Lippia nodiflora</i> .	11
Table 1.4	Co-morbidities of hyperuricemia.	24
Table 2.1	List of chemicals and reagents.	35
Table 2.2	List of instruments.	36
Table 3.1	¹ H- and ¹³ C-NMR chemical shifts of arenarioside (1) (MeOD, 500 MHz) in comparison with the assignments reported by Andary <i>et al.</i> (1985) (DMSO-d ₆ , ¹³ C at 90.53 MHz and ¹ H at 360 MHz).	73
Table 3.2	¹ H- and ¹³ C-NMR chemical shifts, heteronuclear single quantum coherence (HSQC) and heteronuclear multiple-bond correlation (HMBC) of arenarioside (1).	74
Table 3.3	¹ H- and ¹³ C-NMR chemical shifts of verbascoside (2) (MeOD, 500 MHz) in comparison with the assignments reported by Ersöz <i>et al.</i> (2002) (CD ₃ OD, ¹³ C at 125 MHz and ¹ H at 500 MHz).	82
Table 3.4	¹ H- and ¹³ C-NMR chemical shifts, heteronuclear single quantum coherence (HSQC) and heteronuclear multiple-bond correlation (HMBC) of verbascoside (2).	83
Table 3.5	¹ H- and ¹³ C-NMR chemical shifts of 6-hydroxyluteolin (3) (MeOD, 500 MHz) in comparison with the assignments reported by Bai <i>et al.</i> (2010) (DMSO-d ₆ , ¹³ C at 100 MHz and ¹ H at 400 MHz).	88
Table 3.6	¹ H- and ¹³ C-NMR chemical shifts, heteronuclear single quantum coherence (HSQC) and heteronuclear multiple-bond correlation (HMBC) of 6-hydroxyluteolin (3).	89
Table 3.7	The UV and visible spectral shifts data for 3 in the methanol and after the addition of UV shift reagents.	89

Table 3.8	^1H - and ^{13}C -NMR chemical shifts of 6-hydroxyluteolin-7- <i>O</i> -glycoside (4) (MeOD, 500 MHz) in comparison with the assignments reported by Lu and Foo (2000) (DMSO- d_6 , ^{13}C at 75 MHz and ^1H at 300 MHz).	95
Table 3.9	^1H - and ^{13}C -NMR chemical shifts, heteronuclear single quantum coherence (HSQC) and heteronuclear multiple-bond correlation (HMBC) of 6-hydroxyluteolin-7- <i>O</i> -glycoside (4).	96
Table 3.10	The UV and visible spectral shifts data for 4 in methanol and after the addition of UV shift reagents.	96
Table 3.11	^1H - and ^{13}C -NMR chemical shifts of nodifloretin (5) (MeOD, 500 MHz) in comparison with the assignments reported by Bai <i>et al.</i> (2010) (DMSO- d_6 , ^{13}C at 100 MHz and ^1H at 400 MHz)	101
Table 3.12	^1H - and ^{13}C -NMR chemical shifts, heteronuclear single quantum coherence (HSQC) and heteronuclear multiple-bond correlation (HMBC) of nodifloretin (5).	102
Table 3.13	The UV and visible spectral shifts for 5 in methanol and after the addition of UV shift reagents.	102
Table 3.14	Calibration parameters, linear ranges, and limits of detection and quantification ($n = 3$) of chemical standards (1-5) of the <i>Lippia nodiflora</i> .	104
Table 3.15	Recovery, intra-day, and inter-day precision and accuracy values of bioactive compounds (1-5) of the <i>Lippia nodiflora</i> in phytochemical analysis.	106
Table 3.16	Content of the bioactive compounds (1-5) in the (i) methanolic extract of the <i>Lippia nodiflora</i> whole plant and its fractions, (ii) methanolic extract of the different parts of <i>Lippia nodiflora</i> , and (iii) water and hot water extracts of the <i>Lippia nodiflora</i> whole plant.	113
Table 3.17	Relative distribution of the compounds (1-5) in the (i) methanolic extract of the <i>Lippia nodiflora</i> whole plant and its fractions, (ii) methanolic extract of the different parts of <i>Lippia nodiflora</i> , and (iii) water and hot water extracts of the <i>Lippia nodiflora</i> whole plant.	114

Table 3.18	The intra-day and inter-day mean percentage inhibition, standard deviation, and coefficient of variance values of the reference standard allopurinol in the <i>in vitro</i> XOD inhibitory assay.	115
Table 3.19	The preliminary evaluation of the <i>in vitro</i> XOD inhibitory activity of the <i>Lippia nodiflora</i> methanol extract, fractions, sub-fractions, and chemical constituents.	116
Table 3.20	The <i>in vitro</i> XOD inhibitory activity of the <i>Lippia nodiflora</i> methanol extract and fractions.	117
Table 3.21	The <i>in vitro</i> XOD inhibitory activity of the <i>Lippia nodiflora</i> chemical constituents.	117
Table 3.22	Acute effects of <i>Lippia nodiflora</i> methanol extract, fractions, or chemical constituents on the serum uric acid level of the potassium oxonate- and hypoxanthine-induced hyperuricemic rats.	122
Table 3.23	Effect of repeated administration of the most active fraction and chemical constituent of the <i>Lippia nodiflora</i> on the serum uric acid level of the hyperuricemic rats.	123
Table 3.24	Effect of the methanol extract and the most active fraction of the <i>Lippia nodiflora</i> on the serum uric acid level of the normouricemic rats.	124
Table 3.25	Effects of <i>Lippia nodiflora</i> methanol extract, fractions, and chemical constituents on the rat liver XOD and XDH activities of hyperuricemic rats.	129
Table 3.26	Effects of <i>Lippia nodiflora</i> methanol extract, fractions, and chemical constituents on the urinary uric acid excretion and clearance of the hyperuricemic rats.	134
Table 3.27	The effect of oral administration of the methanol extract at doses of 2000 and 5000 mg/kg on the rat body weight.	135
Table 3.28	The effect of oral administration of the fraction F3 at doses of 2000 and 5000 mg/kg on the rat body weight.	135
Table 3.29	Calibration parameters, linear ranges, and limits of detection and quantification of chemical standards (1-5) of the <i>Lippia nodiflora</i> .	136

Table 3.30	Recovery, intra-day, and inter-day precision and accuracy values of compounds (1-5) of the <i>Lippia nodiflora</i> in pharmacokinetic study.	138
Table 3.31	Pharmacokinetic parameters of the compounds (1-5) in rat plasma after intravenous (2 mg/kg each of 1-5) or oral (20 mg/kg each of 1-5) administration of a compounds mixture.	145

LIST OF FIGURES

		Page
Figure 1.1	<i>Lippia nodiflora</i> .	4
Figure 1.2	Purine biosynthesis <i>de novo</i> and salvage pathways (Choi <i>et al.</i> , 2005).	16
Figure 1.3	Uric acid degradation pathway (Florkin, 1949).	18
Figure 1.4	The chemical structures of the (A) benzbromarone, (B) probenecid, and (C) sulfinpyrazone.	27
Figure 1.5	The chemical structures of the (A) allopurinol, (B) febuxostat, and (C) topiroxostat.	28
Figure 1.6	Outline of the present study.	34
Figure 2.1	Schematic diagram on the bioactivity-guided isolation of the bioactive chemical constituents from the <i>Lippia nodiflora</i> .	40
Figure 2.2	Flow chart of the establishment of potassium oxonate- and hypoxanthine-induced hyperuricemic rat model.	48
Figure 2.3	Experimental designs for the evaluation of serum uric acid lowering activities of <i>Lippia nodiflora</i> plant samples in hyperuricemic rats upon single-dose and repeated-dose administration.	50
Figure 2.4	Experimental designs for the evaluation of serum uric acid lowering activities of <i>Lippia nodiflora</i> plant samples in normouricemic rats.	51
Figure 2.5	Experimental designs for the evaluation of rat liver XOD and XDH inhibitory activities of <i>Lippia nodiflora</i> plant samples.	55
Figure 2.6	Experimental designs for the evaluation of uricosuric activities of <i>Lippia nodiflora</i> plant samples in hyperuricemic rats.	58
Figure 3.1	Chemical structure of arenarioside (1).	65
Figure 3.2	¹ H- ¹ H COSY correlations of 1 .	71

Figure 3.3	^1H - ^{13}C HMBC correlations of 1 .	71
Figure 3.4	Selected ^1H - ^1H NOESY correlations of 1 .	72
Figure 3.5	Chemical structure of verbascoside (2).	76
Figure 3.6	^1H - ^1H COSY correlations of 2 .	80
Figure 3.7	^1H - ^{13}C HMBC correlations of 2 .	81
Figure 3.8	Selected ^1H - ^1H NOESY correlations of 2 .	81
Figure 3.9	Chemical structure of 6-hydroxyluteolin (3).	85
Figure 3.10	^1H - ^1H COSY and ^1H - ^{13}C HMBC correlations of 3 .	86
Figure 3.11	Chemical structure of 6-hydroxyluteolin-7- <i>O</i> -glycoside (4).	91
Figure 3.12	^1H - ^1H COSY correlations of 4 .	93
Figure 3.13	^1H - ^{13}C HMBC correlations of 4 .	93
Figure 3.14	Selected ^1H - ^1H NOESY correlations of 4 .	94
Figure 3.15	Chemical structure of nodifloretin (5).	98
Figure 3.16	^1H - ^1H COSY and ^1H - ^{13}C HMBC correlations of 5 .	99
Figure 3.17	Selected ^1H - ^1H NOESY correlations of 5 .	99
Figure 3.18	HPLC chromatogram of mixed standards (10 $\mu\text{g/mL}$ each of 1-5) isolated from <i>Lippia nodiflora</i> (t_R of 1 = 16.78 min, t_R of 2 = 20.77 min, t_R of 3 = 27.36 min, t_R of 4 = 13.13 min, t_R of 5 = 33.35 min).	103
Figure 3.19	HPLC chromatograms of (A) blank solvent system and (B) methanol extract of <i>Lippia nodiflora</i> at a concentration of 100 $\mu\text{g/mL}$.	107
Figure 3.20	HPLC chromatograms of (A) fraction F1; (B) fraction F2; (C) fraction F3; (D) fraction F4 of the methanolic extract of <i>Lippia nodiflora</i> whole plant at a concentration of 100 $\mu\text{g/mL}$.	108
Figure 3.21	HPLC chromatograms of methanolic extracts of different parts of the <i>Lippia nodiflora</i> (A) roots; (B) stems; (C) leaves; (D) flowers at a concentration of 100 $\mu\text{g/mL}$.	109

