EVALUATION ON POLAR QUASSINOIDS OF *EURYCOMA LONGIFOLIA* JACK AS POTENTIAL ANTI-BENIGN PROSTATIC HYPERPLASIA AGENTS

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2024

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by

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

August 2024

ACKNOWLEDGEMENT

Many people have actively helped and contributed directly and indirectly in completion of my research. I will always truly remember them in my heart.

My thanks go first and foremost to my supervisor, Dr. Lee Chong Yew for his trusted, invaluable guidance and support in all aspect of my work. His patience and hard work are deeply appreciated. His insightful comments and suggestions have always inspired me and have been of immense help to me in maintaining the excellence of the research. I honestly say that without my supervisor support my research would have been a daunting task. I will forever be thankful to him for everything he offered to me.

I owe special thanks to my co-supervisor, Professor Chan Kit Lam for his valuable suggestions throughout my research. His support led to the development of three new compounds.

I would like to thank my co-supervisor, Dr. Vikneswaran Murugaiyah for his valuable help in my biological works. His suggestions helped me to overcome many impediments I encountered during the animal studies I would like to thank Mr. Khor Boon Keat for his major contribution to my biological research by performing cell culture work.

I also thank the Discipline of Pharmaceutical Chemistry faculties for allowing me to use various analytical instruments such as NMR, Preparative HPLC, Analytical HPLC, LC-MS, UV, and IR Spectrometers, which significantly helped me to have good results.

My working with the people in the Pharmacy School has been a wonderful experience. I would especially like to acknowledge with thanks to colleagues Ms. Wan Jie, Ms. Lam, Mr. Al-Ameen, Dr. Wakas, Dr. Kaiser, Dr. Chin Hoe, Dr. Madjeed, Dr. Selastin, and Mr. Selva for their support in various ways that immensely helped me to complete my research.

I would also like to thank the Ministry of Higher Education of Malaysia for the Fundamental Research Grant Scheme for the funding of this research. Finally, the research would not have been possible without the unfailing support of my family members. Their financial support, patience and encouragements have made this endeavor possible. They kept me going when I was tired and frustrated.

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LIST OF SYMBOLS

J	Coupling constant
m/z	Mass-to-charge ratio
δ	Chemical shift
δ_c	Chemical shift of carbon
δ_H	Chemical shift of proton
s	singlet
d	doublet
dd	Doublet of doublets
td	Triplet of doublets
t	Triplet
dt	Doublet of triplets
m	Multiplet
br s	Broad singlet
Hz	Hertz
MHz	Megahertz
ppm	Parts per million
$[a]_D^{19}$	Specific optical rotation at 19° C and sodium D line
Glu	Glucose unit
$\Delta^{x,y}$	Olefin at positions x and y of the scaffold
w/w	Weight in grams per total weight of 100 g
w/v	Weight in grams per total volume of 100 mL

LIST OF ABBREVIATIONS

BPH	Benign prostate hyperplasia
^{13}C NMR	Carbon-13 nuclear magnetic resonance
CLogP	Logarithm of partition coefficient by group contribution
COSY	Correlated Spectroscopy
DHT	Dihydrotestosterone
ESI	Electron Spray Ionization
FBS	Fetal bovine serum
FTIR	Fourier transformed infrared spectroscopy
^{1}H NMR	Proton nuclear magnetic resonance
H&E	Haematoxylin and eosin
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
HSQC	Heteronuclear Single Quantum Correlation
KBr	Potassium bromide
LUTS	Lower urinary tract syndrome
MS/MS	Tandem mass spectrometry
NOESY	Nuclear Overhauser Effect Spectroscopy
PBS	Phosphate buffered saline
PI	Prostate index
p.o.	Given orally
PSA	Prostate serum antigen
PW	Prostate weight

QSAR	Quantitative structure activity relationship
s.c.	subcutaneously
TAF2	Tongkat Ali fraction 2
tPSA	Topological polar surface area
TP	Testosterone propionate
UV	Ultraviolet spectroscopy

PENILAIAN KE ATAS KUASINOID BERKUTUB DARIPADA EURYCOMA LONGIFOLIA JACK YANG BERPOTENSI SEBAGAI AGEN ANTI-HIPERPLASIA PROSTAT BENIGNA

ABSTRAK

Kuasinoid *Eurycoma longifolia* Jack menunjukkan aktiviti anti-proliferatif terhadap sel kanser prostat. Hipotesis kajian ini ialah sebatian kelas kuasinoid juga aktif terhadap sel prostat hyperplastik benigna (BPH). Maka, objektif tesis ini ialah pertamanya, untuk memencilkan kuasinoid yang diketahui dan baharumelalui fraksinasi sistematik terhadap fraksi polar tumbuhan berkenaan, dan keduanya, menentukan aktiviti kuasinoid berkenaan sebagai agen anti-hiperplasia prostat benigna yang berpotensi, secara *in vitro* dan *in vivo*. Fraksinasi sistematik terhadap ekstrak etanol akar *E. longifolia* menghasilkan empat pecahanutama iaitu TAF1, TAF2, TAF3, dan TAF4, dalam turutan kekutuban menurun. Fraksi kedua paling polar, TAF2 didapati mengandungi sebahagian besar kuasinoid yang disasarkan untuk beberapa langkah pemencilan. Hasilnya, sembilan sebatian kuasinoid yang diketahui dan tiga sebatian yang belum dilaporkan telahberjaya dipencilkan iaitu **71**, suatu kuasinoid C_{19} jenis pikrasinolid; **72**, suatu glikosida kuasinoid C_{20}; dan **73**, suatu kuasinoid C_{20}. Struktur sebatian tersebut serta konfigurasi masing-masing dikenalpasti melalui teknik-teknik spektroskopi. Dua belas kuasinoid ini seterusnya dinilai aktiviti merencat pertumbuhan sel dalam sel hyperplasia prostat benigna BPH-1 serta sel fibroblas kulit Hs27 yang mewakili sel bukan BPH. Eurikomanon (**3**), 13α,21-dihidroeurikomanon (**12**) dan 13(α)21-epoksieurikomanon (**15**) merupakankuasinoid paling poten merencat pertumbuhan sel BPH-1 pada 10 µM, berbanding finasterida, ubat anti-BPH yang digunakan sebagai kawalan positif. Akan tetapi, kuasinoid tersebut didapati juaga toksik

terhadap sel Hs27. Berbeza dengan **3**, **12** dan **15**, kuasinoid **71** menunjukkan aktiviti sedehana terhadap sel BPH-1 tetapi tidak toksik terhadap sel Hs27. Analisis hubungan struktur-aktiviti mendapati moiti keton α,β-tak epu dalam gelang A penting bagi aktiviti kuasinoid serta peranan lipofilikam dalam menentukan aktiviti kuasinoid dalam kedua-dua sel BPH-1 dan Hs27. TAF2, sumber kepada dua belas kuasinoid dalam kajian ini dan eurikomanon (**3**) dipilih bagi penilaian selanjutnya dalam suatu model tikus BPH diujakan testosteron selama 28 hari. Eurikomanon pada dos oral 5 mg/kg didapti berkesan menurunkan berat prostat ($P < 0.05$) dan nisbah berat prostat:badan ($P< 0.001$) tikus BPH. Tikus BPH yang diberi TAF2 (25 mg/kg) juga menunjukkan penurunan berat prostat ($P < 0.01$). Finasterida (10 mg/kg) bagaimanapun, lebih berkesan dalam menurunkan berat prostat dan nisbah berat prostat:berat badan tikus BPH berbanding eurikomanon. Ini mencadangkan bahawa kedua-dua drug mepunyai mekanisme tindakan dan farmaokinetik yang berbeza. Slaid tisu prostat menunjukkan eurikomanone secara signifikan membaikpulih ciri histopatologi prostat kepada ciri normal. Dapatan kajian ini memberikan bukti bagi menyokong pembangunan selanjutnya kuasinoid polar *Eurycoma longifolia* sebagai fitoterapi anti-BPH. Syor-syor dicadangkan bagi menambaik kuasinoid.

