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

VIKNESWARAN A/L MURUGAIYAH

UNIVERSITI SAINS MALAYSIA

2008

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

by

VIKNESWARAN A/L MURUGAIYAH

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

JUNE 2008

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

ACKNOWLEDGEMENTS

In writing this thesis, I have relied on the active help of many people. First of all, I wish to extend my heartfelt gratitude to my supervisor, Professor Dr. Chan Kit Lam for his invaluable guidance, thoughtful comments, inspiring ideas and commitments throughout the years. I would like to record my thanks to the Ministry of Science, Technology and Innovation, Malaysia, Universiti Sains Malaysia and School of Pharmaceutical Sciences for providing PASCA scholarship, financial support and the necessary facilities for the completion of the project.

My gratitude also goes to Professors K. Takeya from School of Pharmacy, Tokyo University of Pharmacy & Life Sciences and H. Morita from Faculty of Pharmaceutical Sciences, Hoshi University and Associate Professor Osamu Shirota from Faculty of Pharmaceutical Sciences, Tokushima Bunri University for running mass spectra of the isolated compounds and nuclear magnetic resonance of niranthin. My special thanks to Mr. Peter Sprenger from Bruker Biospin, Thailand for running nuclear magnetic resonance of phyllanthin, hypophyllanthin and phyltetralin.

My sincere appreciation is also extended to staff of School of Pharmaceutical Sciences for their continual support and invaluable assistance in many different aspects throughout my study. My thanks also go to the staff of Lam Wah Ee Hospital, Penang for providing the support in running the blood analyses. My sincere thanks to my jovial and helpful colleagues who are too numerous to mention for their great support and encouragements. Their friendship has meant more than words can express. My greatest debt is owed to my parents for raising me up to where I am now, for their unconditional love and for giving me the hope, inspiration and determination to move on. Lastly I would like to thank all the individuals that have contributed directly or indirectly towards the completion of my study.

TABLE OF CONTENTS

CHAPTER TWO : MATERIALS AND METHODS

SECTION I CHEMISTRY

SECTION II PHARMACOLOGY

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

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

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

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

ABSTRAK

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

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

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

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

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

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

ABSTRACT

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

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

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

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

CHAPTER ONE

INTRODUCTION

1.1 Hyperuricemia: A global scenario and its management

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

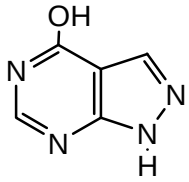
Hyperuricemia is often associated with a number of human diseases (Ruilope and Garcia-Puig, 2001). Classically, hyperuricemia is a major risk factor for gout, urolithiasis and uric acid nephropathy. In addition, it has also been linked with other diseases such as diabetes mellitus, preeclampsia, hypertension,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

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

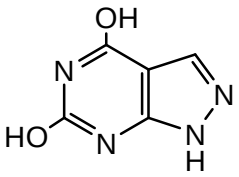
XO inhibitors reduce the blood uric acid level by inhibition of XO enzyme that is responsible for the formation of uric acid from purines. Consequence to the inhibition, the blood and urinary concentrations of uric acid are reduced and there is a simultaneous increase in the excretion of the more soluble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

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

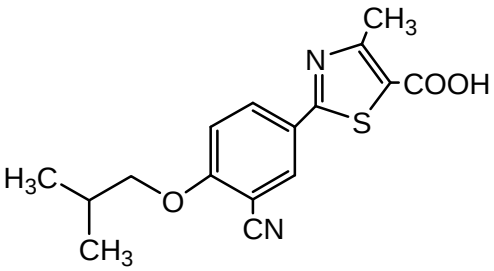
Allopurinol itself is metabolized by the XO enzyme, to its active metabolite oxypurinol (**2**). Although the half-life of allopurinol is 1 to 3 hours, its metabolite has a longer half-life ranging from 18 to 33 hours, thus prolonging the therapeutic effectiveness of allopurinol administered as a single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



1 allopurinol



2 oxypurinol

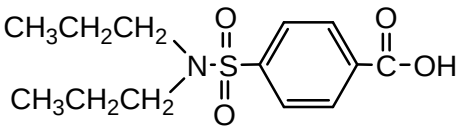


3 febuxostat

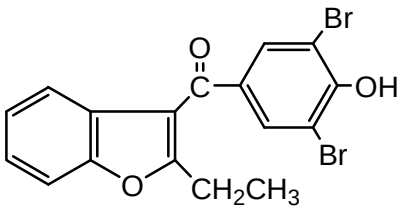
Recently, febuxostat (**3**) [2-(3-cyano-4-isobutoxyphenyl)-4-methylthiazole-5-carboxylic acid], a selective inhibitor of XO was developed in Japan. Febuxostat may become an alternative effective drug to allopurinol for use in the treatment of hyperuricemia and gout (Takano *et al*., 2005; Komoriya *et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