Figure 3.22	HPLC chromatograms of water extracts of <i>Lippia nodiflora</i> whole plant (A) water extract; (B) hot water extract at a concentration of 100 µg/mL.	110
Figure 3.23	Effect of (i) intraperitoneal administration of potassium oxonate (OA) at 250 mg/kg, (ii) oral administration of hypoxanthine (HX) at 500 mg/kg, or (iii) intraperitoneal administration of potassium oxonate and oral administration of hypoxanthine at 250 mg/kg and 500 mg/kg, respectively, on the rat serum uric acid level when compared to their baseline values and the normal control. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$, ^d $P < 0.0001$ significantly different compared to their baseline values. ^e $P < 0.05$, ^f $P < 0.01$, ^g $P < 0.001$, ^h $P < 0.0001$ significantly different compared to normal control.	119
Figure 3.24	Effect of intraperitoneal administration of potassium oxonate (OA) 250 mg/kg and oral administration of different doses of hypoxanthine (HX) at 250, 500, or 1000 mg/kg on the rat serum uric acid level when compared to their baseline values and the normal control. ^a $P < 0.01$, ^b $P < 0.001$, ^c $P < 0.0001$ significantly different compared to their baseline values. ^d $P < 0.01$, ^e $P < 0.001$, ^f $P < 0.0001$ significantly different compared to normal control.	120
Figure 3.25	The effect of protein concentration of the cytosolic fractions on the XOD and XDH activities in the presence of a constant xanthine concentration of 50 µM and incubation time of 30 min. Values were expressed as mean \pm SD for six replicates.	126
Figure 3.26	The effect of incubation time on the XOD and XDH activities in the presence of a constant protein concentration of 0.25 mg/mL and xanthine final concentration of 50 µM. Values were expressed as mean \pm SD for six replicates.	126
Figure 3.27	The effect of xanthine concentration on the XOD and XDH activities in the presence of a constant protein concentration of 0.25 mg/mL and incubation time of 30 min. Values were expressed as mean \pm SD for six replicates.	127

Figure 3.28	The effect of NAD^+ concentration on the XDH activity in the presence of a constant protein concentration of 0.25 mg/ml, xanthine concentration of 70 μM , and incubation time of 30 min. Values were expressed as mean \pm SD for six replicates.	127
Figure 3.29	Molecular interaction between (a) 3 or (b) allopurinol and XOD.	132
Figure 3.30	HPLC chromatogram from the analysis of arenarioside (1), verbascoside (2), 6-hydroxyluteolin (3), 6-hydroxyluteolin-7- <i>O</i> -glycoside (4), and nodifloretin (5). (A) Blank rat plasma; (B) mixed standards (10 $\mu\text{g/mL}$ each of 1-5) isolated from <i>Lippia nodiflora</i> ; (C) rat plasma spiked with 1-5 (5 $\mu\text{g/mL}$ each); (D) rat plasma at 1 h after intravenous administration of mixed standards (2 mg/kg each of 1-5).	139
Figure 3.31	Mean plasma concentration-time profiles (mean \pm SEM, $n = 6$) of (A) phenylethanoid glycosides (1,2) and (B) flavonoids (3-5) after intravenous administration of a mixture consisting of bioactive compounds 1-5 (2 mg/kg of each compound).	141
Figure 3.32	Mean plasma concentration-time profiles (mean \pm SEM, $n = 6$) of (A) phenylethanoid glycosides (1,2) and (B) flavonoids (3-5) after oral administration of a mixture consisting of bioactive compounds 1-5 (20 mg/kg of each compound).	143

LIST OF ABBREVIATIONS

$[M+Na]^+$	sodium adduct ion
$[M+H]^+$	pseudomolecular ion
ADT	autodock tools
AHS	allopurinol hypersensitivity syndrome
AMP	adenosine monophosphate
ANOVA	analysis of variance
APRT	adenine phosphoribosyltransferase
ARASC	animal research and service centre
$AUC_{0-\infty}$	area under the plasma concentration-time curve to infinity
AUC_{0-t}	area from time zero to the last sampling time
$AUC_{t-\infty}$	area from the last sampling time to infinity
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CHF	congestive heart failure
CKD	chronic kidney disease
CL	body clearance
C_{max}	peak concentration
C_0	theoretical concentration at time zero
CO ₂	carbon dioxide
CoA	coenzyme A
COSY	homonuclear correlation spectroscopy
CV	coefficient of variation
ddH ₂ O	double distilled water
DEPT	distortionless enhancement by polarization transfer
DNA	deoxyribonucleic acid
F	absolute oral bioavailability
FAD	flavin adenine dinucleotide

g	gram
<i>g</i>	relative centrifugal force
G6Pase	glucose-6-phosphatase
GLUT9	glucose transporter 9
GMP	guanosine monophosphate
HCl	hydrochloric acid
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence spectroscopy
HX	hypoxanthine
Hz	hertz
IC ₅₀	half maximal inhibitory concentration
ICH	international conference on harmonisation
IMP	inosine monophosphate
IR	infrared
KBr	potassium bromide
<i>K_e</i>	elimination rate constant
LC-MS	liquid chromatography-mass spectrometer
LOD	limit of detection
LOQ	limit of quantification
M	molarity
MeOH	methanol
mg	milligram
mM	milimolar
MS	mass spectra
N	normality
NAD ⁺	nicotinamide adenine dinucleotide
NADP ⁺	nicotinamide adenine dinucleotide phosphate

NaOH	sodium hydroxide
NH ₃	ammonia
NHANES	National Health and Nutrition Examination Survey
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser enhancement spectroscopy
O ₂	oxygen
OA	potassium oxonate
°C	degree celcius
OECD	Organization for Economic Co-Operation and Development
PDB	Protein Data Bank
PRPP	5-phosphoribosyl-1-pyrophosphate
r^2	coefficient of determination
RNA	ribonucleic acid
SEM	standard error of the mean
$t_{1/2}$	half-life
T2DM	type 2 diabetes mellitus
TCM	Traditional Chinese Medicine
T_{\max}	time to reach C_{\max}
ESI-MS-TOF	time of flight mass spectrometry electrospray
t_R	retention time
URAT1	urate transporter 1
URATv1	voltage-driven urate efflux transporter
UV	ultraviolet
v/v	volume by volume
V_d	volume of distribution
w/v	weight per volume
w/w	weight per weight
WHO	World Health Organization
XDH	xanthine dehydrogenase

XOD	xanthine oxidase
XOR	xanthine oxidoreductase
α	alpha
β	beta

LIST OF APPENDICES

		Page
Appendix 1a	MS spectrum of arenarioside (1).	199
Appendix 1b	UV spectrum of arenarioside (1).	200
Appendix 1c	IR spectrum of arenarioside (1).	201
Appendix 1d	¹ H-NMR spectrum of arenarioside (1).	202
Appendix 1e	¹³ C-NMR spectrum of arenarioside (1).	203
Appendix 1f	DEPT-Q spectrum of arenarioside (1).	204
Appendix 1g	COSY spectrum of arenarioside (1).	205
Appendix 1g(i)	Expansion on COSY correlations of arenarioside (1).	206
Appendix 1g(ii)	Expansion on COSY correlations of arenarioside (1).	207
Appendix 1g(iii)	Expansion on COSY correlations of arenarioside (1).	208
Appendix 1g(iv)	Expansion on COSY correlations of arenarioside (1).	209
Appendix 1g(v)	Expansion on COSY correlations of arenarioside (1).	210
Appendix 1h	HSQC spectrum of arenarioside (1).	211
Appendix 1h(i)	Expansion on HSQC correlations of arenarioside (1).	212
Appendix 1h(ii)	Expansion on HSQC correlations of arenarioside (1).	213
Appendix 1h(iii)	Expansion on HSQC correlations of arenarioside (1).	214
Appendix 1h(iv)	Expansion on HSQC correlations of arenarioside (1).	215
Appendix 1h(v)	Expansion on HSQC correlations of arenarioside (1).	216
Appendix 1i	HMBC spectrum of arenarioside (1).	217
Appendix 1i(i)	Expansion on HMBC correlations of arenarioside (1).	218
Appendix 1i(ii)	Expansion on HMBC correlations of arenarioside (1).	219
Appendix 1i(iii)	Expansion on HMBC correlations of arenarioside (1).	220