EVALUATION ON POLAR QUASSINOIDS OF *EURYCOMA LONGIFOLIA* JACK AS POTENTIAL ANTI-BENIGN PROSTATIC HYPERPLASIA AGENTS

ABSTRACT

The quassinoids of *Eurycoma longifolia* Jack have shown promising antiproliferative activity against prostate cancer cells. This study hypothesized that this class of compounds would also be active against benign prostate hyperplastic (BPH) cells. Hence, this thesis aimed firstly, to isolate known and novel quassinoids by systematic fractionation of the polar fraction of the plant, and secondly, to determine the activity of the quassinoids as potential anti-benign prostate hyperplasia agents, *in vitro* and *in vivo*. Systematic fractionation of 70% alcoholic extract of *E. longifolia* roots through Diaion HP20 resin column produced four major fractions TAF1(10% Methanol in water), TAF2(30% Methanol in water), TAF3(50% Methanol in water), and TAF4(100% Methanol in water), in decreasing order of polarity. The second most polar fraction TAF2, found to contain a majority of the targeted quassinoids, was further pursued through a series of fractionation and purification steps, resulting in the isolation of nine known quassinoids and three hitherto unreported compounds: **71**, a picrasinolide-type C_{19} quassinoid; **72**, a C_{20} quassinoid glycoside; and **73**, a C_{20} quassinoid. The structures of the compounds were confirmed and their configurations assigned by an array of spectroscopic techniques. The twelve quassinoids were evaluated *in vitro* in a benign prostate hyperplasia cell line BPH-1 for their ability to inhibit cell growth. They were tested in parallel in a human skin fibroblasts Hs27 which represented non-BPH cells. Eurycomanone (**3**), 13α,21-dihydroeurycomanone (**12**) and 13α,21-epoxyeurycomanone (**15**) were the most potent quassinoids inhibiting BPH-1 cell growth at 10 µM, relatively better than finasteride, an anti-BPH medication used as

the positive control. However, the three quassinoids were also similarly toxic in the HS27 fibroblasts. In contrast, quassinoid **71** showed moderate activity against BPH-1 but no significant toxicity to the HS27 cells. Structure-activity relationship analysis revealed the importance of an α,β-unsaturated ketone within the ring A of the scaffold for potent activity and the contribution of lipophilicity towards antiproliferative activity in both cell lines. The plant fraction which bore the twelve quassinoids, TAF2 and eurycomanone (**3**) were selected for further evaluation in a 28-day testosterone-induced rat model of BPH. Eurycomanone at an oral dose of 5 mg/kg was efficacious at reducing the prostate weights ($P < 0.05$) and prostate-to-body weight ratios ($P < 0.001$) of the BPH rats. BPH rats treated with TAF2 (25 mg/kg) also showed significant reduction in prostate weights ($P < 0.01$). Finasteride (10 mg/kg) however, showed a better efficacy, reducing prostate weight and prostate-to-body weight ratio to larger extents than eurycomanone, suggesting a difference in mechanism of action and pharmacokinetics between the two drugs. The prostate tissue slides revealed eurycomanone markedly restored the histopathological features of the prostate tissue to those of the normal prostate, consistent with the prostate weight reduction observed. The present findings of this study provided evidence to support further development of the *Eurycoma longifolia* polar quassinoids as anti-BPH phytomedicine. Recommendations were made for structural improvement of the quassinoid in view of the present findings.

CHAPTER 1

INTRODUCTION

1.1 Benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH), also known as prostate enlargement refers to the nonmalignant growth and increase in size of the prostate gland. BPH is one of the most common diseases among aging men that negatively impacts their health-related quality of life and raises the need for considerable medical intervention and expenses (Patel & Parsons, 2014). Disease prevalence has been shown to increase with advancing age. Histologic prevalence of BPH could be seen as early in men in their 40s (20 %), reaches 50 to 60 % for men in their 60s and is about 80 to 90 % for men in their 70s and 80s (Lim, 2017). Indeed, when living long enough, most men will develop some histologic features that are consistent with BPH.

The development of BPH is characterized by an increased proliferation of both stromal and epithelial cells within the prostate transition zone (surrounding the urethra) (Figure 1.1). This leads to the compression of the urethra and the development of bladder outflow obstruction which is a major cause in the manifestation of a set of symptoms known as lower urinary symptoms (LUTS).

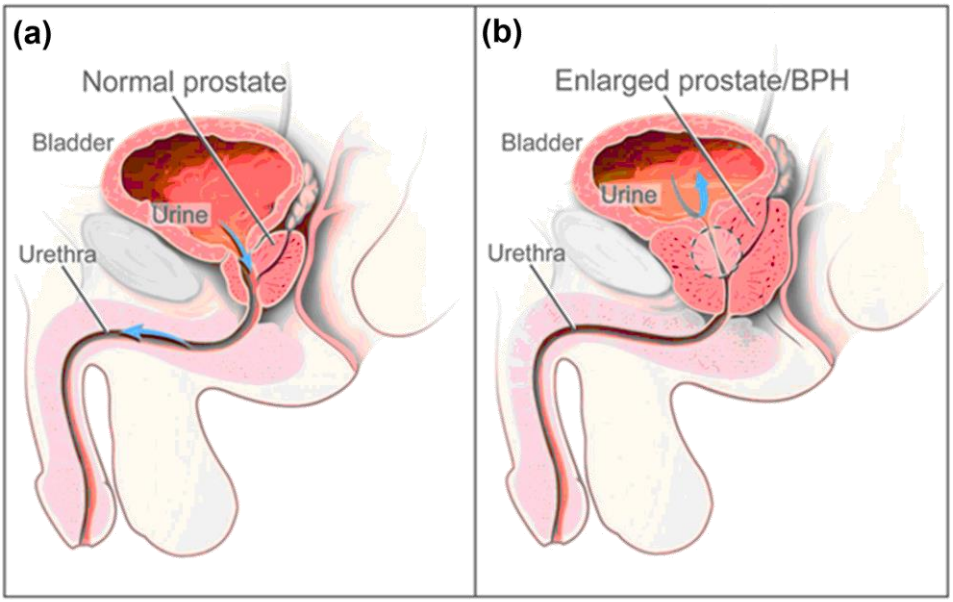


Figure 1.1. Anatomy of the lower urinary tract and the prostate gland in (a) normal condition and in (b) BPH. (Source: National Cancer Institute Visual Online, 2008).

The LUTS symptom complex may be divided into obstructive and irritative symptoms (Roehrborn, 2005). Men with BPH experience obstructive symptoms such as urinary hesitancy, straining, weak flow, prolonged voiding, partial or complete urinary retention, and ultimately overflow incontinence. The often more bothersome irritative symptoms include frequency, urgency with urge incontinence, night urination, painful urination, small voided volumes. The prevalence of LUTS also correlates closely with advancing age indicating that histologic hyperplasia has to develop with time to an extent sufficient to cause bladder obstruction and manifestation of LUTS. Although less common to occur, BPH may also lead to severe complications such as sudden and complete urinary retention requiring insertion of a catheter for urine removal, development of stones in the bladder or kidney, bladder wall damage or decompensation, blood in the urine, urinary tract infection, renal dysfunction, incontinence and erectile dysfunction (Speakman & Cheng, 2014; Tubaro *et al.*, 2003).

Not all men with obstructive or irritative voiding symptoms will be bothered by these symptoms, and thus would not seek medical attention. In many cases, these symptoms are accepted as a natural occurrence of aging, and men have learned to live with them. Also, the threshold for men to seek for consultation with a health care provider for LUTS differs widely within and between ethnic groups. Ultimately, when men become significantly bothered by the symptoms, they will usually seek medical consultation and treatment.

1.2 Pathogenesis of BPH

Despite the prevalence of BPH in men of advanced age, the pathogenesis of the condition is only partially understood. For example, it is largely unknown why some men develop a 4-gram prostate and others a 200-g prostate, while a small proportion of the population do not develop BPH despite their old age. The mechanisms leading to the development of BPH are likely multifactorial and involve several pathways.

The hormonal pathway is notable and has been the basis for current BPH treatment namely the 5-α reductase inhibitors. The prostate like other sex-accessory tissues, is stimulated in its growth and secretory function by the constant presence of hormones and growth factors (Madersbacher, Sampson & Culig, 2019). The presence of the male hormone testosterone is under the control of the hypothalamus-pituitary-testicular axis. Testosterone originating from the testes and the adrenal gland is the major hormone stimulating the growth of the prostate gland. Testosterone levels remain fairly constant between 25 to 60 years of age at an average of 600 ng/mL but starts to decline gradually with old age. Despite testosterone being the major androgen in the blood, it appears to function as a prohormone. The more potent androgen driving prostate growth is dihydrotestosterone (DHT), produced by the enzyme 5α-reductase from testosterone. Beside DHT, the female hormone estrogen, generated by the enzyme

aromatase from the aromatization of testosterone also plays a role in prostate hyperplasia (Figure 1.2).