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

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



4 probenecid



5 benzbromarone

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

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

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

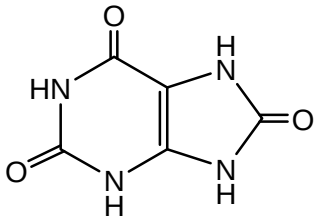
Besides the two classes of drugs, other pharmacological agents may also be used for the treatment of hyperuricemia and gout. Losartan and fenofibrate, in addition to their principal pharmacological activities, have blood uric acid lowering effect. Both of them diminish uric acid reabsorption at the proximal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

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

1.2 Literature review

1.2.1 Biochemistry and physiology of uric acid

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



6 Uric acid

1.2.1.1 Biosynthesis and regulation of uric acid formation

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

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

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

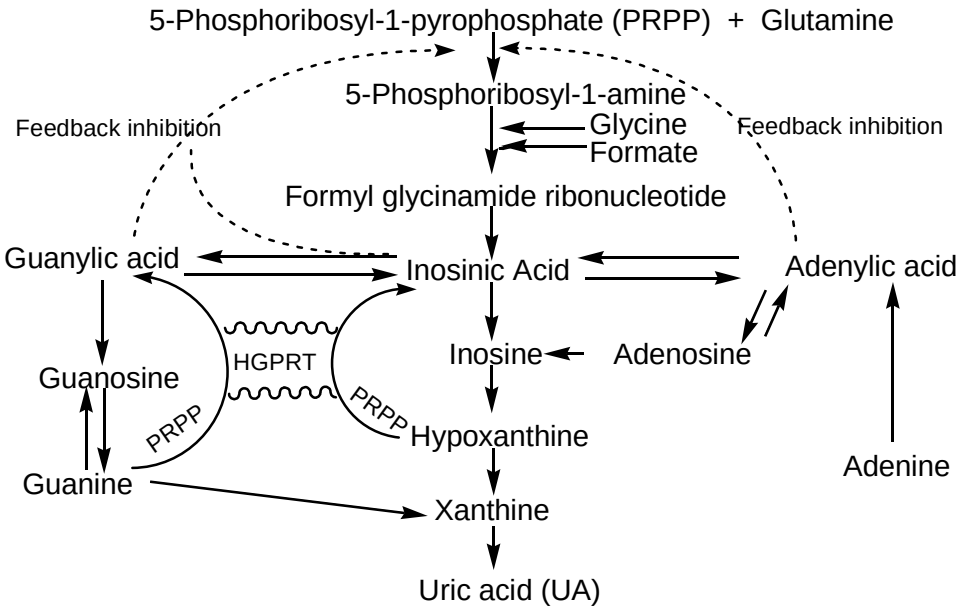


Figure 1.1 Pathways for biosynthesis of purines and the formation of uric acid (Seegmiller, 1976).

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

In mammals, the liver and intestine have the highest XOR activity (Pritsos, 2000; Krenitsky *et al*., 1986). The primary structure, catalytic properties and cofactor requirements of XOR are highly conserved with a 90 % homology among rat, mouse and human XOR enzymes (Pritsos, 2000). The XOR enzymes catalyze the oxidation of hypoxanthine to xanthine and xanthine to uric acid. However, their mechanisms of action are different in that the XD reduces NAD^+ (nicotinamide adenine dinucleotide) by a direct two-electron reduction whereas XO reduces molecular oxygen by a single electron. During the process, the substrates hypoxanthine and xanthine bind to the molybdenum site, and the electron acceptors NAD^+ and O_2 interact with the FAD cofactor (Pritsos, 2000; Fujimoto *et al*., 2000, Mondal *et al*., 2000).

1.2.1.2 Degradation of uric acid

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

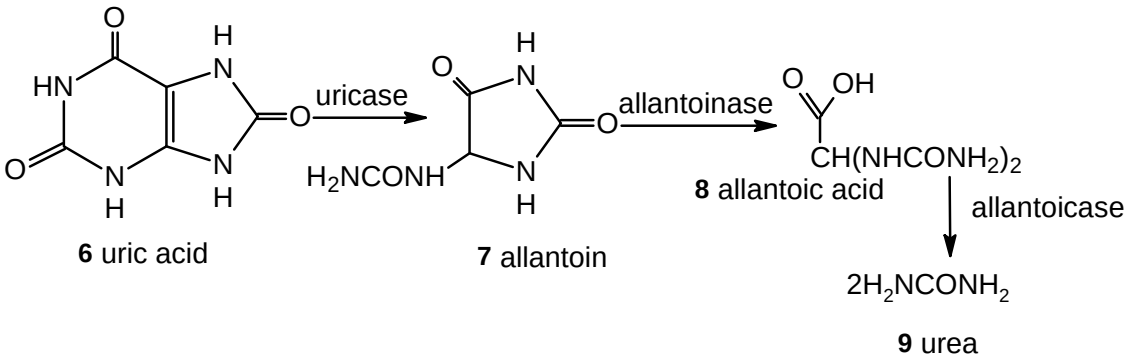


Figure 1.2 Enzymatic degradation of uric acid (Hitchings, 1978).