Appendix 1i(iv)	Expansion on HMBC correlations of arenarioside (1).	221
Appendix 1i(v)	Expansion on HMBC correlations of arenarioside (1).	222
Appendix 1i(vi)	Expansion on HMBC correlations of arenarioside (1).	223
Appendix 1i(vii)	Expansion on HMBC correlations of arenarioside (1).	224
Appendix 1i(viii)	Expansion on HMBC correlations of arenarioside (1).	225
Appendix 1i(ix)	Expansion on HMBC correlations of arenarioside (1).	226
Appendix 1i(x)	Expansion on HMBC correlations of arenarioside (1).	227
Appendix 1i(xi)	Expansion on HMBC correlations of arenarioside (1).	228
Appendix 1j	NOESY spectrum of arenarioside (1).	229
Appendix 2a	MS spectrum of verbascoside (2).	230
Appendix 2b	UV spectrum of verbascoside (2).	231
Appendix 2c	IR spectrum of verbascoside (2).	232
Appendix 2d	¹ H-NMR spectrum of verbascoside (2).	233
Appendix 2e	¹³ C-NMR spectrum of verbascoside (2).	234
Appendix 2f	DEPT-Q spectrum of verbascoside (2).	235
Appendix 2g	COSY spectrum of verbascoside (2).	236
Appendix 2h	HSQC spectrum of verbascoside (2).	237
Appendix 2i	HMBC spectrum of verbascoside (2).	238
Appendix 2j	NOESY spectrum of verbascoside (2).	239
Appendix 3a	MS spectrum of 6-hydroxyluteolin (3).	240
Appendix 3b	UV spectrum of 6-hydroxyluteolin (3).	241
Appendix 3c	IR spectrum of 6-hydroxyluteolin (3).	242
Appendix 3d	¹ H-NMR spectrum of 6-hydroxyluteolin (3).	243
Appendix 3e	¹³ C-NMR spectrum of 6-hydroxyluteolin (3).	244
Appendix 3f	DEPT-Q spectrum of 6-hydroxyluteolin (3).	245

Appendix 3g	COSY spectrum of 6-hydroxyluteolin (3).	246
Appendix 3h	HSQC spectrum of 6-hydroxyluteolin (3).	247
Appendix 3i	HMBC spectrum of 6-hydroxyluteolin (3).	248
Appendix 3j	NOESY spectrum of 6-hydroxyluteolin (3).	249
Appendix 4a	MS spectrum of 6-hydroxyluteolin-7- <i>O</i> -glycoside (4).	250
Appendix 4b	UV spectrum of 6-hydroxyluteolin-7- <i>O</i> -glycoside (4).	251
Appendix 4c	IR spectrum of 6-hydroxyluteolin-7- <i>O</i> -glycoside (4).	252
Appendix 4d	¹ H-NMR spectrum of 6-hydroxyluteolin-7- <i>O</i> -glycoside (4).	253
Appendix 4e	¹³ C-NMR spectrum of 6-hydroxyluteolin-7- <i>O</i> -glycoside (4).	254
Appendix 4f	DEPT-Q spectrum of 6-hydroxyluteolin-7- <i>O</i> -glycoside (4).	255
Appendix 4g	COSY spectrum of 6-hydroxyluteolin-7- <i>O</i> -glycoside (4).	256
Appendix 4h	HSQC spectrum of 6-hydroxyluteolin-7- <i>O</i> -glycoside (4).	257
Appendix 4i	HMBC spectrum of 6-hydroxyluteolin-7- <i>O</i> -glycoside (4).	258
Appendix 4j	NOESY spectrum of 6-hydroxyluteolin-7- <i>O</i> -glycoside (4).	259
Appendix 5a	MS spectrum of nodifloretin (5).	260
Appendix 5b	UV spectrum of nodifloretin (5).	261
Appendix 5c	IR spectrum of nodifloretin (5).	262
Appendix 5d	¹ H-NMR spectrum of nodifloretin (5).	263
Appendix 5e	¹³ C-NMR spectrum of nodifloretin (5).	264
Appendix 5f	DEPT-Q spectrum of nodifloretin (5).	265

Appendix 5g	COSY spectrum of nodifloretin (5).	266
Appendix 5h	HSQC spectrum of nodifloretin (5).	267
Appendix 5i	HMBC spectrum of nodifloretin (5).	268
Appendix 5j	NOESY spectrum of nodifloretin (5).	269
Appendix 6a	The intra-day percentage inhibition, standard deviation, and coefficient of variance values of the reference standard allopurinol in the <i>in vitro</i> XOD inhibitory assay (Section 3.3, Table 3.18).	270
Appendix 6b	The inter-day percentage inhibition, standard deviation, and coefficient of variance values of the reference standard allopurinol in the <i>in vitro</i> XOD inhibitory assay (Section 3.3, Table 3.18).	270
Appendix 6c	The percentage inhibition values of <i>in vitro</i> XOD inhibitory activity of the <i>Lippia nodiflora</i> methanol extract, fractions, sub-fractions, and chemical constituents (Section 3.3, Table 3.19).	271
Appendix 6d	The IC ₅₀ values of <i>in vitro</i> XOD inhibitory activity of the <i>Lippia nodiflora</i> methanol extract and fractions (Section 3.3, Table 3.20).	271
Appendix 6e	The IC ₅₀ values of <i>in vitro</i> XOD inhibitory activity of the <i>Lippia nodiflora</i> chemical constituents and allopurinol (Section 3.3, Table 3.21).	273
Appendix 7a	The effects of intravenous administration of potassium oxonate (250 mg/kg) or oral administration of hypoxanthine (500 mg/kg) or a combination of both on the rat serum uric acid level (Section 3.4.1, Figure 3.23; Section 3.4.2, Figure 3.24).	274
Appendix 7b	The effects of intravenous administration of potassium oxonate (250 mg/kg) and oral administration of different doses of hypoxanthine (250 and 1000 mg/kg) (Section 3.4.2, Figure 3.24).	276

Appendix 8a	The effects of <i>Lippia nodiflora</i> methanol extract, fractions, or chemical constituents on the serum uric acid level of the hyperuricemic rats. (Section 3.5.1, Table 3.22).	277
Appendix 8b	The repeated administration effect of the most active fraction and chemical constituent of the <i>Lippia nodiflora</i> on the serum uric acid level of the hyperuricemic rats (Section 3.5.2, Table 3.23).	279
Appendix 8c	The effects of the methanol extract and the most active fraction of the <i>Lippia nodiflora</i> on the serum uric acid level of the normouricemic rats (Section 3.5.3, Table 3.24).	281
Appendix 8d	The effects of different protein concentrations of cytosolic fractions on the XOD and XDH activities (Section 3.5.4, Figure 3.25).	281
Appendix 8e	The effects of different incubation times on the XOD and XDH activities (Section 3.5.4, Figure 3.26).	281
Appendix 8f	The effects of different xanthine concentrations on the XOD and XDH activities (Section 3.5.4, Figure 3.27).	282
Appendix 8g	The effects of different NAD ⁺ concentrations on the XDH activity (Section 3.5.4, Figure 3.28).	282
Appendix 8h	The protein concentration of cytosolic fraction obtained from the hyperuricemic rat treated with the <i>Lippia nodiflora</i> methanol extract, fractions, and chemical constituents (Section 3.5.4).	282
Appendix 8i	The effects of <i>Lippia nodiflora</i> methanol extract, fractions, and chemical constituents on the XOD and XDH activities in the liver of hyperuricemic rats (Section 3.5.4, Table 3.25).	284
Appendix 9a	The molecular binding interactions of 3 and allopurinol toward XOD (Section 3.6, Figure 3.29).	289
Appendix 10a	The effects of <i>Lippia nodiflora</i> methanol extract, fractions, and chemical constituents on the urine output of hyperuricemic rats (Section 3.7).	290

Appendix 10b	The effects of <i>Lippia nodiflora</i> methanol extract, fractions, chemical constituents on the urinary uric acid excretion and clearance of the hyperuricemic rats (Section 3.7, Table 3.26).	292
Appendix 11a1	The plasma concentration of the chemical constituent 1 after intravenous administration of a mixture consisting of bioactive compounds 1-5 (2 mg/kg of each compound) (Section 3.10, Figure 3.31).	297
Appendix 11a2	The plasma concentration of the chemical constituent 2 after intravenous administration of a mixture consisting of bioactive compounds 1-5 (2 mg/kg of each compound) (Section 3.10, Figure 3.31).	297
Appendix 11a3	The plasma concentration of the chemical constituent 3 after intravenous administration of a mixture consisting of bioactive compounds 1-5 (2 mg/kg of each compound) (Section 3.10, Figure 3.31).	298
Appendix 11a4	The plasma concentration of the chemical constituent 4 after intravenous administration of a mixture consisting of bioactive compounds 1-5 (2 mg/kg of each compound) (Section 3.10, Figure 3.31).	298
Appendix 11a5	The plasma concentration of the chemical constituent 5 after intravenous administration of a mixture consisting of bioactive compounds 1-5 (2 mg/kg of each compound) (Section 3.10, Figure 3.31).	299
Appendix 11b1	The plasma concentration of the chemical constituent 1 after oral administration of a mixture consisting of bioactive compounds 1-5 (20 mg/kg of each compound) (Section 3.10, Figure 3.32).	299
Appendix 11b2	The plasma concentration of the chemical constituent 2 after oral administration of a mixture consisting of bioactive compounds 1-5 (20 mg/kg of each compound) (Section 3.10, Figure 3.32).	300
Appendix 11b3	The plasma concentration of the chemical constituent 3 after oral administration of a mixture consisting of bioactive compounds 1-5 (20 mg/kg of each compound) (Section 3.10, Figure 3.32).	300