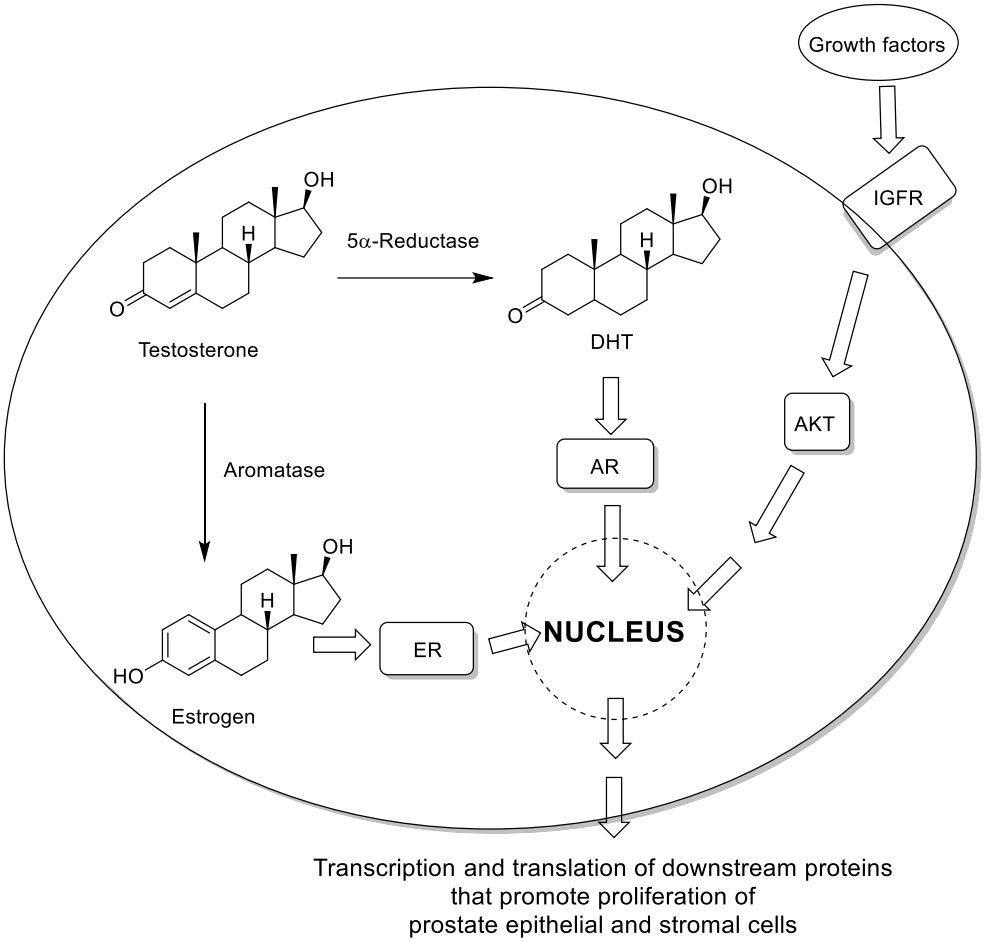


Figure 1.2. The hormonal pathway contributing to BPH. AR: Androgen receptor, ER: Estrogen receptor, IGFR: Insulin-like growth factor receptor, AKT: Protein kinase B.

The observation of chronic inflammation co-existing with BPH histologic changes in pathological tissue specimens has led to the suspicion that inflammation plays an important role in fueling prostate hyperplasia and the development of BPH and LUTS (De Nunzio, Presicce & Tubaro, 2016). Chronic inflammation localized at the prostate leads to the accumulation of immunocompetent cells, mainly T-lymphocytes and macrophages which produce cytokines, interleukins and growth factors and participate in the inflammatory process. The prostate epithelial and stromal cells themselves possess cytokine receptors on their cell membrane and also participate in creating the local inflammatory microenvironment. The origin for this prostate inflammation remains a subject of argument and is likely to be multifactorial. The inflammatory process may be triggered by viral or bacterial antigens, chemical irritants such as uric acid crystals and the co-existence of metabolic disorders such as insulin resistance, hypertension, obesity and dyslipidemia (Figure 1.3).

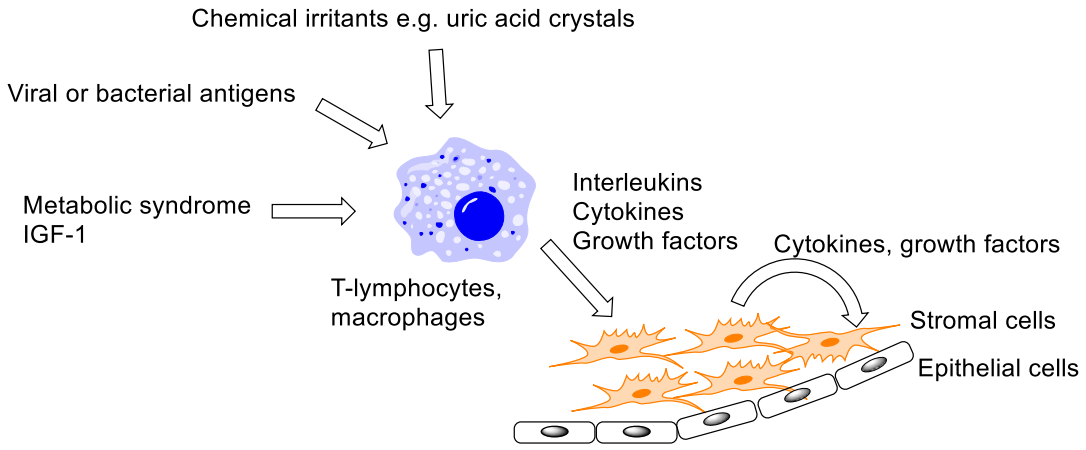


Figure 1.3. The inflammatory pathway contributing to BPH.

The third major contributor to the development of BPH are the presence of metabolic diseases. Preclinical and clinical studies have indicated that several age-related metabolic disorders such as diabetes mellitus, insulin resistance, obesity, hypertension and dyslipidemia

are important determinants to the development of BPH (Ngai et al., 2017). In the Baltimore Longitudinal Study of Aging cohort, each 1 kg/m^2 increase in body mass index (BMI) corresponded to a 0.4-mL increase in prostate volume (Parsons et al., 2006). Obese participants in the study had a 3.5-fold increased risk of developing BPH versus non-obese participants. The underlying pathophysiology linking metabolic disorders to BPH is not completely understood, but systemic inflammation, insulin resistance leading sympathetic nervous system activation and an increase in prostate smooth muscle tone, and pelvic atherosclerosis (resulting from hypertension and dyslipidemia) may play a role in BPH development.

1.3 Current pharmacological treatment modalities for BPH

The LUTS of prostate hyperplasia are mainly due to two factors: the dynamic overactive tone of the prostatic smooth muscles and bladder neck, and the static enlarged prostatic growth that causes mechanical obstruction. Current mainstream drug therapy modalities for BPH aim to alleviate the LUTS of the disease by addressing these two factors (Yu et al., 2020).

α1-Adrenergic receptor antagonist or α1-blockers such as doxazocin, terazosin and tamsulosin are among the most commonly prescribed medications to address overactive smooth muscles of the prostate. This class of drugs act by blocking the α1-receptors that are highly expressed in the smooth muscles of the prostate, bladder, and urethra, leading to smooth muscle relaxation. α1-Blockers can significantly improve the urinary symptoms of BPH including voiding and storage symptoms with the effects seen within a few weeks. Since these drugs only target the α1-receptor, they do not have any effect in reducing the size of the enlarged prostate. As with agents that act on the autonomic nervous system, α1-Blockers come with potential cardiovascular side effects such as orthostatic hypotension, dizziness, and in some cases, ejaculatory dysfunction.