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

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

1.2.1.3 Uric acid disposal

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

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

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

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

Substances that decrease uric acid excretion	*Substances that increase uric acid excretion*
Lactate	Probenecid
Acetoacetate	Sulfinpyrazone
β-Hydoxybutyrate	Benzbromarone
Nicotinate	Losartan (antihypertensive drug)
Pyrazinamide/pyrazinoate	Tienilic acid (diuretic)

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

1.2.2 Hyperuricemia

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

Table 1.2: Classification of hyperuricemia

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

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

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

1.2.2.1 Experimental hyperuricemia in rodents

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

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

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

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

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

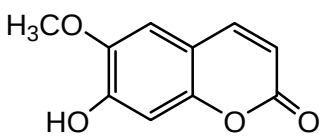
1.2.3 Role of medicinal plants and natural products in hyperuricemia

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

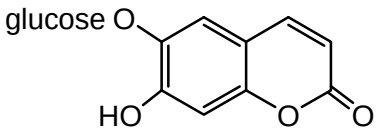
Diverse medicinal plants and natural products have been investigated as inhibitors of XO enzyme. Natural XO inhibitors from *in vitro* studies were reported from a variety of plants used as traditional herbal medicines such as *Coccinia grandis* and *Vitex negundo* in India (Umamaheswari *et al*., 2007),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

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

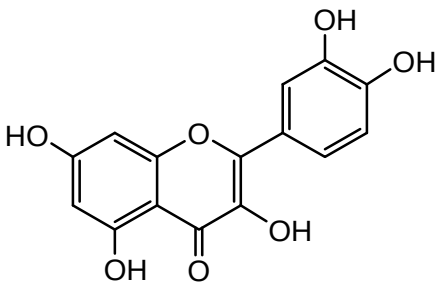
Scopoletin (**10**) isolated from *Erycibe obtusifolia* (Ding *et al*., 2005), aesculin (**11**) from *Fraxinus rhynchophylla* (Kong *et al*., 2002), quercetin (**12**) and rutin (**13**) from *Biota orientalis* (Zhu *et al*., 2004) exhibited a potent antihyperuricemic effect after administration in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



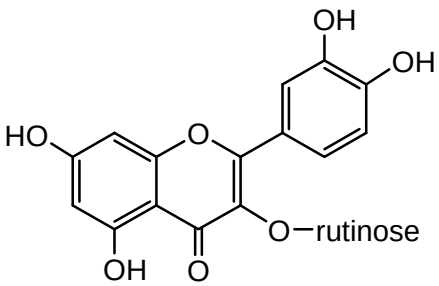
10 scopoletin



11 aesculin



12 quercetin



13 rutin

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

1.2.4 *Phyllanthus niruri* L.

1.2.4.1 Botanical aspects and geographical distributions

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Euphorbiales
Family	:	Euphorbiaceae
Genus	:	*Phyllanthus*
Species	:	*niruri*