Appendix 11b4	The plasma concentration of the chemical constituent 4 after oral administration of a mixture consisting of bioactive compounds 1-5 (20 mg/kg of each compound) (Section 3.10, Figure 3.32).	301
Appendix 11b5	The plasma concentration of the chemical constituent 5 after oral administration of a mixture consisting of bioactive compounds 1-5 (20 mg/kg of each compound) (Section 3.10, Figure 3.32).	301
Appendix 11c1	The pharmacokinetic parameters of the chemical constituent 1 in rat plasma after intravenous (2 mg/kg of each compound) or oral (20 mg/kg of each compound) administration of a compounds mixture comprising bioactive compounds 1-5 (Section 3.10, Table 3.31).	302
Appendix 11c2	The pharmacokinetic parameters of the chemical constituent 2 in rat plasma after intravenous (2 mg/kg of each compound) or oral (20 mg/kg of each compound) administration of a compounds mixture comprising bioactive compounds 1-5 (Section 3.10, Table 3.31).	303
Appendix 11c3	The pharmacokinetic parameters of the chemical constituent 3 in rat plasma after intravenous (2 mg/kg of each compound) or oral (20 mg/kg of each compound) administration of a compounds mixture comprising bioactive compounds 1-5 (Section 3.10, Table 3.31).	304
Appendix 11c4	The pharmacokinetic parameters of the chemical constituent 4 in rat plasma after intravenous (2 mg/kg of each compound) or oral (20 mg/kg of each compound) administration of a compounds mixture comprising bioactive compounds 1-5 (Section 3.10, Table 3.31).	305
Appendix 11c5	The pharmacokinetic parameters of the chemical constituent 5 in rat plasma after intravenous (2 mg/kg of each compound) or oral (20 mg/kg of each compound) administration of a compounds mixture comprising bioactive compounds 1-5 (Section 3.10, Table 3.31).	306

KAJIAN FITOKIMIA *LIPPIA NODIFLORA* (L.) MICHX DAN AKTIVITI ANTI-HIPERURISEMIKNYA

ABSTRAK

Lippia nodiflora telah digunakan secara tradisional dalam Ayurveda, Unani, Sindh, dan Perubatan Tradisional Cina untuk rawatan sakit lutut sendi, lithiasis, diuresis, penyakit urinari dan bengkak. Dalam kajian ini, ekstrak metanol *L. nodiflora* menunjukkan aktiviti perencatan xantina oksidase (XOD) secara *in vitro* baik. Pemeringkatan berpandukan bioaktiviti ekstrak metanol menghasilkan empat pecahan (F1-F4) dengan F3 dikenal pasti sebagai pecahan yang paling poten. Penulenan F3 selanjutnya menghasilkan lima sebatian bioaktif, termasuk dua feniletanoid glikosida arenariosida (**1**) dan verbaskosida (**2**), dan tiga flavonoid 6-hidroksiluteolin (**3**), 6-hidroksiluteolin-7-*O*-glikosida (**4**), dan nodifloretin (**5**), di mana **1** dan **4** diasingkan untuk kali pertama daripada *L. nodiflora*. Ekstrak metanol, pecahan-pecahan, dan juzuk kimia tersebut kemudiannya diuji untuk aktiviti antihiperurisemik berpotensi secara *in vivo* dengan menggunakan model tikus hiperurisemik diaruh oleh kalium oxonate dan hipoksantina. Rawatan oral ekstrak metanol menurunkan paras asid urik serum tikus hiperurisemik dengan berkesan dan bersandarkan dos. F3 menunjukkan kesan penurunan asid urik serum tikus yang tertinggi. Sebatian **3** merupakan sebatian yang paling poten antara sebatian-sebatian yang telah diasingkan di mana ia dapat menurunkan paras asid urik serum tikus hiperurisemik dengan signifikan dan bersandarkan dos. Namun begitu, **3** tidak

menurunkan paras asid urik serum tikus hiperurisemik rendah daripada kawalan normal walaupun pada dos tertinggi yang diberikan. Pemberian berulang F3 atau **3** kepada tikus hiperurisemik selama 10 hari secara berterusan menyebabkan kesan penurunan asid urik serum yang signifikan dan progresif dalam tikus hiperusemik. Berbeza dengan allopurinol, ekstrak metanol dan F3 tidak menurunkan paras asid urik serum tikus normorurisemik. Tambahan pula, tiada kesan toksik diperhatikan dalam tikus-tikus yang diberi 5000 mg/kg ekstrak metanol atau F3, menunjukkan profil keselamatan mereka yang menggalakkan. Selepas itu, mekanisme aktiviti antihiperurisemik mereka ditafsirkan dengan menggunakan kajian *in vivo* perencatan XOD dan xantina dehidrogenase (XDH) hati tikus dan kajian urikosurik. Menariknya, **3** berupaya untuk merencat aktiviti kedua-dua XOD dan XDH dalam hati tikus ke tahap yang setanding dengan allopurinol. Pengedokan molekul **3** mendedahkan bahawa **3** berinteraksi dengan XOD dengan susunan yang sama seperti allopurinol tetapi dengan tenaga pengikat bebas yang lebih rendah daripada allopurinol. Di samping itu, F4 meningkatkan penyingkiran asid urik tikus hiperurisemik secara signifikan. Sementara itu, satu kaedah kromatografi cecair berprestasi tinggi yang mudah dan boleh dipercayai dengan pengesanan ultralembayung telah dibangunkan dan disahkan untuk penentuan serentak lima sebatian bioaktif tersebut. Kaedah tersebut kemudiannya berjaya diaplikasikan untuk analisis fitokimia dan kajian farmakokinetik bagi **1-5** dalam sampel tumbuhan *L. nodiflora* dan sampel plasma tikus, masing-masing, untuk kali pertama. Batang kayu didapati memaparkan jumlah feniletanoid glikosida dan flavonoid yang tertinggi. Biokeperolehan oral untuk

sebatian **1, 2, 3, 4,** dan **5** didapati rendah dan tidak lengkap dengan anggaran nilai biokeperolehan oral mutlak 5.22, 2.10, 5.97, 3.13, dan 0.93 %, masing-masing. Diambil bersama, aplikasi berpotensi *L. nodiflora* terhadap hiperurisemia dalam tikus berdasarkan kegunaan tradisional telah ditunjukkan dalam kajian ini untuk kali pertama. Kesan antihiperurisemik yang dimiliki oleh *L. nodiflora* disumbangkan terutamanya oleh aktiviti perencatan XOD and XDH hati dan sebahagiannya oleh kesan urikosurik. Flavonoid adalah terutamanya bertanggungjawab untuk kesan penurunan asid urik oleh *L. nodiflora* dengan bertindak sebagai perencat XOD.

PHYTOCHEMICAL STUDIES OF *LIPPIA NODIFLORA* (L.) MICHX AND ITS ANTI-HYPERURICEMIC ACTIVITY

ABSTRACT

Lippia nodiflora has been traditionally used in the Ayurvedic, Unani, Sindh, and Traditional Chinese Medicine for the treatment of knee joint pain, lithiasis, diuresis, urinary disorder and swelling. In the present study, methanol extract of *L. nodiflora* showed promising xanthine oxidase (XOD) inhibitory activity *in vitro*. Bioactivity-guided fractionation of methanol extract yielded four fractions (F1-F4) with F3 being identified as the most potent fraction. Further purification of F3 afforded five bioactive compounds, including two phenylethanoid glycosides arenarioside (**1**) and verbascoside (**2**), and three flavonoids 6-hydroxyluteolin (**3**), 6-hydroxyluteolin-7-*O*-glycoside (**4**), and nodifloretin (**5**), of which **1** and **4** were first time isolated from *L. nodiflora*. The methanol extract, fractions, and chemical constituents were then tested for potential antihyperuricemic activity *in vivo* using potassium oxonate- and hypoxanthine-induced hyperuricemic rat model. Oral treatment with methanol extract effectively and dose-dependently reduced the serum uric acid level of hyperuricemic rats. F3 exhibited the highest rat serum uric acid lowering effect. Compound **3** was established as the most potent of the isolated chemical constituents whereby it significantly and dose-dependently reduced the serum uric acid level of hyperuricemic rats. Nonetheless, **3** did not lower the serum uric acid level of hyperuricemic rats below that of the normal control even at the

highest dose given. Repeated administration of F3 or **3** to the hyperuricemic rats for 10 continuous days resulted in a significant and progressive serum uric acid lowering effect in hyperuricemic rats. In contrast to allopurinol, the methanol extract and F3 did not reduce serum uric acid level of normoruricemic rats. Furthermore, no toxic effect was observed in rats administered with 5000 mg/kg of methanol extract or F3, indicating their favorable safety profile. Subsequently, their mechanism(s) of antihyperuricemic activity were elucidated using *in vivo* rat liver XOD and xanthine dehydrogenase (XDH) inhibitory and uricosuric studies. Interestingly, **3** was able to inhibit both XOD and XDH activities in rat liver to an extent comparable to the allopurinol. Molecular docking of **3** revealed that **3** interacted with XOD in a manner similar to allopurinol but with a free energy of binding lower than allopurinol. On the other hand, F4 significantly increased the uric acid excretion of hyperuricemic rats. Meanwhile, a simple and reliable high performance liquid chromatography with ultraviolet detection method was developed and validated for the simultaneous determination of the five bioactive compounds. The method was then successfully applied for the phytochemical analysis and pharmacokinetic study of **1-5** in *L. nodiflora* plant samples and rat plasma samples, respectively, for the first time. Stems were found to contain the highest total content of phenylethanoid glycosides and flavonoids. The oral bioavailability of the compound **1**, **2**, **3**, **4**, and **5** was found to be low and incomplete with estimated absolute oral bioavailability values of 5.22, 2.10, 5.97, 3.13, and 0.93 %, respectively. Taken together, the potential application of *L. nodiflora* against hyperuricemia in rat in accordance with its traditional uses has

been demonstrated in the present study for the first time. The antihyperuricemic effect possessed by *L. nodiflora* was contributed mainly by liver XOD and XDH inhibitory activities and partially by uricosuric effect. Flavonoids are mainly accountable for the uric acid lowering effect of *L. nodiflora* by acting as XOD inhibitor.

CHAPTER 1

INTRODUCTION

1.1 Medicinal plants as potential source for drug discovery against hyperuricemia

A medicinal plant is defined by the World Health Organization (WHO) as a plant which is capable of inducing a pharmacological effect when it is applied in a particular form and by any means to the humans (Silva *et al.*, 2011). Medicinal plants, particularly higher plants, once served as the main source of medicaments to humankind. Today, medicinal plants continue to retain their significance as important source of novel and known chemical constituents useful as medicinal agents and leads for the production of synthetic or semi-synthetic organic medicinal agents (Balandrin *et al.*, 1993).