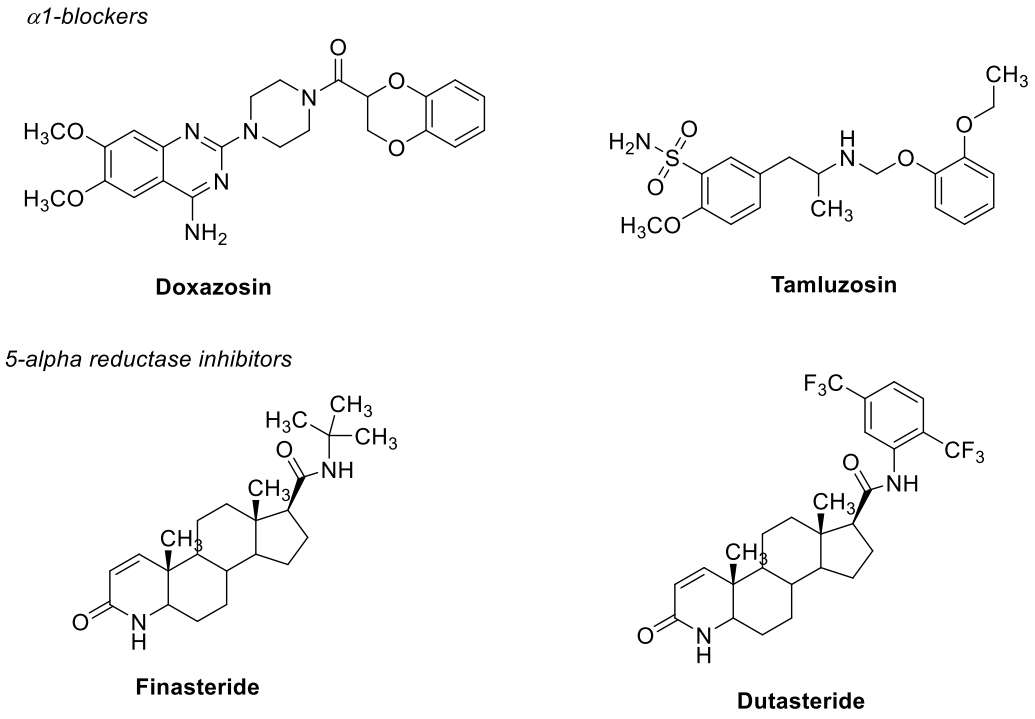


Figure 1.4. Structures of current drugs to treat BPH.

5α-Reductase inhibitors represent the second class of drugs frequently used to treat BPH. The high levels of DHT in the prostate have been implicated for the uncontrolled growth of the prostate tissue. DHT, a potent androgen hormone that drives prostate cell proliferation is generated from testosterone by the enzyme 5α-reductase. 5α-Reductase inhibitors are agents that inhibit this enzyme thus leading to the reduction of DHT. Examples of these drugs are finasteride and dutasteride. These drugs are effective at slowing the proliferation of the prostate cells, reducing the prostate serum antigen (PSA) levels and prostate volumes. They are thus most suitable for patients with moderate to severe LUTS with an overtly enlarged prostate. Studies have shown that 5α-reductase inhibitors are more efficacious at slowing further prostate enlargement, deterioration of LUTS, and preventing the occurrence of acute urinary retention,

a complication that potentially occurs as the disease worsens (Kaplan et al., 2011). However, potent modification of the androgens as a mechanism of action of these agents are also accompanied by some hormone-related, unpleasant adverse effects. These include decreased libido, gynecomastia (enlarged breast), and erectile dysfunction.

1.4 Potential phytomedicines for BPH

Population based surveys have found that considerable number of men experiencing LUTS do not consult health care providers to seek medical treatment for their disease (Speakman et al., 2015). Urinary tract problems being associated with shame and embarrassment may be partly the reason. Some patients simply accept these symptoms as an inevitable part of the aging process. While others may have reservations with the medical treatments prescribed for BPH due to the undesirable side effects these medications may bring about.

Alternatively, men with LUTS would tend to seek a plant-based remedy or phytomedicine. The phytomedicine may be in the form of a crude plant extract, herbal teas, an extract standardized to certain chemical constituents, or the chemical constituents themselves to which the therapeutic effect has been assigned. The general perception that phytomedicine is "natural" and therefore is devoid of side effects have encouraged self-medication with plant extracts to treat the LUTS.

Two of the most studied phytomedicines for BPH are the extracts of the saw palmetto (*Seronoa repens*) and pumpkin (*Cucurbita pepo*) seeds. The mechanism of action of the plant extracts are not well understood. Proposed pharmacological effects include inhibition of 5α-reductase, anti-inflammatory, anti-androgenic, anti-estrogenic or action on various receptors (Fornara et al., 2020). Saw palmetto and pumpkin seed extracts contain phytosterols, mainly β-sitosterol and other Δ^5 sterols (Figure 1.5). But it seems unlikely that these standard Δ^5 sterols could have contributed to the pharmacological effect against LUTS and BPH since the typical

daily doses of phytosterols taken with these extracts are less than 100 mg, far below the dietary intake of β-sitosterol of 250 to 300 mg daily.

Among plant extracts for BPH that contain phytosterols, pumpkin seed stood out as an exception in containing Δ^7 sterols, a distinct group of sterols not found in the standard diet. Δ^5- and Δ^7-sterols have different chemical structures pertaining to the double-bond position in the tetracyclic ring system and the lipophilic branched side chains. Chemical analysis of the sterol content in several plants extracts for the treatment of BPH have found that only pumpkin seed soft gel extract contained significant amount of these unique Δ^7 sterols (Muller & Bracher, 2015). Experiments to elucidate the mode of action of the plant extracts have therefore focused on these structurally unique phytosterols. Postulated mechanism of actions of Δ^7 sterols include binding to the cytoplasmic androgen receptor and targeting the 5α- reductase to inhibit DHT production. Not surprisingly, the inhibitory effect of the Δ^7 sterols were weaker than anti-androgen drugs such as finasteride. This coincides with the clinical experience with pumpkin seed extracts which did not influence the PSA values, had a milder effect, and did not bring about the typical side effects seen with finasteride.

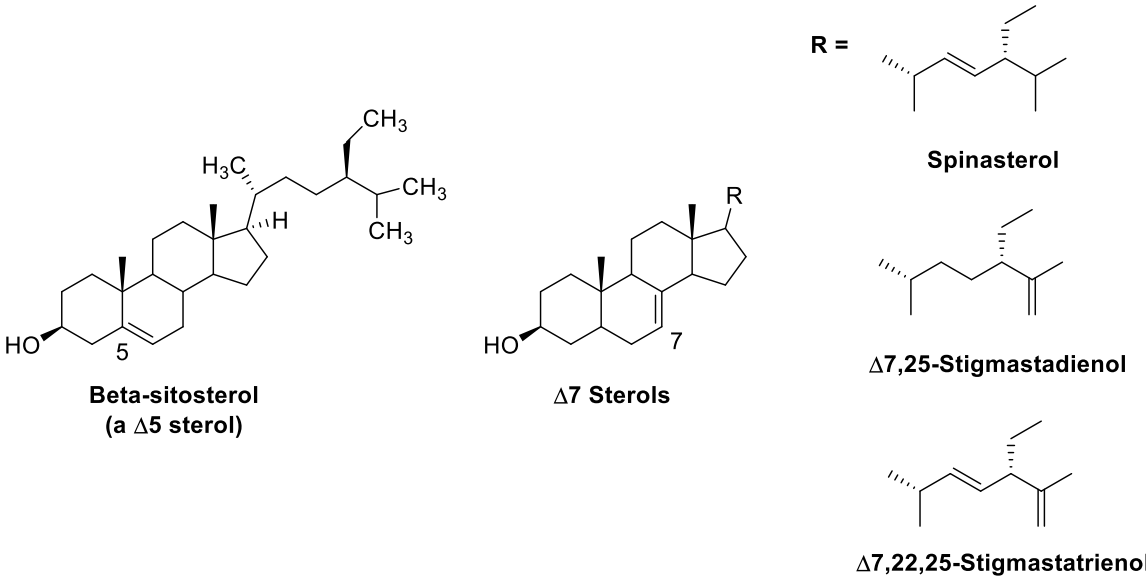


Figure 1.5. Structures of phytosterols contained in plant extracts for the treatment of BPH such as saw palmetto (*Seronoa repens*) and pumpkin (*Cucurbita pepo*) seeds.

1.5 *Eurycoma longifolia* Jack

Eurycoma longifolia Jack (Simaroubaceae), known by local ethnic populations of South East Asia as "Tongkat Ali" (Malaysia), "Pasak Bumi" (Indonesia), "Tung Saw" (Thailand) and "Cây Bá Bệnh" (Vietnam) is traditionally used by the local people for its aphrodisiac properties and general reproductive health benefits (Bhat & Karim, 2010; Khanijio & Jiraungkoorskul, 2016; Rehman, Choe & Yoo, 2016). The plant thrives very well in the tropical climate, commonly found along the hilly jungle slopes and widely distributed in primary, secondary, evergreen and mixed deciduous forests in Malaysia, Sumatra, Borneo, the Philippines and Indochina (Kuo et al., 2003).