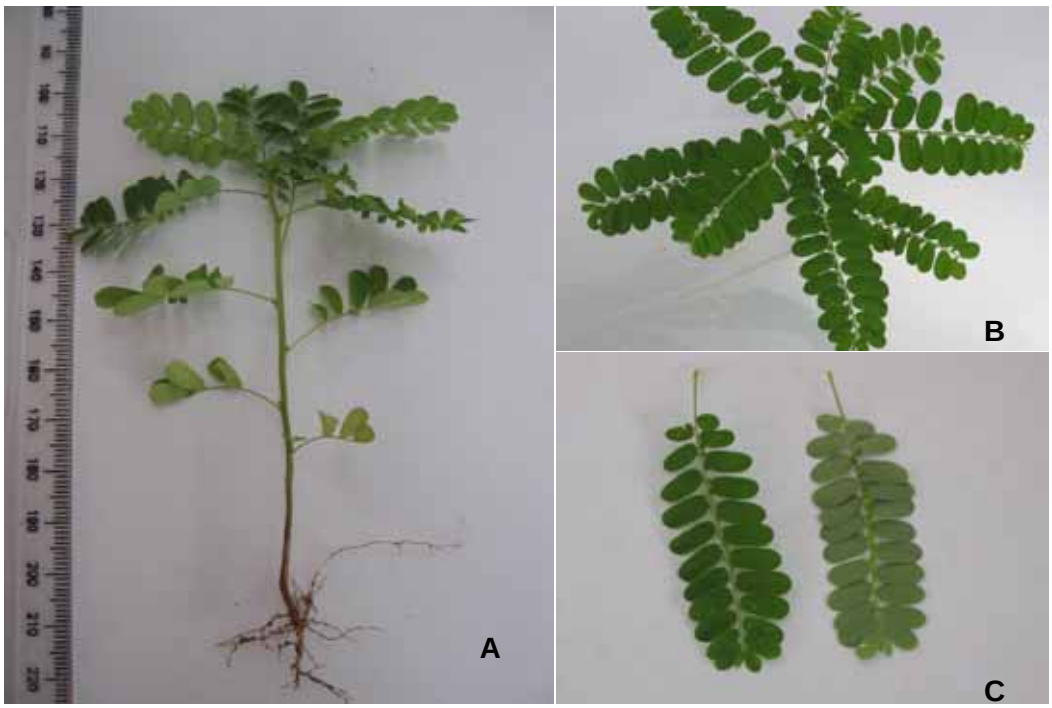


Figure 1.3 *Phyllanthus niruri* L.; (A) whole plant (B) aerial part (C) leaves.

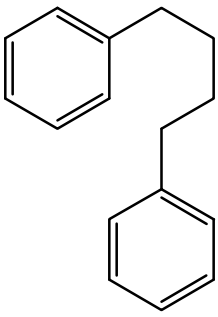
Phyllanthus niruri L., known locally as “dukong anak”, is found in most tropical and subtropical regions, commonly in fields, grasslands and forests. It is a small herb that grows up to 60 cm in height and can easily be differentiated from shrub species such as *P. pulcher* or *P. reticulatus*. The plant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

1.2.4.2 Chemical constituents of Phyllanthus niruri L.

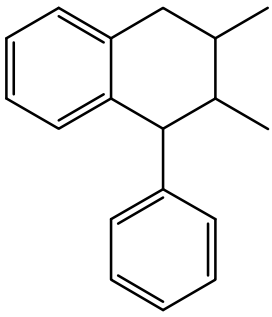
P. niruri has been the subject of much phytochemical studies since the mid 1960s. Different classes of organic compounds with various medical interest have been reported, the major being the lignans, tannins, polyphenols, alkaloids, flavonoids, terpenoids and steroids (Calixto *et al*., 1998). The following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

Lignans

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



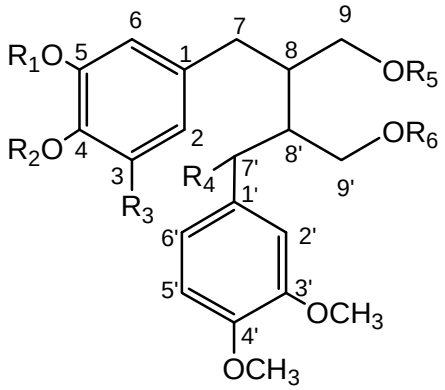
1,4-diarylbutane skeleton



1-aryltetralin skeleton

- *Diarylbutane lignans*

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



14 phyllanthin $R_1 = R_2 = CH_3$ $R_3 = R_4 = H$ $R_5 = R_6 = CH_3$

15 niranthin $R_1 + R_2 = CH_2$ $R_3 = CH_3$ $R_4 = H$ $R_5 = R_6 = CH_3$

16 seco-isolariciresinol trimethyl ether $R_1 = R_2 = CH_3$ $R_3 = R_4 = R_5 = H$ $R_6 = CH_3$

17 hydroxyniranthin $R_1 + R_2 = CH_2$ $R_3 = CH_3$ $R_4 = OH$ $R_5 = R_6 = CH_3$

19 2,3-desmethoxyseco-isolintetralin $R_1 + R_2 = CH_2$ $R_3 = R_4 = R_5 = R_6 = H$

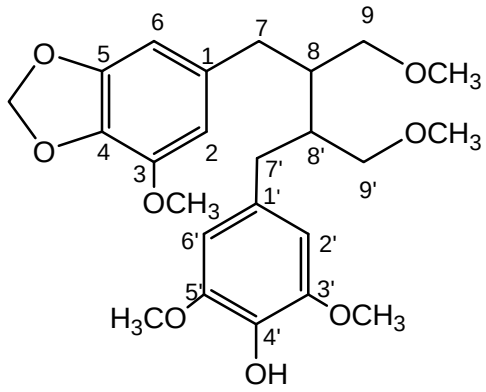
20 2,3-desmethoxyseco-isolintetralin diacetate $R_1 + R_2 = CH_2 R_3 = R_4 = H$ $R_5 = R_6 = COCH_3$