It has been reported that the medical indications of approximately 80 % of the plant-derived bioactive chemical constituents used as commercial drugs were the same or related to the traditional uses of their respective plants (Farnsworth *et al.*, 1985 cited in Fabricant and Farnsworth, 2001). For example, the plant *Colchicum autumnale* has been described in the Ebers Papyrus of 1500 B.C. and used to treat painful articular attacks and gouty arthritis. Of which its compound, colchicine found to exhibit activity reflecting the traditional uses of the plant and it is now a popular clinically used agent for hyperuricemia and gout therapy (Graham and Roberts, 1953).

The exploration and use of medicinal plants and their phytochemicals as potential antihyperuricemic agents dated back to thousand years ago since hyperuricemia-related diseases such as gout, osteoarthritis, rheumatism, bone and joint disorders, painful articular attacks, and joint pain are widely described in the ethnomedicine literature like Ayurveda (5000-3000 B.C.), Traditional Chinese Medicine (TCM, 2000 B.C.), and Ebers Papyrus (1500 B.C.) (Gourie-Devi *et al.*, 1991; Graham and Roberts, 1953; Lozano, 2014; Williamson, 2015).

To date, various medicinal plants and their phytochemicals have been reported to be active against hyperuricemia by inhibiting xanthine oxidase *in vitro* or *in vivo*. For medicinal plants, the examples include *Larix laricina* from northeastern North America (Owen and Johns, 1999), *Clerodendrum floribundum*, *Eremophila maculata*, and *Stemodia grossa* from Australia (Sweeney *et al.*, 2001), *Cinnamomum cassia*, *Chrysanthemum indicum*, and *Lycopus europaeus* from China (Kong *et al.*, 2000), *Strychnos nux-vomica*, *Coccinia grandis*, and *Vitex negundo* from India (Umamaheswari *et al.*, 2007), *Averrhoa carambola*, *Carica papaya*, *Dimocarpus longan malesianus*, *Manikara zapota*, and *Salacca zalacca* from Malaysia (Azmi *et al.*, 2012). Whereas, the examples for phytochemicals include cinnamaldehyde isolated from *Cinnamomum osmophloeum* (Wang *et al.*, 2008), iocineraflavone isolated from *Lonicera hypoglauc*a (Chien *et al.*, 2009), 4'-methylether robustaflavone, robustaflavone, eriodictyol, and amentoflavone isolated from *Selaginella labordei* (Tan *et al.*, 2009), and riparsaponin isolated from *Homonoia riparia* (Xu *et al.*, 2014).

These examples clearly show that the search for new medicinal plants with antihyperuricemic activity and alternative uric acid lowering agents from medicinal plants are still ongoing.

1.2 *Lippia nodiflora*

Lippia nodiflora (L.) Michx, also known as *Phyla nodiflora*, is one of the medicinal plants recorded in the Ayurvedic, Unani, Sindh, and Traditional Chinese Medicine (TCM) systems (Khare, 2007; Umberto Quattrocchi, 2012; Yang *et al.*, 2003). It has been extensively used as a traditional medicine to treat illness by the local community, has a very long history for human use, and has a broad array of reported pharmacological activities (Siddiqui *et al.*, 2009).

1.2.1 Taxonomy classification of *Lippia nodiflora*

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Lamiales
Family: Verbenaceae
Genus: *Phyla*
Species: *nodiflora*

(Adopted from Sharma and Singh, 2013)



Figure 1.1: *Lippia nodiflora*.

1.2.2 Morphology of *Lippia nodiflora*

Lippia nodiflora is a fast growing, creeping, prostrate, much branched, and appressed medifixed hairs covered perennial herb (Figure 1.1) (Gadhvi *et al.*, 2012; Munir, 1993). It can grow up to a height of approximately 20-30 cm when in competition with other species (Sharma and Singh, 2013). The stems of the plant are 2-3 cm thick, 30-95 cm long, and green to purple in color when young but becoming grey and woody with age (Leigh and Walton, 2004). The leaves are cuneate-spathulate to abovate in shape, 10-30 mm long, 5-12 mm wide, dentate at the upper margins, tapering gradually below to a short petiole or subsessile, with sharp antrorse teeth on the upper half, and usually arise in pairs in the opposite direction at the stem nodes (Leigh and Walton, 2004; Mandaville, 1990; Munir, 1993). Roots are fibrous, branched, brown in color, 2-10 cm in length, and 1-1.5 mm in diameter while nodal roots are smaller, 0.5-1 cm in length and unbranched (The Ayurvedic Pharmacopoeia of India, Volume V, Part I, 1989). The plant flowers are rose-purple to nearly white in color and produce in the spikes or heads on the slender auxiliary peduncles which are mostly 1.5-3.0 cm long (Foin and Unger, 1991; Munir, 1993).

1.2.3 Ethnobotanical uses of *Lippia nodiflora*

The use of *Lippia nodiflora* as a folk medicine for treating various ailments has been recorded in medicinal systems and regions or countries as presented in Table 1.1.

Table 1.1: Ethnobotanical uses of *Lippia nodiflora*.

Medicinal system	Ethnobotanical uses	References
Ayurveda, Unani, and Sindh	<ul style="list-style-type: none">• knee joint pain• urinary disorder• diuresis• burning sensation during urination• liver tonic• jaundice• gasointestinal disorders• skin disorders• atherosclerosis diseases• blood purification• pneumonia• blood dysentery• spasmolytic• cough• cold• headache• fever• indigestion• febrifuge• aphrodisiac• menstrual disorders• as a demulcent in cases of venereal diseases	Khare, 2007 Narendra <i>et al.</i> , 2012 Shanmugasundaram <i>et al.</i> , 1983 Umberto Quattrocchi, 2012
Traditional Chinese Medicine (TCM)	<ul style="list-style-type: none">• removing wind, heat, and swelling• detoxicating• stranguria• sore throat• tonsillitis• suppurative infections on the body surface• dysentery of heat type• ulcerative gingivitis• herpes zoster	Yang <i>et al.</i> , 2003

Table 1.1: Continued.

Region/ Country	Ethnobotanical uses	References
United States of America	<ul style="list-style-type: none"> • colds • grippe • bronchitis • coughs • asthma • orthopaedic aid 	Pascual <i>et al.</i> , 2001 Speck, 1941 in Austin, 2004
Taiwan	<ul style="list-style-type: none"> • treating inflammatory diseases like hepatitis and dermatitis • toxic • irregular menses • sore throat 	Li, 2006 Yen <i>et al.</i> , 2012
Africa	<ul style="list-style-type: none"> • malaria • arthritis • osteoarticular pains • diuresis • vermifuge • antiseptic • antitussive • respiratory diseases • bronchitis • dyspepsia • gonorrhoea • analgesic • inflammation • antipyretic • hookworm 	Forestieri <i>et al.</i> , 1996 Mukherjee, 1991 Pascual <i>et al.</i> , 2001
India	<ul style="list-style-type: none"> • spermatorrhoea • jaundice • diuresis • asthma • as 'laehiums' for paralysis • displacement of joint • cut wound and external wound • gastrointestinal problems 	Ambesta, 1986 in Nyman <i>et al.</i> , 1998 Chitravadivu <i>et al.</i> , 2009 Prusti and Behera, 2007 Savithramma <i>et al.</i> , 2007 Muralidharan and Narasimhan, 2012

Table 1.1: Continued.

Region/ Country	Ethnobotanical uses	References
Pakistan	<ul style="list-style-type: none"> • skin diseases • as cosmetic • micturition • dysuria • bleeding biles • indigestion in children • hepatitis • abscess • as cooling agent • as a demulcent in cases of venereal diseases • antidote for snake bite 	Abbasi <i>et al.</i> , 2010 Qureshi and Bhatti, 2008 Khan <i>et al.</i> , 2013 Marwat <i>et al.</i> , 2011
Sindh	<ul style="list-style-type: none"> • abscess • hepatitis • leishmaniasis • as an antidote to snake or scorpion sting 	Rahman <i>et al.</i> , 2012
Bangladesh	<ul style="list-style-type: none"> • eczema • rheumatoid arthritis • nervous disorders • gonorrhoea • constipation • heat stroke • fever • spasms • headache • dizziness • pain in back or waist due to rheumatism • limb pain 	Rahmatullah <i>et al.</i> , 2011 Biswas <i>et al.</i> , 2010
Nepal	<ul style="list-style-type: none"> • headache • fever • cough • cold 	Taylor, 1996

1.2.4 Phytochemicals of *Lippia nodiflora*

Since 1959, over 50 chemical constituents have been isolated from *L. nodiflora* as listed in the Table 1.2.

Table 1.2: Chemical constituents isolated from *Lippia nodiflora*.

Class of compounds	Name of chemical constituent	References
Flavonoids	• nodifloretin (or 6-hydroxy-3'-methoxyluteolin or batatifolin)	Barua <i>et al.</i> , 1969 Barua <i>et al.</i> , 1971
	• 6-hydroxyluteolin-7- <i>O</i> -apioside	Barnabas <i>et al.</i> , 1980
	• luteolin-7- <i>O</i> -glucoside	
	• hispidulin-7-sulfate	Tomás-Barberán <i>et al.</i> , 1987
	• hispidulin-7,4'-disulfate	
	• jaceosidin-7,4'-disulfate	
	• nepetin-3',4'-disulfate	
	• nodifloretin-6,7-disulfate	
	• 6-hydroxyluteolin-6,7-disulfate	
	• nodifloretin-7-sulfate	
	• 6-hydroxy-luteolin-6-sulfate	
	• 6-hydroxyluteolin-7-sulfate	
	• jaceosidin-7-sulfate	
	• nepetin-7-sulfate	
	• hispidulin-4'-sulfate	
	• hispidulin	
	• jaceosidin	
	• demethoxycentaureidin (or 5,7,3'-trihydroxy-6,4'-dimethoxy flavone)	Khalil <i>et al.</i> , 1995
	• ganzalitosin I (or 5-hydroxy-3',4',7-trimethoxy flavones)	Sudha and Srinivasan, 2014

Table 1.2: Continued.