E. longifolia is a medium size, slender shrub or tree. An adult or mature tree may reach up to a height of 10 meters (Plate 1.1). The plant is often unbranched with reddish brown petioles and each petiole is crowned by an umbrella-like rosette of leaves (Corner, 1951). The petioles can reach up to 1 meter in length, with 30 to 40 even pinnate leaflets that are dark

green in colour (Plate 1.2). The flowers are wide, about 0.5 cm, hairy and purplish crimson in colour. The petals of the flowers are small and have very fine pubescence. The fruits are about 1.0 to 1.5 cm in length, hard, ovoid in shape and yellowish brown in colour when young and will turn brownish red when ripe.

Plate 1.1. A mature *Eurycoma longifolia* (Taken on 24 April 2014, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang, Malaysia).

1.1

1.2

Plate 1.2. Petioles and leaflets of *Eurycoma longifolia.* (Source: Water colour painting by William Farquhar, National Museum of Singapore).

The roots of *Eurycoma longifolia* used for ethnopharmacological purposes contain a group of chemical compounds called the quassinoids which are unique to the Simaroubaceae family (Chung, Chan & Lee, 2021) in substantial quantities (5 to 10 % w/w of the plant material) Since these compounds represent the major constituents of the plant, it is not surprising that many studies (Rehman, Choe & Yoo, 2016) have identified the quassinoids being the contributor to the various pharmacological effects shown by the plant.

1.6 Basic quassinoid scaffolds and the nomenclature used to describe their structures

Quassinoids, the bitter-tasting constituents of Simaroubaceous plants including *Eurycoma longifolia* are structurally complex and highly oxygenated degraded triterpenoids characterized by a polycyclic architecture with a δ-lactone (six-membered ring) or a γ-lactone (five-membered ring). Based on the number of carbons in their basic skeletons, they may be classified as C_{25}, C_{22}, C_{20}, C_{19} and C_{18} quassinoids (Curcuni & Braz-Filho, 2006). The researchers who discovered them have followed an informal but relatively consistent convention in naming the compounds based their structures. The following describes several basic skeletons of the quassinoids and their naming convention.

The C_{19} eurycomalactones are essentially tetracyclic with a γ-lactone positioned adjacent to ring C (Figure 1.6). The C_{20} eurycoman-2-one or eurycoman-2-ol derivatives possess a δ-lactone fused between rings B and C. In contrast, the C_{20} klaineanone derivatives have the same skeleton as the eurycomanones except for the absence of an oxymethylene bridge connecting carbon 8 to carbon 11. The C_{19} longilactones share features similar to the klaineanones except for possessing a γ-lactone instead of a δ-lactone.

Eurylactones are scaffolds where a furanone ring juts out from the main skeleton or what is termed as a picranolide skeleton (Figure 1.6). The smallest scaffolds in terms of carbon numbers are the C_{18} eurycolactones which share the same feature as eurycomalactones except for the lack of one carbon. The myriad quassinoids found from *E. longifolia* differ between each other by the functional groups, frequently hydroxyls, attached to these basic skeletons.

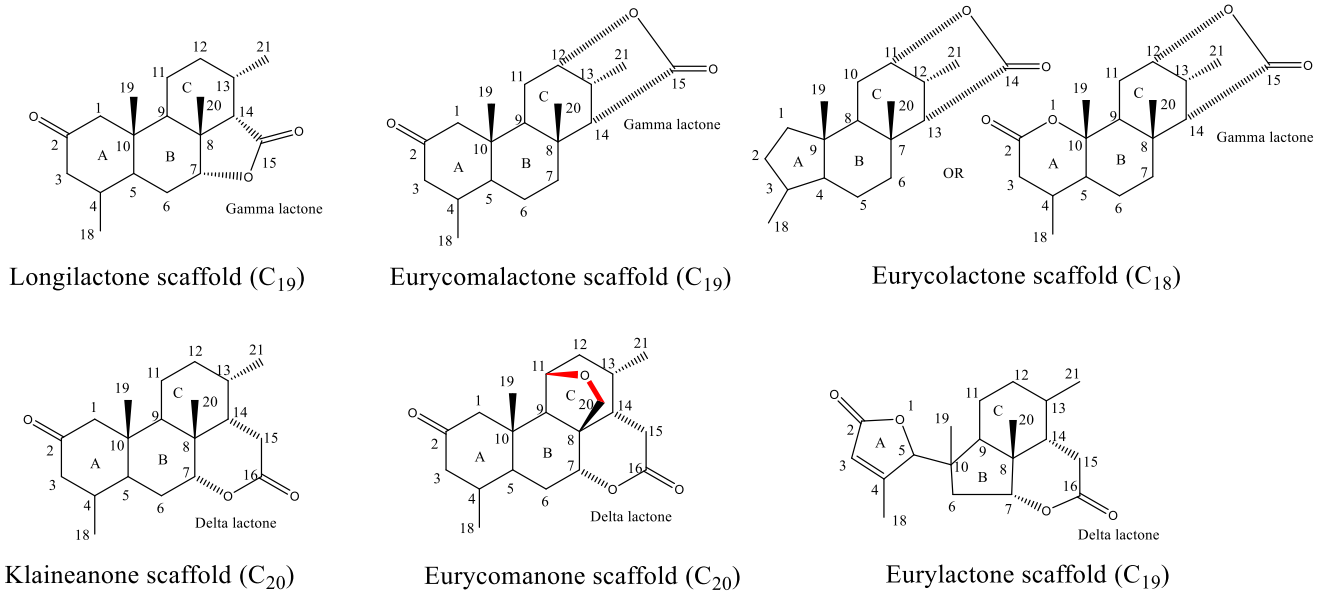


Figure 1.6 Basic scaffolds of the quassinoids and their nomenclature.

1.7 A review of quassinoids isolated from *Eurycoma longifolia* from 1970 until 2020

Until 1985, only eight quassinoids were isolated from *Eurycoma longifolia*, of which two were with a C_{18} skeleton, four with a C_{19} skeleton and the other two with a C_{20} scaffold. These quassinoids included eurycomalactone (**1**) and 3,4-dihydroeurycomalactone (**2**) isolated from the bark by Le Van and Nguyen Ngoc (1970), eurycomanone (**3**) and eurycomanol (**4**) from the roots by Darise et al. (1982), laurycolactone A (**5**) and laurycolactone B (**6**) by Nguyen Ngoc et al. (1982), and 5,6-dehydroeurycomalactone (**7**) and 6-hydroxy-5,6-dehydroeurycomalactone (**8**) by Bates et al. (1984). In 1989, Chan et al. isolated eurycomanol-2-*O*-β-D-glucopyranoside (**9**) from the roots of the plant. Upon closer examination of the plant material, another quassinoid with a C_{20} skeleton, 13β,18-dihydroeurycomanol (**10**) was isolated (Chan et al., 1991). (Figure 1.6).

Examination of the *E. longifolia* root extract by Morita et al. (1990) led to the isolation of four new quassinoids: longilactone (**11**), 13α,21-dihydroeurycomanone (**12**), 13β,21-

dihydroxyeurycomanone (**13**) and 14,15β-dihydroxyklaineanone (**14**). Tada et al. (1991) isolated a new C_{20} quassinoid, pasakbumin B or 13(α)21-epoxyeurycomanone (**15**), with an epoxy bridge linking the 13 and 21 positions.

In 1992, another quassinoid, 6α-hydroxyeurycomalactone (**16**) was isolated by Itokawa et al. from the bark of the plant. The subsequent year saw the same group isolating a quassinoid with an unusual 1,2-seco-1-nor-6(5-10)-abeo-picran-2,5-olide skeleton, eurylactone B (**17**) and two other novel quassinoids (**18)** and (**19)** (Itokawa et al., 1993a). They went on to isolate three more quassinoids, eurylactone A (**20**), (**21)** and (**22)**. (Itokawa et al., 1993b).

Examination of the leaves of the plant by Morita et al. (1993) resulted in the isolation of two new quassinoids, 6-dehydrolongilactone (**23**) and 7α-hydroxyeurycomalactone (**24**). The leaves of the plant also yielded several *O*-acetylated derivatives (**25** – **29**).