21 linnanthin $R_1 = R_2 = R_3 = CH_3$ $R_4 = H$ $R_5 = R_6 = CH_3$

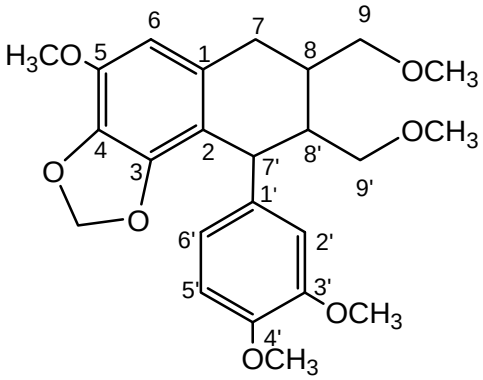
22 demethylenedioxyniranthin $R_1 = R_2 = H$ $R_3 = CH_3$ $R_4 = H$ $R_5 = R_6 = CH_3$

- *Aryltetralin lignans*

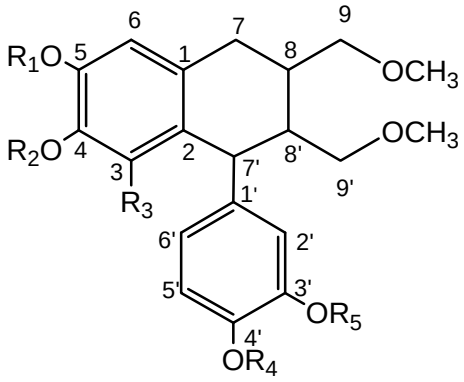
Hypophyllanthin (**23**) (Row and Srinivasulu, 1964), nirtetralin (**24**), phyltetralin (**25**) (Anjaneyulu *et al*., 1973), lintetralin (**26**) (Ward *et al*., 1979), isolintetralin (**27**) (Huang *et al*., 1992), neonirtetralin (**28**) (Wei *et al*., 2002).



18 nirphyllin



23 hypophyllanthin



24 nirtetralin $R_1 + R_2 = CH_2$ $R_3 = OCH_3$ $R_4 = R_5 = CH_3$

25 phyltetralin $R_1 = R_2 = CH_3$ $R_3 = H$ $R_4 = R_5 = CH_3$

26 lintetralin $R_1 = R_2 = CH_3$ $R_3 = H$ $R_4 + R_5 = CH_2$

27 isolintetralin $R_1 + R_2 = CH_2$ $R_3 = H$ $R_4 = R_5 = CH_3$

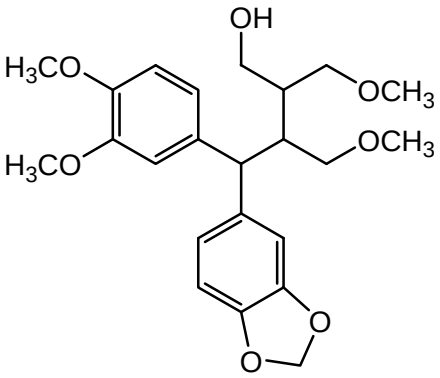
28 neonirtetralin $R_1 + R_2 = CH_2$ $R_3 = OCH_3$ $R_4 = R_5 = CH_3$

- *Other lignans*

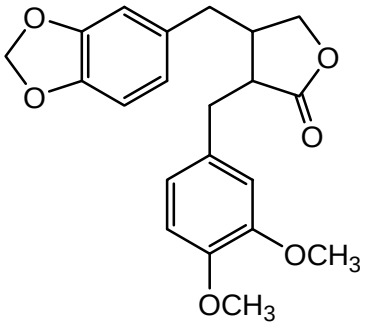
Seco-4-hydroxylintetralin (**29**), dibenzylbutyrolactone (**30**) (Satyanarayana *et al*., 1988), hinokinin (**31**) (Huang *et al*., 1992).

- *Neolignan*

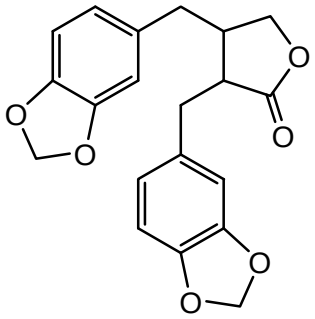
Phyllnirurin (**32**) (Singh *et al*., 1989a).