Class of compounds	Name of chemical constituent	References
Flavonoids	<ul style="list-style-type: none"> • 3,7,4',5'-tetrahydroxy-3'-methoxyflavone • 4'-hydroxywogonin • onopordin • cirsiolol • larycitrin • 5,7,8,4'-tetrahydroxy-3'-methoxyflavone 	Lin <i>et al.</i> , 2014
Phytosterol	<ul style="list-style-type: none"> • β-sitosterol glucoside • stigmasterol glucoside • β-sitosterol • 4', 5'-dimethoxybenzoxystigmasterol • stigmasterol 	Barua <i>et al.</i> , 1969 Barua <i>et al.</i> , 1971 Akhtar, 1993 Siddiqui <i>et al.</i> , 2009
Triterpene	<ul style="list-style-type: none"> • 3β-19α-dihydroxy-urs-1,20-(30)-diene • ursolic acid • pomolic acid • Lippiacin 	Akhtar, 1993 Siddiqui <i>et al.</i> , 2007
Quinol	<ul style="list-style-type: none"> • halleridone (or benzofuranone renglyolone) • hallerone 	Ravikanth <i>et al.</i> , 2000
Iridoid	<ul style="list-style-type: none"> • loganin • catalpol 	Akhtar, 1993
Phenylethanoid glycosides	<ul style="list-style-type: none"> • acteoside (or verbascoside or kusagin in or russetinol or stereospermin) • 2'-O-acetylchinacoside 	Khalil <i>et al.</i> , 1995

Table 1.2: Continued.

Class of compounds	Name of chemical constituent	References
Others	• nodifloridin A	Joshi and Bhakuni, 1959
	• nodifloridin B	
	• nodiflorin A	Joshi and Bhuwan, 1970
	• nodiflorin B	
	• cornoside	Rimpler and Sauerbier, 1986
	• α -ethyl-galactose	Akhtar, 1993

As shown in the Table 1.2, majority of the chemical constituents isolated from *L. nodiflora* belonged to the class of flavonoids.

1.2.5 Pharmacological properties of *Lippia nodiflora*

Lippia nodiflora has been reported to be active against a wide array of biological activities as listed in the Table 1.3.

Table 1.3: Reported pharmacological effects of *Lippia nodiflora*.

Reported biological activities	References
• Analgesic	Forestieri <i>et al.</i> , 1996
• Angiotensin converting enzyme inhibitory	Nyman <i>et al.</i> , 1998
• Antiatherosclerotic	Shanmugasundaram <i>et al.</i> , 1983
• Anticancer	Vanajothi <i>et al.</i> , 2012 Sankaranarayanan <i>et al.</i> , 2013
• Antidiabetic	Balamurugan and Ignacimuthu, 2011 Balamurugan <i>et al.</i> , 2011
• Antihepatotoxic	Durairaj <i>et al.</i> , 2008 Sampathkumar <i>et al.</i> , 2008 Balamurugan <i>et al.</i> , 2011 Narendra <i>et al.</i> , 2012 Sudha <i>et al.</i> , 2013 Arumanayagam and Arunmani, 2015
• Antihyperlipidemia	Balamurugan and Ignacimuthu, 2011 Balamurugan <i>et al.</i> , 2011
• Antihypertensive	Akhtar, 1993 Gadhvi <i>et al.</i> , 2012 Gadhvi <i>et al.</i> , 2015
• Antiinflammatory	Forestieri <i>et al.</i> , 1996 Balakrishnan <i>et al.</i> , 2010 Jabeen <i>et al.</i> , 2015 Ahmed <i>et al.</i> , 2004

Table 1.3: Continued.

Reported biological activities	References
<ul style="list-style-type: none"> Antimicrobial 	Durairaj <i>et al.</i> , 2007 Sivakumar, 2008 Jeya and Veerapagu, 2011 Ravikumar and Sudha, 2011 Malathi <i>et al.</i> , 2011 Zare <i>et al.</i> , 2012 Priyadarshni <i>et al.</i> , 2013 Regupathi <i>et al.</i> , 2014 Priya and Ravindhran, 2015 Taylor, 1996 Balakrishna <i>et al.</i> , 1996 Hsueh <i>et al.</i> , 2010 Jeyachandran <i>et al.</i> , 2010 Patel <i>et al.</i> , 2011 Salve and Bhuktar, 2012 Thamaraiselvi <i>et al.</i> , 2013 Ullah <i>et al.</i> , 2013 Jabeen <i>et al.</i> , 2015 Anitha <i>et al.</i> , 2013 Regupathi and Chitra, 2015 Pirzada <i>et al.</i> 2005 Manimegalai and Ambikapathy, 2012 Wang and Huang, 2005 Simonsen <i>et al.</i> , 2001 Kavitha <i>et al.</i> , 2012 Mako and Noor, 2006
<ul style="list-style-type: none"> Antioxidant 	Ashokkumar <i>et al.</i> , 2008 Ashokkumar <i>et al.</i> , 2009 Durairaj <i>et al.</i> , 2008 Shukla <i>et al.</i> , 2009b Lin <i>et al.</i> , 2014
<ul style="list-style-type: none"> Antipyretic 	Forestieri <i>et al.</i> , 1996
<ul style="list-style-type: none"> Antiulcer 	Sumalatha, 2012

Table 1.3: Continued.

Reported biological activities	References
• Kidney disorders	Dodoala <i>et al.</i> , 2010 Ashok kumar <i>et al.</i> , 2008 Shukla <i>et al.</i> , 2009a Gadhvi <i>et al.</i> , 2015
• Larvicidal	Sivakumar, 2008
• Lipid peroxide scavenging	Durairaj <i>et al.</i> , 2007
• Melanogenesis inhibitory	Yen <i>et al.</i> , 2012
• Neuropharmacological	Turaskar <i>et al.</i> , 2011
• Skin whitening	Ko <i>et al.</i> , 2014

In addition to the activities listed, Dodoala *et al.* (2010) showed that *L. nodiflora* ethanol extract also exhibited antiurolithiatic effect. The extract significantly prevented the formation of the calcium oxonate stone dissolved the pre-formed calcium oxolate stone in the kidney of rats induced with gentamycin and calculi producing diet. The antiurolithiatic activity of the extract was due to its ability to increase the urinary pH and excretion of the calcium and oxolate, and also to reduce the urine supersaturation with the calculogenic ions.

On the other hand, methanol extract of *L. nodiflora* whole plant was found to possess diuretic properties by significantly increasing the urine volume of the treated rats with enhanced excretion of the Na^+ , Ca^{2+} , and Cl accompanied by the excretion of the K^+ in a dose-dependent manner as demonstrated by Ashok kumar *et al.* (2008). Besides, Shukla *et al.* (2009a) showed that both methanol and aqueous extracts of aerial parts of *L. nodiflora* possess significant diuretic potential by increasing the

urine volume, urinary concentration of the Na⁺ and K⁺ ions in an *in vivo* Lipschitz rat model.

Recently, Gadhvi *et al.* (2015) reported that *L. nodiflora* methanol extract exhibited kidney protective effect in the deoxycorticosteroneacetate-induced hypertensive rats. It significantly improved the serum creatinine level of the treated rats and the histopathology studies on the rat renal tissues of the treated rats showed fewer inflammatory cells with architecture close to normal control.

The antiurolithiatic, diuretic, and kidney protective activities of *L. nodiflora* described above suggested that it may possess activity against hyperuricemia.

1.3 Hyperuricemia

Hyperuricemia is a symptom of abnormally high uric acid concentration in blood. It arises when the serum uric acid level is greater than 6.0 mg/dL (350 µmol/L) in women and 7.0 mg/dL (450 µmol/L) in men (Fam, 1990; Sachs *et al.*, 2009). Uric acid is formed from the metabolism of purine nucleotides (Wright, 1995).

1.3.1 Nucleotides and purine nucleotides

A nucleotide is a compound that contains either a purine (purine nucleotide) or pyrimidine (pyrimidine nucleotide) base. Purine nucleotides serve as building blocks for deoxyribonucleic acid (DNA), ribonucleic acid (RNA), sources of energy, extra- and intra-cellular messengers, secondary messengers, allosteric enzyme effectors, neurotransmitters, antioxidants, and precursors of coenzymes (Baranowska-Bosiacka *et al.*, 2004; Cannella and Mikuls, 2005; Halabe and Sperling, 1994). Purine

nucleotides can be synthesized *de novo*, or reconstructed from already existing free purine bases through the salvage pathway (Baranowska-Bosiacka *et al.*, 2004).

1.3.2 Purine metabolism: *de novo* synthesis and salvage pathways of purine nucleotides

The synthesis of purine nucleotides occurs mainly in the liver (Moriwaki, 2014) and requires a source of ribose-5-phosphate which is produced from the glucose-6-phosphate via pentose phosphate pathway (also known as hexose monophosphate shunt and phosphogluconate pathway) (Cohen and Roth, 1953; Larrabee, 1989). Ribose-5-phosphate formed is then converted into 5-phosphoribosyl-1-pyrophosphate (PRPP) by PRPP synthetase (Figure 1.2). PRPP is the starting substrate for both purine biosynthesis *de novo* and salvage pathways. In the presence of the second substrate, L-glutamine, *de novo* synthesis pathway uses PRPP to generate the first purine nucleotides called inosine monophosphate (IMP) and from IMP to the other purine nucleotides called adenosine monophosphate (AMP) and guanosine monophosphate (GMP) (Gilbert, 2000; Wyngaarden, 1974).