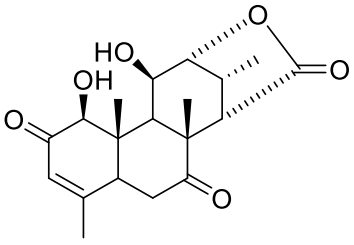
The following decade witnessed investigations that yielded several novel structures from *E. longifolia*. Ang et al. (2000), focusing on the roots of the plant isolated three novel quassinoids which were named eurycolactone A (**30**), eurycolactone B (**31**) and eurycolactone C (**32**). Eurycolactone B (**31**) was unusual as being the only quassinoid found in nature that was chlorinated. Further investigation has led to several more similar congeners (**33** – **35**) (Ang et al., 2002). An epimeric derivative, 12-*epi*-11-dehydroklaineanone (**36**) was obtained by Jiwajinda et al. (2001) from the leaves along with several known quassinoids and were identified to possess plant growth inhibitory activity. Eurycomaoside (**37**), a C_{19}-type glycoside which had its sugar moiety located at position 1 of the scaffold, was isolated by Bedir et al. (2003). An extensive investigation of the roots of *E. longifolia* by Kuo et al. in 2004 yielded eurycomalide A (**38**), eurycomalide B (**39**), 13β,21-dihydroxyeurycomanol (**40**), 5α,14β,15β-trihydroxyklaieanone (**41**) and iandinone (**42**), a 15-epimer of 13,21-dihydroeurycomanone (**12**). They also isolated eleven previously reported quassinoids. Interestingly, the study also

found the trio of pasakbumins A, B, and C (eurycomanone (**3**), 13α (21)-epoxyeurycomanone (**15**) and 13,21-dihydroeurycomanone (**12**), respectively) were particularly anti-proliferative against MCF-7 breast cancer cell lines. While the C_{18} quassinoids eurycomalactone (**1**), 6-dehydrolongilactone (**23**), and laurycolactone B (**6**) exhibited anti-proliferative activity against both A-549 lung carcinoma and MCF-7 cell lines. Eurycomanone (**3**) and 13α (21)-epoxyeurycomanone (**15**) were the only quassinoids that showed appreciable degree of anti-malarial activity against chloroquine-resistant *Plasmodium falciparum* W2 and D6 clones (Kuo et al., 2004). In 2009, a foray made by Miyake et al. has yielded ten new quassinoids with diverse scaffolds: two eurycomanone-like C_{20} compounds (**43**, **44**), one klaineanone type C_{20} compound (**45**), six eurycomalactone type structures (**46** to **51**) and one picrasanolide type C_{19} compound (**52**). In 2010, Teh et al. added a new longilactone derivative **53** into the collection of known quassinoids from their foray into the roots of *E. longifolia.*

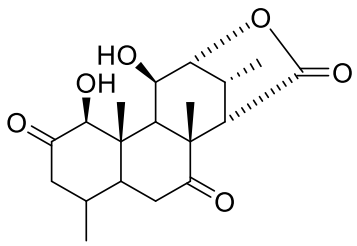
2014 appeared to be a fruitful year for the discovery of new quassinoids. Tran et al. isolated eurycomalide C (**54**), a new addition to the series isolated by Kuo et al. in 2004 along with eleven known quassinoids. The C_{19} and C_{20} quassinoids in this study, particularly eurycomalactone (**1**), 14,15β-dihydroxyklaineanone (**14**), and 13,21-dihydroeurycomanone (**12**) were found to be potent NF-κB inhibitors suggesting their potential as anti-inflammatory agents. Three new eurycolactones E, F and G (**55** – **57**), and eurycomalide D (**58**) and eurycomalide E (**59**) together with ten known quassinoids were isolated from the roots of the plant (Park et al., 2014). In this study, the C_{19} and C_{20} quassinoids eurycomalactone (**1**), eurycomanone (**3**), 14,15β-dihydroxyklaineanone (**14**), and 13,21-dihydroeurycomanone (**12**) again emerged with good anti-proliferative activity in three cancer cell lines confirming previous finding (Kuo et al., 2004). Later in May 2019, Dang et al. added three members to the eurycomalide group of compounds (**60**, **61**, and **62**). Meng et al. (2014) isolated four new C_{20} quassinoids (**63** – **66**) including three with a lactone replacing the usual oxymethylene bridge

of eurycomanone. Having a second lactone to their structures, the researchers named them 5-3iso-eurycomadilactone (**64**), eurycomadilactone (**65**), 13-epi-eurycomadilactone (**66**). Evaluation of the compounds on several cancer cell lines again confirmed eurycomanone (**3**) and **63** with better anti-proliferative activity.

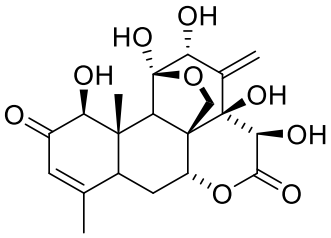
In March 2020, Yang et al. reported the isolation of six novel quassinoids from *Eurycoma longifolia*. Two of these, **67** and **68**, are the first members of a new class of quassinoids with an unusual C_{26} skeleton while compound **69**, with C_{20} scaffold exhibited an unusual cage-like and densely functionalized 2,5-dioxatricyclo [5.2.2.04,8] undecane core. One compound **70** showed potent anti-feedant activity against the diamondback moth larvae.



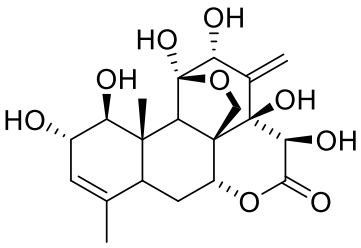
Eurycomalactone (**1**)



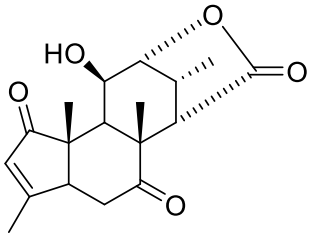
3,4-Dihydroeurycomalactone (**2**)



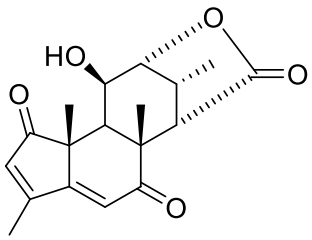
Eurycomanone (**3**)
(Pasakbumin A)



Eurycomanol (**4**)

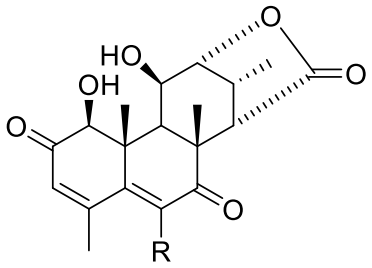


Laurycolactone A (**5**)



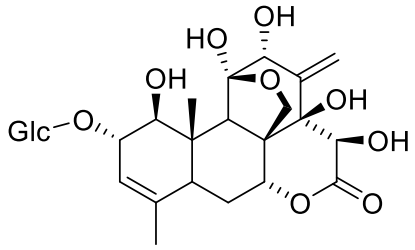
Laurycolactone B (**6**)

Figure 1.7. Continued

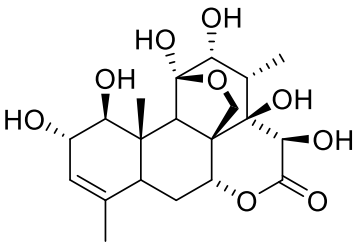


5,6-Dehydroeurycomalactone, R = H (**7**)

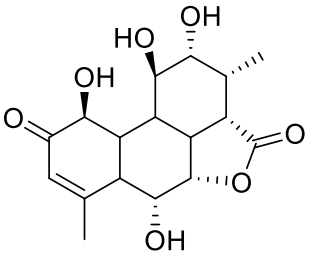
6-hydroxy-5,6-dehydroeurycomalactone, R = OH (**8**)



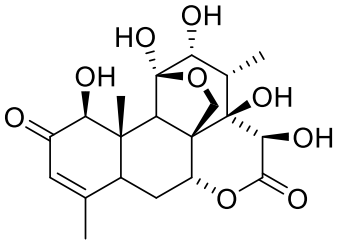
Eurycomanol-2-*O*-β-glucopyranoside (**9**)



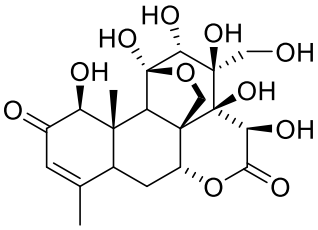
13β,18-Dihydroeurycomanol (**10**)



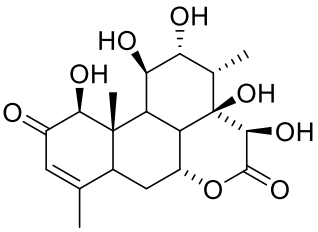
Longilactone (**11**)



