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

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

by

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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 form 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_o	theoretical concentration at time zero
CO_2	carbon dioxide
CoA	coenzyme A
COSY	homonuclear correlation spectroscopy
CV	coefficient of variation
ddH_2O	double distilled water
DEPT	distortionless enhancement by polarization transfer
DNA	deoxyribonucleic acid
F	absolute oral bioavailability
FAD	flavin adenine dinucleotide

g	gram
g	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
K_e	elimination rate constant
LC-MS	liquid chromatography-mass spectrometer
LOD	limit of detection
LOQ	limit of quantification
M	molarity
MeOH	methanol
mg	miligram
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
^oC	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

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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 activiti 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.

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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 hypoglauca* (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 nodilflora*

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: Lamialas

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, prostate, 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	• knee joint pain • urinary disorder • diuresis • burning sensation during urination • liver tonic • jaundice • gasointestinal disorders • skin disorders • atherosclerosis diseases • blood purification • pneumonia • blood dysentry • spasmolytic • cough • cold • headache • fever • indigestion • febrifuge • aphrodisiac • menstrual disorders • as a demulcent in cases of venereal diseases	Khare, 2007 Narendra *et al*., 2012 Shanmugasundaram *et al*., 1983 Umberto Quattrocchi, 2012
Traditional Chinese Medicine (TCM)	• removing wind, heat, and swelling • detoxicating • stranguria • sore throat • tonsillitis • suppurative infections on the body surface • dysentry of heat type • ulcerative gingivitis • herpes zoster	Yang *et al*., 2003

Table 1.1: Continued.

Region/ Country	Ethnobotanical uses	References
United States of America	• colds • grippe • bronchitis • coughs • asthma • orthopaedic aid	Pascual *et al*., 2001 Speck, 1941 in Austin, 2004
Taiwan	• treating inflammatory diseases like hepatitis and dermatitis • toxic • irregular menses • sore throat	Li, 2006 Yen *et al*., 2012
Africa	• malaria • arthritis • osteoarticular pains • diuresis • vermifuge • antiseptic • antitussive • respiratory diseases • bronchitis • dyspepsia • gonorrhoea • analgesic • inflammation • antipyretic • hookworm	Forestieri *et al*., 1996 Mukherjee, 1991 Pascual *et al*., 2001
India	• spermatorrhoea • jaundice • diuresis • asthma • as 'laehiums' for paralysis • displacement of joint • cut wound and external wound • gastrointestinal problems	Ambesta, 1986 in Nyman *et al*., 1998 Chitravadivu *et al*., 2009 Prusti and Behera, 2007 Savithramma *et al*., 2007 Muralidharan and Narasimhan, 2012

Table 1.1: Continued.

Region/ Country	Ethnobotanical uses	References
Pakistan	• 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 *et al*., 2010 Qureshi and Bhatti, 2008 Khan *et al*., 2013 Marwat *et al*., 2011
Sindh	• abscess • hepatitis • leishmaniasis • as an antidote to snake or scorpion sting	Rahman *et al*., 2012
Bangladesh	• eczema • rheumatoid arthritis • nervous disorders • gonorrhoea • constipation • heat stroke • fever • spasms • headache • dizziness • pain in back or waist due to rheumatism • limb pain	Rahmatullah *et al*., 2011 Biswas *et al*., 2010
Nepal	• 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 *et al*., 1969 Barua *et al*., 1971
	• 6-hydroxyluteolin-7-*O*-apioside • luteolin-7-*O*-glucoside	Barnabas *et al*., 1980
	• hispidulin-7-sulfate • 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	Tomás-Barberán *et al*., 1987
	• demethoxycentaureidin (or 5,7,3'-trihydroxy-6,4'-dimethoxy flavone)	Khalil *et al*., 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	• 3,7,4',5'-tetrahydroxy-3'-methoxyflavone • 4'-hydroxywogonin • onopordin • cirsiliol • larycitrin • 5,7,8,4'-tetrahydroxy-3'-methoxyflavone	Lin *et al*., 2014
Phytosterol	• β-sitosterol glucoside • stigmasterol glucoside	Barua *et al*., 1969 Barua *et al*., 1971
	• β-sitosterol	Akhtar, 1993
	• 4', 5'-dimethoxybenzoloxystigmasterol • stigmasterol	Siddiqui *et al*., 2009
Triterpene	• 3β-19α-dihydroxy-urs-1,20-(30)-diene • ursolic acid • pomolic acid	Akhtar, 1993
	• Lippiacin	Siddiqui *et al*., 2007
Quinol	• halleridone (or benzofuranone renglyolone) • hallerone	Ravikanth *et al*., 2000
Iridoid	• loganin • catalpol	Akhtar, 1993
Phenylethanoid glycosides	• acteoside (or verbascoside or kusaginin or russetinol or stereospermin) • 2'-*O*-acetylechinacoside	Khalil *et al*., 1995

Table 1.2: Continued.