Whereas, the salvage pathway uses PRPP to react with the free purine bases derived either from the turnover of nucleotides or from the diet to reconstruct purine nucleotides by using two salvage enzymes which are known as hypoxanthine-guanine phosphoribosyltransferase (HGPRT), to catalyze the formation of IMP and GMP, and adenine phosphoribosyltransferase (APRT), to catalyze the formation of AMP (Baranowska-Bosiacka *et al.*, 2004).

Catabolism of the IMP, AMP and GMP purine nucleotides leads to the production of purine nucleosides namely inosine, adenosine and guanosine, then to the formation of purine bases namely hypoxanthine, xanthine and guanine, and ultimately to the generation of uric acid through the activities of xanthine oxidoreductase (XOR) (Choi *et al.*, 2005).

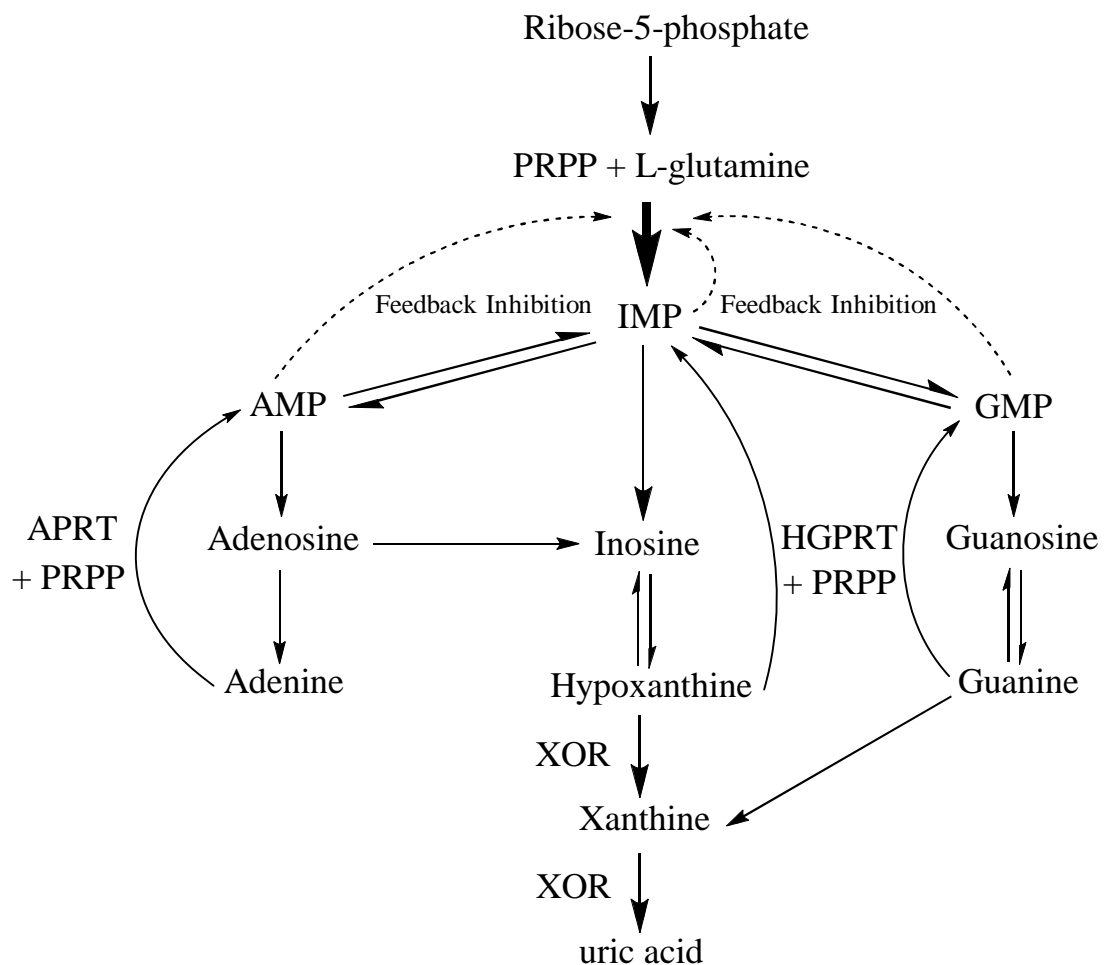


Figure 1.2: Purine biosynthesis *de novo* and salvage pathways (Choi *et al.*, 2005).

1.3.3 Xanthine oxidoreductase

Xanthine oxidoreductase (XOR), a rate-limiting enzyme in purine biosynthesis, exists in two forms as xanthine dehydrogenase (XDH), which is the primary gene

product of XOR and as xanthine oxidase (XOD), which is formed via the post-translational modification of XDH. XDH requires the presence of the cofactor nicotinamide adenine dinucleotide (NAD^+) as its primary electron acceptor, yet XOD is unable to bind to NAD^+ and uses molecular oxygen (O_2) as its electron acceptor (Vorbach *et al.*, 2003). In both forms, xanthine oxidoreductase has a key role in purine catabolism by catalyzing two-steps of sequential oxidative hydroxylation from hypoxanthine to xanthine and from xanthine to the end product of the humans' purine metabolism called uric acid (Fukunari *et al.*, 2004).

1.3.4 Uric acid

Uric acid (2,6,8-trihydroxypurine, $\text{C}_5\text{H}_4\text{N}_4\text{O}_3$) is the end product of purine catabolism in humans due to the loss of the uricase resulted from the various mutations of its gene as a consequence of the hominoids evolution occurred during the Miocene epoch (approximately 8-20 million years ago). This evolution may of importance to allow early hominoid ancestors survive with the food shortage and climate changes that occurred during the mid Miocene (Johnson *et al.*, 2005; Johnson *et al.*, 2011). As a consequence, humans have circulating uric acid levels that are five to twenty folds higher than most other mammals (Bobulescu and Moe, 2012). Normal uric acid range in human is between 3 and 7 mg/dL (Johnson *et al.*, 2013). Therefore, the lack of uricase is the main reason that humans develop hyperuricemia and eventually gout (Álvarez-Lario and Macarrón-Vicente, 2010).

1.3.5 Degradation of uric acid

Uric acid is an intermediary product in most mammals with uricase (Kahn *et al.*, 1997). A complete chain of uricolytic or purinolytic enzymes namely uricase,

allantoinase, allantoicase, and urease is necessary for degrading purines completely (Florkin, 1949; Greene, 1954). The end product of purine degradation or uricolysis varies from species to species depending on the uricolytic enzyme(s) involved (Florkin, 1949 in Noguchi *et al.*, 1979; Kahn *et al.*, 1997). The enzyme uricase plays an important role in the degradation of uric acid by converting a water-insoluble uric acid to a highly water-soluble product called allantoin which is readily eliminated in the urine (Kahn *et al.*, 1997). Allantoinase will then hydrolyzes allantoin to allantoic acid (Florkin, 1949; Greene, 1954; Kuzhivelil and Mohamed, 1998; Osuji and Ory, 1986). Allantoic acid formed is the substrate for enzyme allantoicase to form urea and glyoxylic acid. Enzyme urease will then hydrolyzes urea to carbon dioxide (CO₂) and ammonia (NH₃) (Figure 1.3) (Florkin, 1949; Greene, 1954; Keilin, 1958 in Usuda *et al.*, 1994).

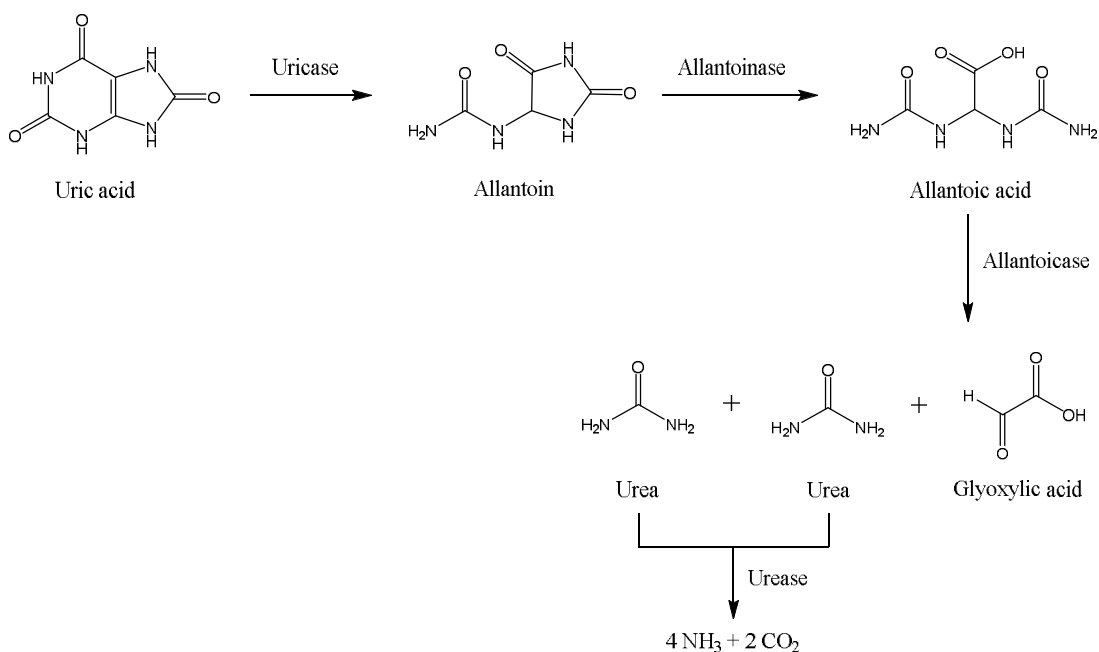


Figure 1.3: Uric acid degradation pathway (Florkin, 1949).