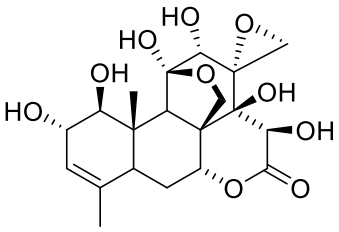
13α,21-Dihydroeurycomanone (**12**)
(Pasakbumin C)



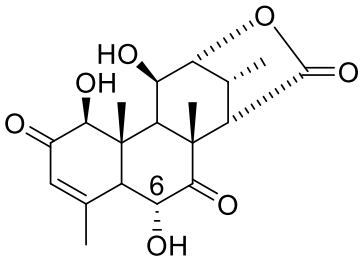
13β,21-Dihydroxyeurycomanone (**13**)
(Pasakbumin D)



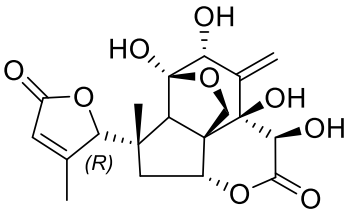
14,15β-Dihydroxyklaineanone (**14**)



13α(21)-Epoxyeurycomanone (**15**)
(Pasakbumin B)



6α-Hydroxyeurycomalactone (**16**)



Eurylactone B (**17**)

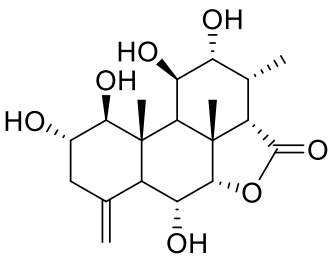


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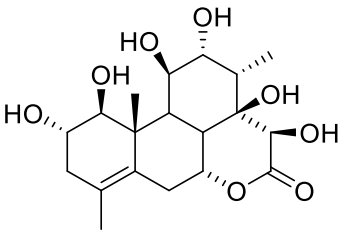
Figure 1.7. Continued



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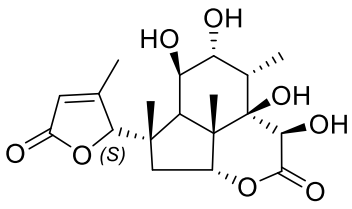
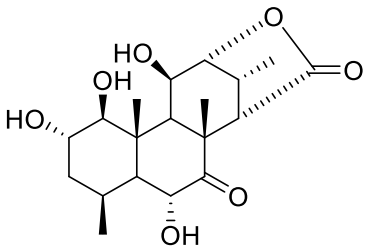


Eurylactone A (**20**)



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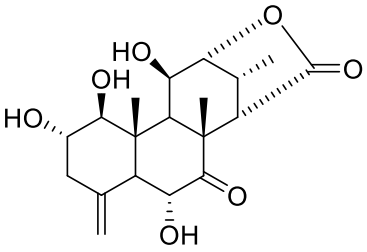

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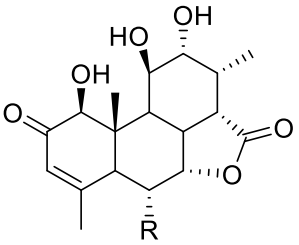
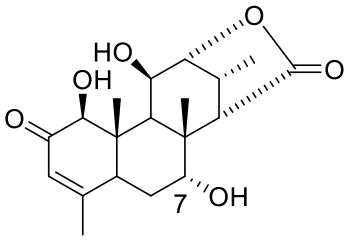
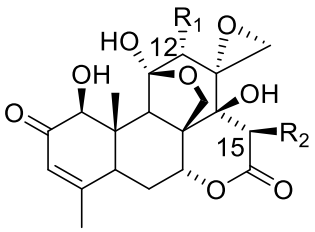


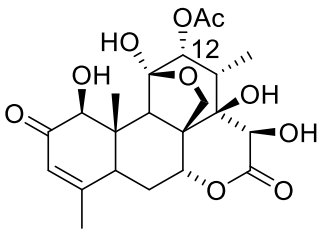
6-Dehydrolongilactone, R = H (**23**)
Eurycolactone F, R = OAc (**35**)



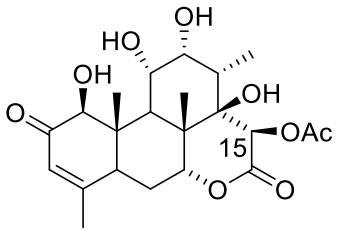
7α-Hydroxyeurycomalactone (**24**)



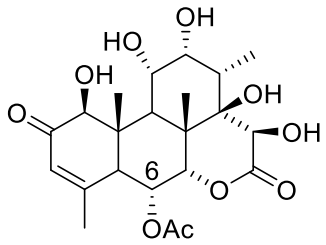
	R_1	R_2
15-*O*-Acetyl-13a(21)-epoxyeurycomaone (**25**)	OH	OAc
12,15-*O*-Diacetyl-13α(21)-epoxyeurycomanone (**26**)	OAc	OAc



12-*O*-Acetyl-13α,21-dihydroeurycomanone (**27**)

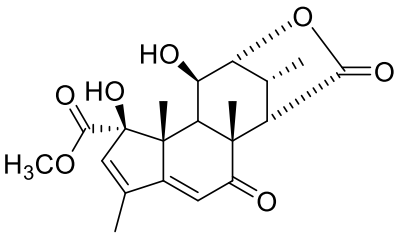


15β-*O*-Acetyl-14-hydroxyklaineanone (**28**)

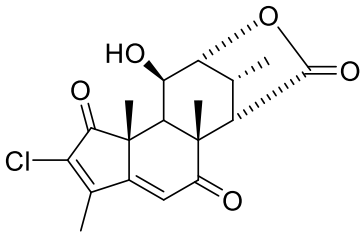


6α-*O*-Acetyl-14,15β-dihydroxyklaineanone (**29**)

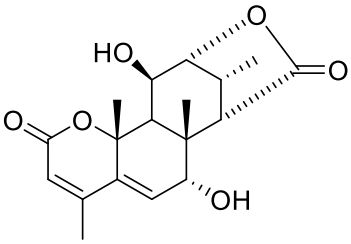
Figure 1.7. Continued



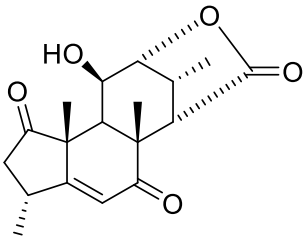
Eurycolactone A (**30**)



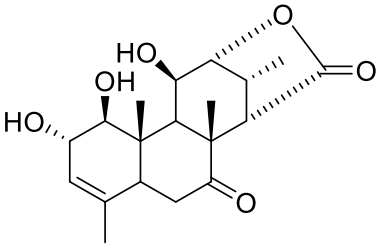
Eurycolactone B (**31**)



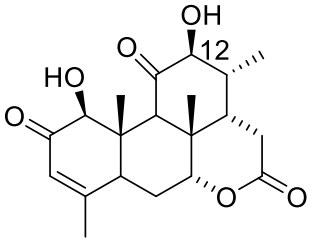
Eurycolactone C (**32**)



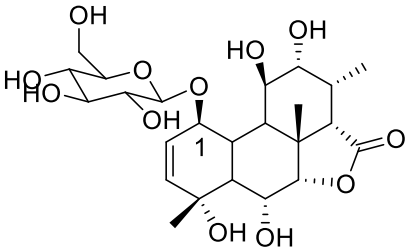
Eurycolactone D (**33**)



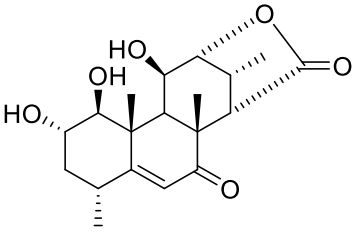
Eurycolactone E (**34**)



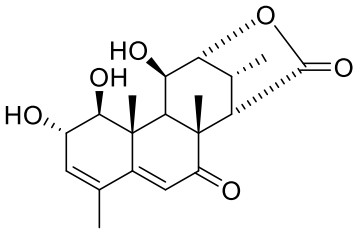
12-*epi*-11-Dehydroklaineanone (**36**)



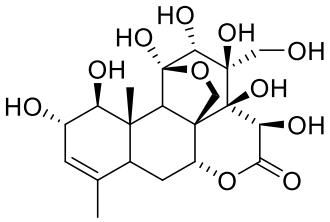
Eurycomaoside (**37**)