Class of compounds	Name of chemical constituent	References
Others	• nodifloridin A • nodifloridin B	Joshi and Bhakuni, 1959
	• nodiflorin A • nodiflorin B	Joshi and Bhuwan, 1970
	• 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 *et al*., 1996
• Angiotensin converting enzyme inhibitory	Nyman *et al*., 1998
• Antiatherosclerotic	Shanmugasundaram *et al*., 1983
• Anticancer	Vanajothi *et al*., 2012 Sankaranarayanan *et al*., 2013
• Antidiabetic	Balamurugan and Ignacimuthu, 2011 Balamurugan *et al*., 2011
• Antihepatotoxic	Durairaj *et al*., 2008 Sampathkumar *et al*., 2008 Balamurugan *et al*., 2011 Narendra *et al*., 2012 Sudha *et al*., 2013 Arumanayagam and Arunmani, 2015
• Antihyperlipidemia	Balamurugan and Ignacimuthu, 2011 Balamurugan *et al*., 2011
• Antihypertensive	Akhtar, 1993 Gadhvi *et al*., 2012 Gadhvi *et al*., 2015
• Antiinflammatory	Forestieri *et al*., 1996 Balakrishnan *et al*., 2010 Jabeen *et al*., 2015 Ahmed *et al*., 2004

Table 1.3: Continued.

Reported biological activities	References
• Antimicrobial	Durairaj *et al*., 2007 Sivakumar, 2008 Jeya and Veerapagu, 2011 Ravikumar and Sudha, 2011 Malathi *et al*., 2011 Zare *et al*., 2012 Priyadarshni *et al*., 2013 Regupathi *et al*., 2014 Priya and Ravindhran, 2015 Taylor, 1996 Balakrishna *et al*., 1996 Hsueh *et al*., 2010 Jeyachandran *et al*., 2010 Patel *et al*., 2011 Salve and Bhuktar, 2012 Thamaraiselvi *et al*., 2013 Ullah *et al*., 2013 Jabeen *et al*., 2015 Anitha *et al*., 2013 Regupathi and Chitra, 2015 Pirzada *et al*. 2005 Manimegalai and Ambikapathy, 2012 Wang and Huang, 2005 Simonsen *et al*., 2001 Kavitha *et al*., 2012 Mako and Noor, 2006
• Antioxidant	Ashokkumar *et al*., 2008 Ashokkumar *et al*., 2009 Durairaj *et al*., 2008 Shukla *et al*., 2009b Lin *et al*., 2014
• Antipyretic	Forestieri *et al*., 1996
• Antiulcer	Sumalatha, 2012

Table 1.3: Continued.