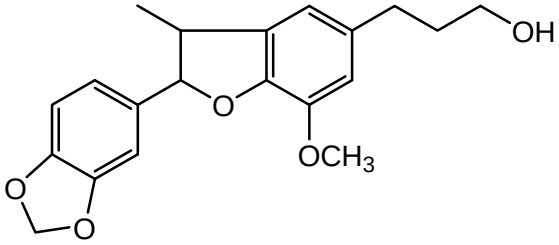
29 seco-4-hydroxylintetralin



30 dibenzylbutyrolactone



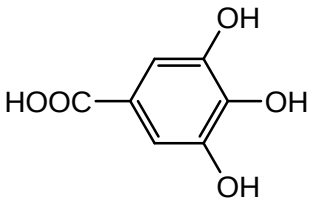
31 hinokinin



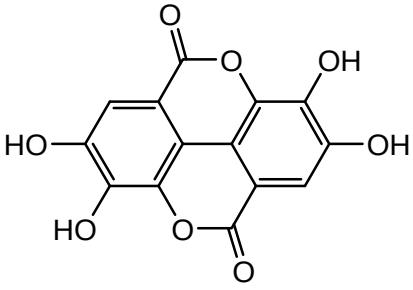
32 phylnirurin

Coumarins, tannins and related polyphenols

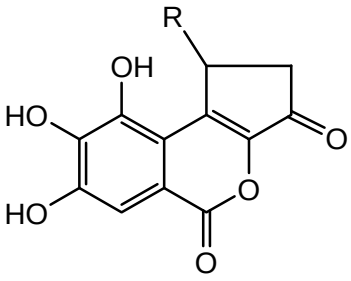
The following coumarins, tannins and polyphenols have been isolated from *P. niruri*: gallic acid (**33**), ellagic acid (**34**), brevifolin carboxylic acid (**35**), ethyl brevifolin carboxylate (**36**) (Shmizu *et al*., 1989), methyl brevifolin carboxylate (**37**) (Iizuka *et al*., 2006), geraniin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



33 gallic acid



34 ellagic acid

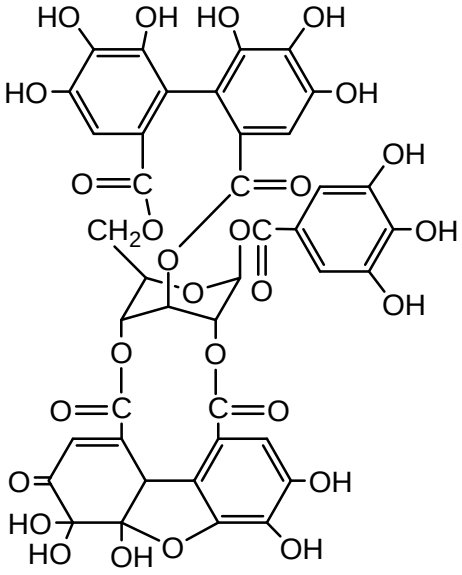


R

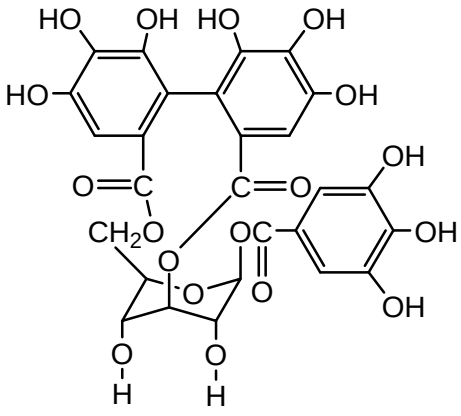
35 brevifolin carboxylic acid COOH

36 ethyl brevifolin carboxylate $COOCH_2CH_3$

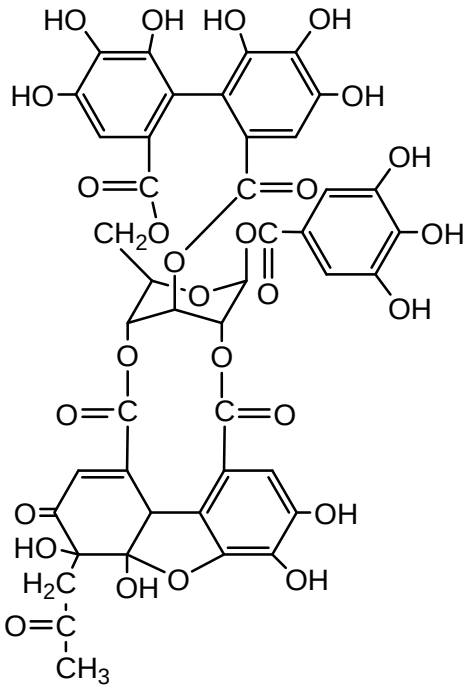
37 methyl brevifolin carboxylate $COOCH_3$