1.3.6 Excretion of uric acid

Since humans lack the enzyme uricase, when in excess, uric acids are either consumed by other pathways or excreted via the kidney and gastrointestinal tract (Cannella and Mikuls, 2005). Gastrointestinal tract is responsible for one-third of the uric acid excretion. The remainder is handled primarily by the kidney which removes uric acid from the blood plasma into urine following a system that includes four components namely glomerular filtration, proximal tubular pre-secretory reabsorption, secretion, and post-secretory reabsorption (Prosper *et al.*, 1993; Barr, 1990).

Glomerular filtration is the first step in the complex process of uric acid excretion (Holechek, 2003). Approximately 95 % of the uric acid is filtered by the glomerular filtration barrier and subsequently undergoes bidirectional movement in the proximal tubule to enter the second step which involves pre-secretory reabsorption at the pre-secretory site (S1 segment of the proximal tubule), whereby approximately 99 % of the uric acid is reabsorbed. During the third step, secretion occurs at the tubular (S2 segment of the proximal tubule) and approximately 50 % of the uric acid is secreted, followed by the post-secretory reabsorption that takes place at the post-secretory site (S3 segment of the proximal tubule), leading to approximately 40-50 % of the secreted uric acid being reabsorbed (Cannella and Mikuls, 2005; Diamond and Paolino, 1973; Ngo and Assimos, 2007; Prosper *et al.*, 1993). Eventually, the uric acid excreted in the urine accounts for approximately 5-10 % of the glomerular filtrate (Gutman and Yu, 1958). Failure of any of these components could result in the development of hyperuricemia (Barr, 1990).

1.4 The etiology of hyperuricemia

Defects in the *de novo* synthesis pathway, salvage pathway, and degradation and excretion of purines and uric acid may result in hyperuricemia, due mainly to the overproduction or underexcretion of uric acid or a combination of the two mechanisms (Dincer *et al.*, 2002; Ghei *et al.*, 2002; Barr, 1990).

1.4.1 Uric acid overproduction

The causes that affect the balance between uric acid production and elimination, leading to hyperuricemia, are multifactorial and include both genetic and environmental factors (den Boer, 2012). Overproduction of uric acid is often associated with abnormalities of the enzyme involved in the purine biosynthesis pathway. These include structural mutants of phosphoribosyl-pyrophosphate (PRPP) synthetase with increased activities, structural mutants of hypoxanthine-guanine phosphoribosyltransferase (HGPRT) with reduced activities, structural mutants of adenine phosphoribosyltransferase (APRT) with reduced activities, and structural mutants of XOR with hyperactivities (Perez-Ruiz and Herrero-Beites, 2014; Sorensen, 2012; Wyngaarden, 1974).

There are a number of drugs that may cause hyperuricemia by stimulating uric acid production. Examples include fructose, xylitol, and theophylline which increase uric acid concentration in the blood by accelerating purine nucleotide degradation. In addition, certain cytotoxic agents such as anthracyclines and doxorubicin derivatives may also increase uric acid production by increasing the turnover rate of cell death (Moriwaki, 2014).

Approximately two-thirds of the total body uric acid is produced endogenously, whilst the remaining one-third is accounted for by diet which serves as an exogenous source of the purine nucleotides (Schumacher, 2008). Therefore, dietary habit like high consumption of ethanol and protein-, fructose- and purine-rich foods such as red meats, organ meats, shellfish, anchovies, and sugared soft drinks are important factors that increase uric acid production (Keith and Gilliland, 2007; Terkeltaub *et al.*, 2009; Villegas *et al.*, 2012).

1.4.2 Uric acid underexcretion

In year 2003, Turner *et al.* showed that uromodulin mutation is one of the metabolic defects, leading to hyperuricemia due to uric acid underexcretion. Later, Zivna *et al.* (2009) reported that deletion or amino acid exchange mutations of a single leucine residue in the signal sequence of renin reduced the expression of renin and other components of the renin-angiotensin system and altered the juxtaglomerular apparatus functionality, leading to nephron dropout and progressive kidney failure and thus uric acid retention. In addition, the deficiency of G6Pase were not only reported to cause uric acid overproduction, they were also a cause for uric acid underexcretion as the degradation of the phosphorylated sugars accumulated due to GTPase mutants leads to the formation of lactate which may compete with uric acid for renal excretion (Cohen *et al.*, 1985).

The use of certain medications may also causes uric acid retention and thus hyperuricemia. Examples include pyrazinamide (Cullen *et al.*, 1956 in Yu, 1974), ethambutol (Postlethwaite *et al.*, 1972 in Yu, 1974), thiazide and loop diuretics such as furosemide and bumetanide (Jutabha *et al.*, 2010; Scott and Higgins, 1992),

cyclosporine (Gores *et al.*, 1988), levadopa (Honda and Gindin, 1972 in Yu, 1974), nicotinic acid or niacin, cytotoxic agents, and low dose aspirin (Moriwaki, 2014; Vázquez-Mellado, 2004).

In addition, uric acid underexcretion may be caused by disease states such as renal insufficiency as 70 % of the uric acid is excreted from the kidney (Ohno, 2011). Therefore, hyperuricemia is common in renal allograft recipients because their renal function and hence the excretion of uric acid are compromised by immunosuppressive drugs and diuretics. Since immunosuppressive drugs such as cyclosporine were used to prevent rejection of the transplantation while diuretics such as thiazide and loop were used to control hypertension and edema in renal transplant recipients (Clive, 2000; Gores *et al.*, 1988).

The consumption of alcoholic beverages causes not only uric acid overproduction but also uric acid underexcretion since alcohol catabolism increases the production of lactate, which is an antiuricosuric agent (Fallen and Fox, 1982).

1.5 Prevalence of hyperuricemia

Hyperuricemia is a health concern with worldwide distribution, reportedly afflicting 5 to 30 % of the general population (Vázquez-Mellado *et al.*, 2004). For instance, the prevalence of hyperuricemia in Japan was 24.4 % during year 1997-2000 (Nagahama *et al.*, 2004). In Thailand, the overall prevalence of hyperuricemia was 10.6 % in year 1999-2000 (Lohsoonthorn *et al.*, 2006). In Northern and Northeastern Chinese provinces, the prevalence of hyperuricemia was 13.7 % during year 2008-2010 (Qiu

et al., 2013). In Malaysia, 21.1 % of the population had rheumatic complaint during year 2007 (Veerapen *et al.*, 2007).

The prevalence of hyperuricemia varies with race, gender, and age, and appears to be increasing worldwide (Singh *et al.*, 2010; Vázquez-Mellado *et al.*, 2004). A higher incidence of hyperuricemia is observed in African Americans likely due to their greater risk of hypertension (Hochberg *et al.*, 1995), and among Filipinos, Maori, Samoans, and other South Pacific Islanders probably due to their genetic and dietary factors that heighten predisposition (Harris, 2013). The occurrence of hyperuricemia is higher in men than in women with a reported men to women ratio ranged from 7:1 to 9:1. However, the ratio tends to equalize with increasing age since the serum uric acid level increases in women post-menopause. Therefore, the ratios of 4:1 and 3:1 were reported for those below and above the age of 65 years old, respectively (Doherty, 2009; Kramer and Curhan, 2002; Singh *et al.*, 2010; Wallace *et al.*, 2004). The likelihood of developing hyperuricemia increases with age, especially over 65 years old (Wallace *et al.*, 2004).

The US National Health and Nutrition Examination Survey (NHANES) conducted during year 2007-2008 revealed that hyperuricemia affected 21.4 % or 43.3 million individuals among adults in United States of America. The survey also showed that the prevalence of hyperuricemia has increased by 3.2 % compared to hyperuricemia prevalence of 18.2 % or 30.5 million recorded in the NHANES-III conducted during year 1988-1994. This signified that the prevalence of the hyperuricemia has been substantially sustained over the past decades and indeed may still be increasing (Rho *et al.*, 2011; Zhu *et al.*, 2011).

1.6 Co-morbidities of hyperuricemia

Hyperuricemia is a known major risk factor for gout. It is also highly associated with, and may predispose to diseases as listed in the Table 1.4.

Table 1.4: Co-morbidities of hyperuricemia.

Co-morbidities	References
• cancer and malignancy	Fini <i>et al.</i> , 2012
• contrast-induced acute kidney injury	Liu <i>et al.</i> , 2013
• coronary, cerebral and peripheral arterial disease	Newland, 1975
• diuresis	Scott and Higgins, 1992
• human immunodeficiency virus infection	Manfredi <i>et al.</i> , 1996 Medina-Rodriguez <i>et al.</i> , 1993 Patel <i>et al.</i> , 2013 Walker <i>et al.</i> , 2006
• hypercholesterolemia	Harris-Jones <i>et al.</i> , 1956 Marinoff <i>et al.</i> , 1962 Schoenfeld and Goldberger, 1963 cited in Fessel, 1972
• hypertriglyceridemia	Berkowitz, 1964
• hyperparathyroidism	Mintz <i>et al.</i> , 1961
• hyperthyroidism	Sato <i>et al.</i> , 1995
• hypoglycemia and hyperglucagonemia	Cohen <i>et al.</i> , 1985
• hypoparathyroidism	Kirschner, 1956 cited in Yu, 1974
• hypothyroidism	Leeper <i>et al.</i> , 1960
• laxative abuse syndrome	Adam and Goebel, 1998 Gupta and Kavanaugh-Danelon, 1989
• lithiasis	Kramer <i>et al.</i> , 2003 Kramer and Curhan, 2002 Vázquez-Mellado, 2004