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

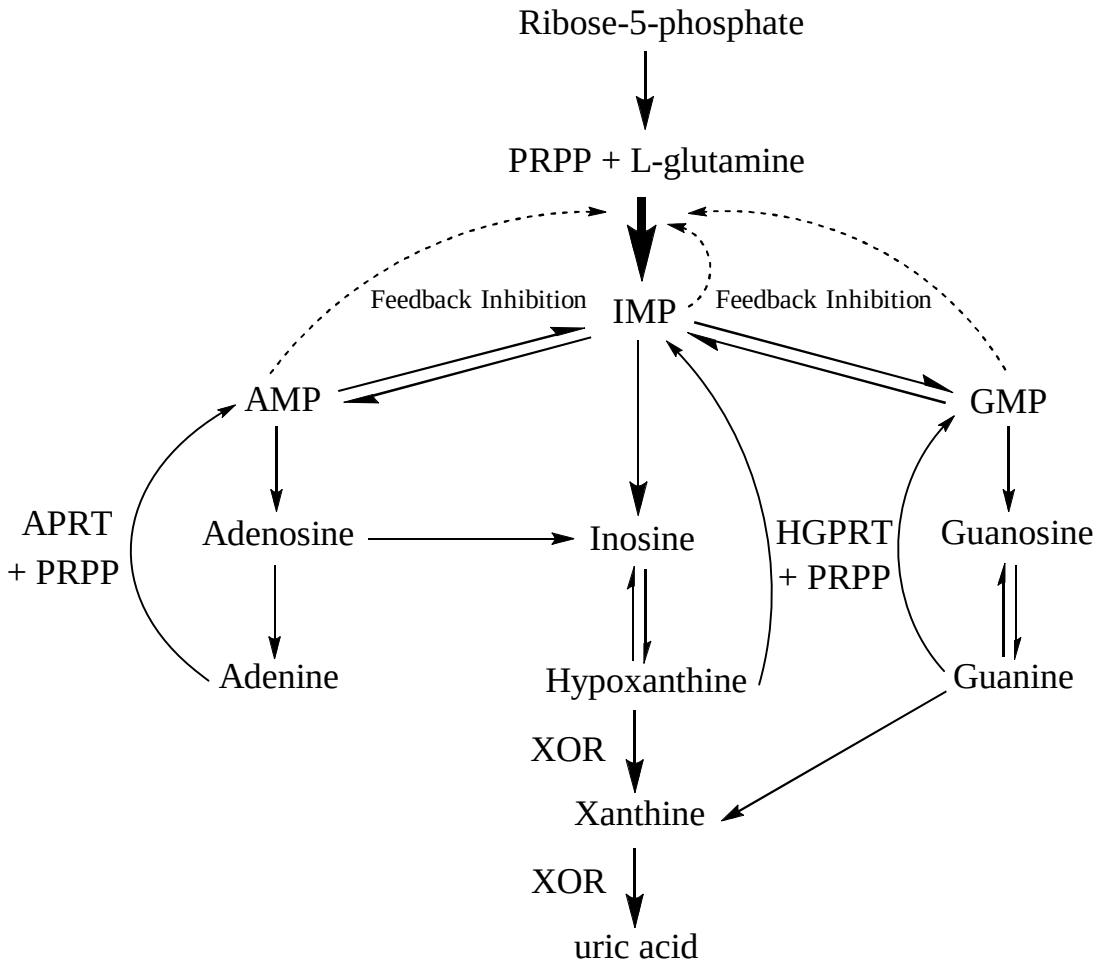

Figure 1.2: Purine biosynthesis *de novo* and salvage pathways (Choi *et al*., 2005).

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, $C_5H_4N_4O_3$) 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 (CO_2) and ammonia (NH_3) (Figure 1.3) (Florkin, 1949; Greene, 1954; Keilin, 1958 in Usuda *et al*., 1994).

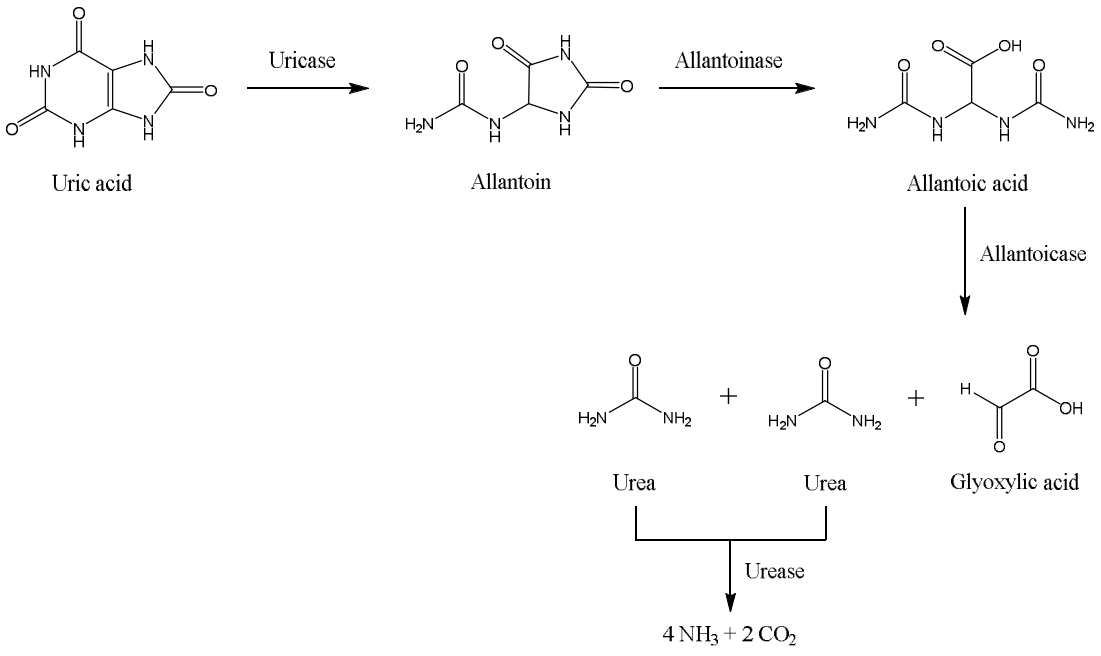

Figure 1.3: Uric acid degradation pathway (Florkin, 1949).

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