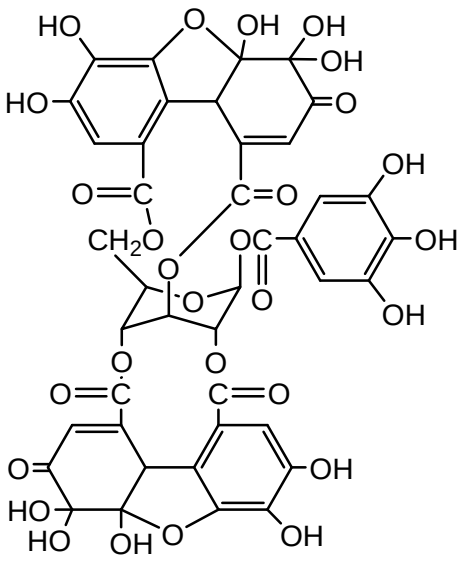
38 geraniin



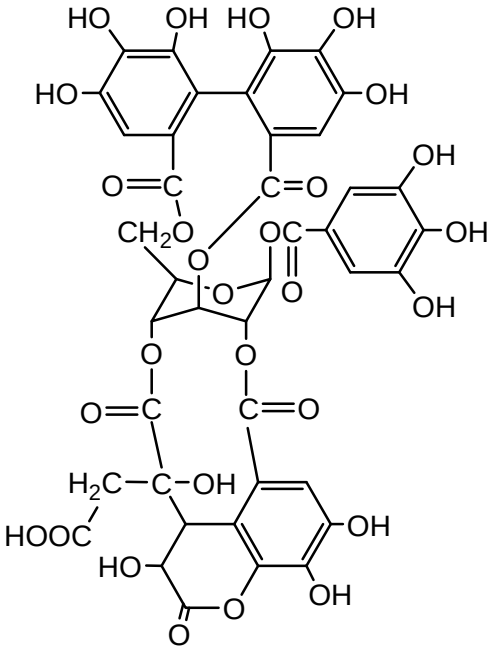
39 corilagin



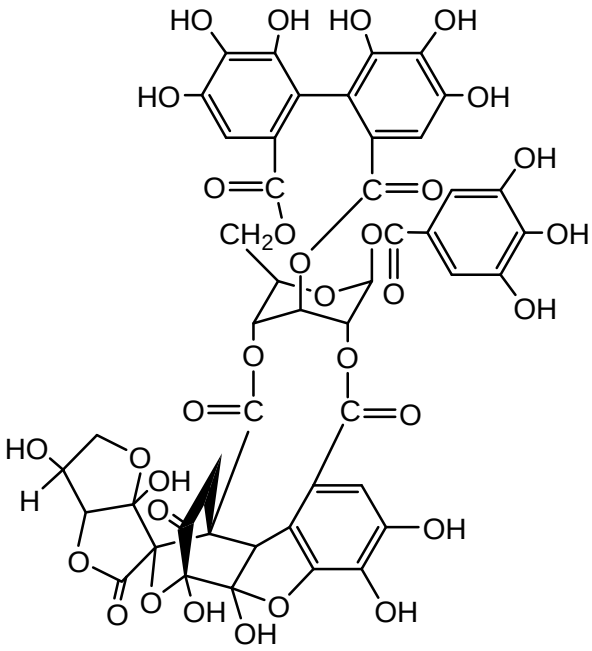
40 phyllanthusiin D



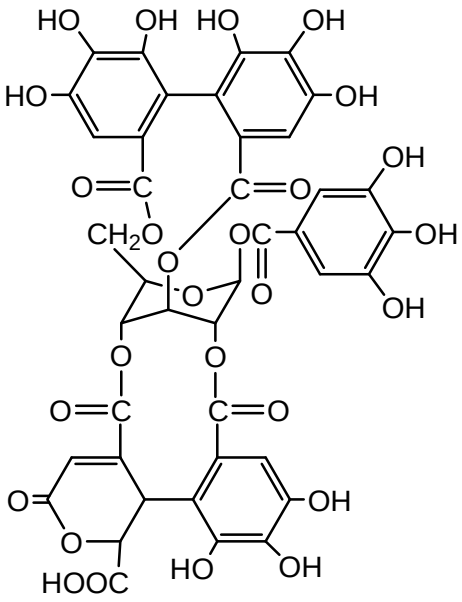
41 amariin



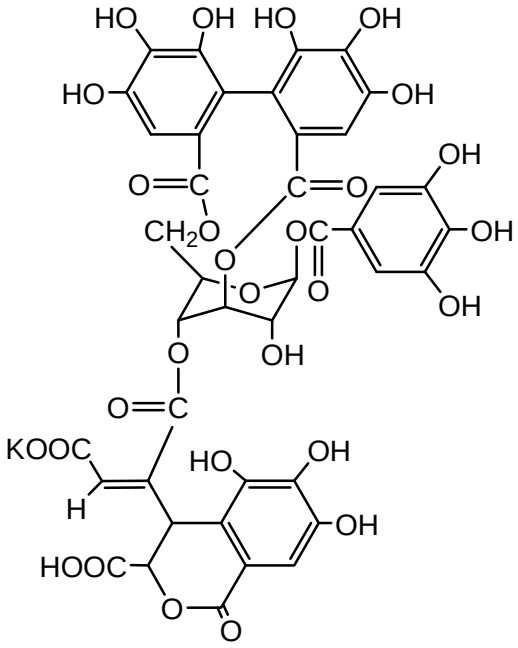
42 amariinic acid



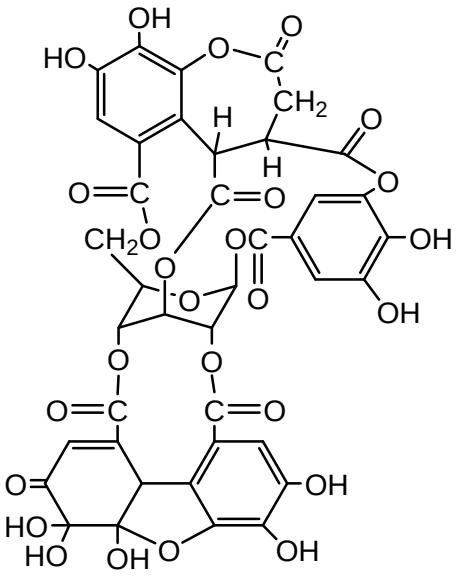
43 elaeocarpusin



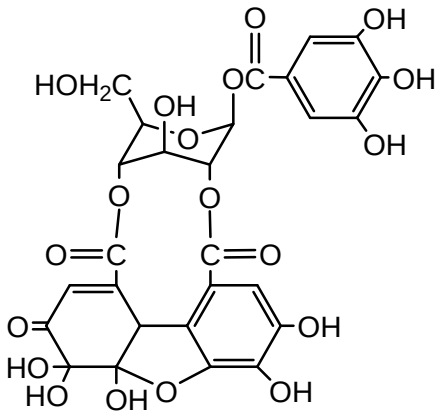
44 geraniinic acid



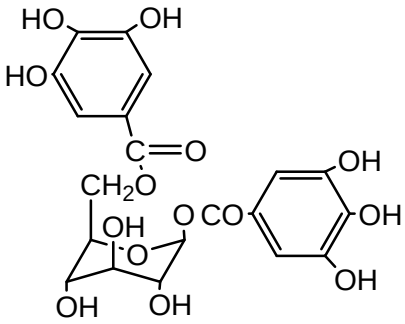
45 repandusinic acid

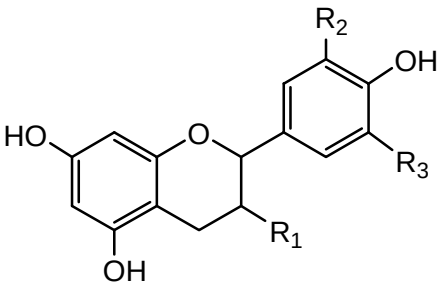


46 amarulone



47 furosin



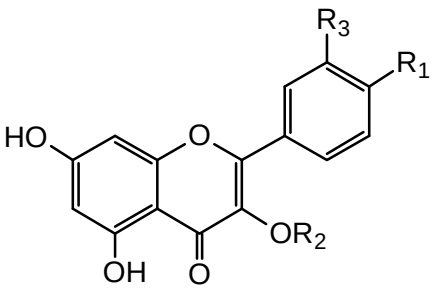


48 1,6-digalloyl glucopyranoside

		R1	R2	R3
49	catechin	OH (β)	OH	H
50	epicatechin	OH (α)	OH	H
51	gallocatechin	OH (β)	OH	OH
52	epigallocatechin	OH (α)	OH	OH
53	epicatechin 3-*O*-gallate	O-gallate (α)	OH	H
54	epigallocatechin 3-*O*-gallate	O- gallate(α)	OH	OH

Flavonoids

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



55	astragalin	R1 = OH	R2 = glucose	R3 = H
56	quercitrin	R1 = OH	R2 = rhamnose	R3 = OH
57	isoquercetin	R1 = OH	R2 = glucose	R3 = OH