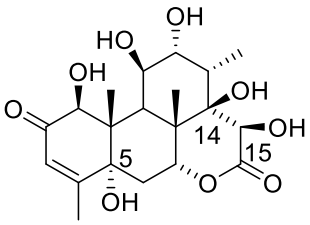
Eurycomalide A (**38**)



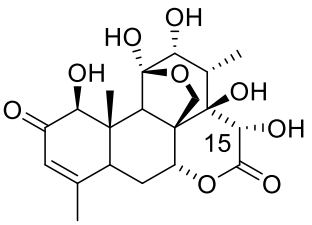
Eurycomalide B (**39**)



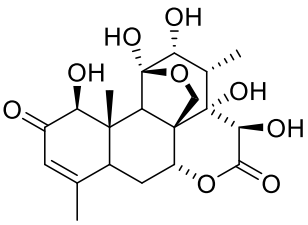
13β,21-Dihydroxyeurycomanol (**40**)



5α,14β,15β-Trihydroxyklaieanone (**41**)



Iandonone (**42**)



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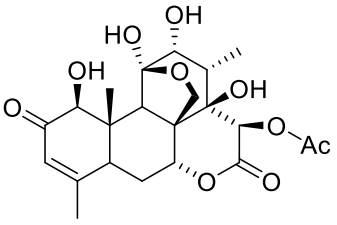

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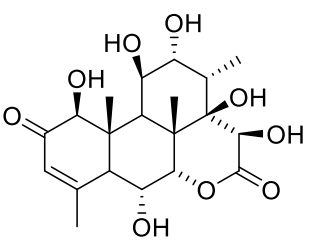

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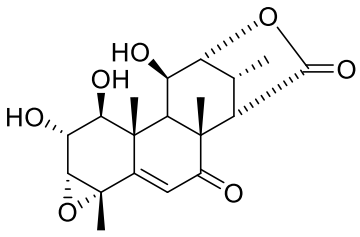
Figure 1.7. Continued



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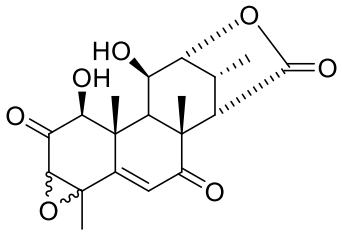

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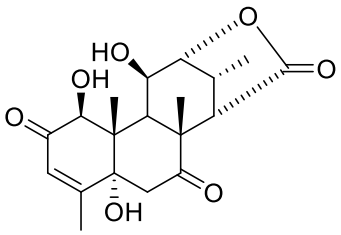

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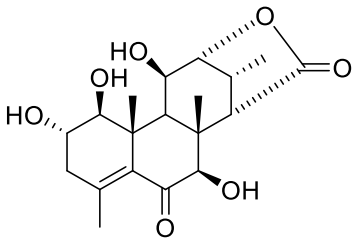

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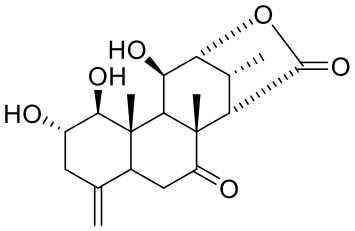

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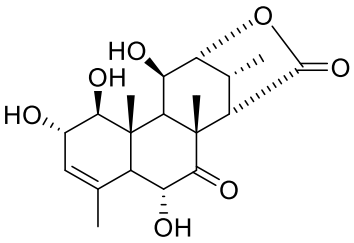

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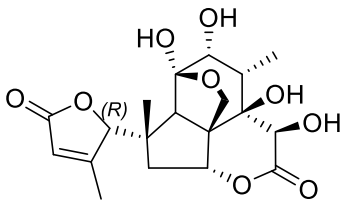

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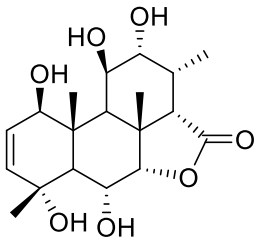

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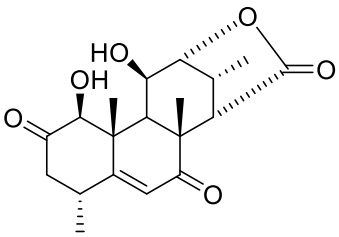
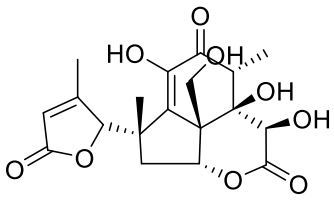
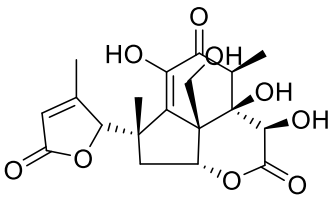


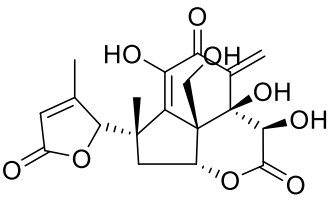
Eurycomalide C (**54**)



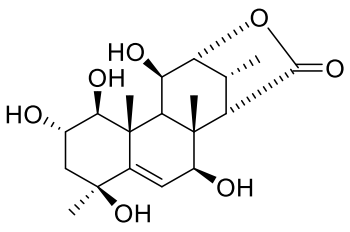
Eurycolactone E **(55)**



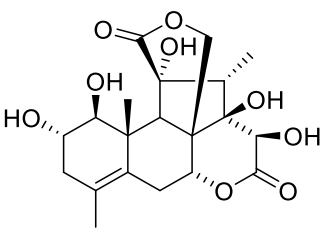
Eurycolactone F (**56**)



Eurycolactone G **(57)**



Eurycomalide D **(58)**



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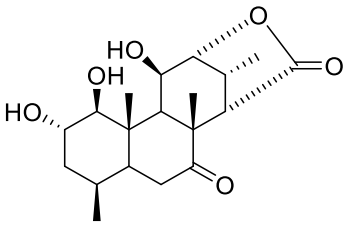
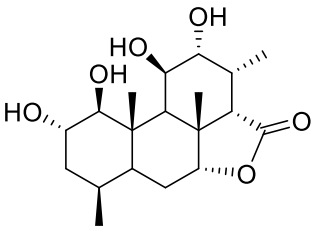
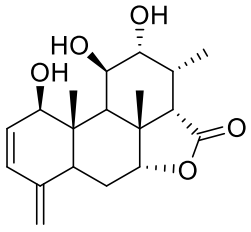


Eurycomalide F **(60)**

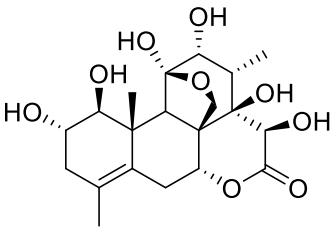
Figure 1.7. Continued.



Eurycomalide G **(61)**



Eurycomalide H **(62)**



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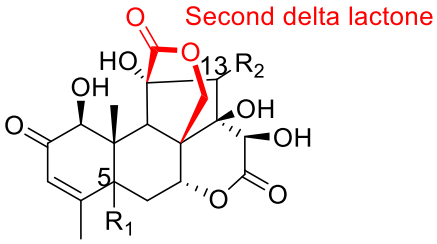
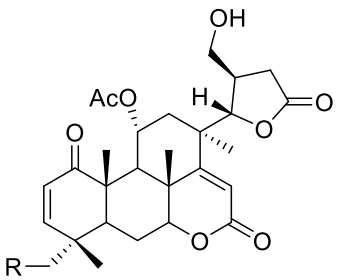
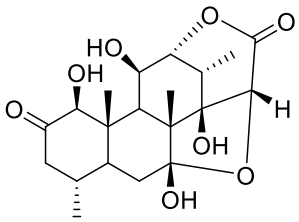


5-Isoeurycomadilactone, R_1 = a-H, R_2 = b-CH_3 (**64**)
Eurycomadilactone, R_1 = b-H, R_2 = b-CH_3 (**65**)
13-epi-Eurycomadilactone, R_1 = b-H, R_2 = a-CH_3 (**66**)



67 R = H
68 R = OH



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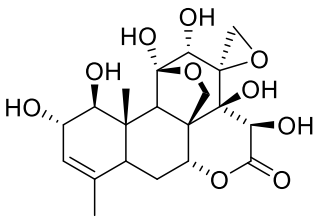

70

Figure 1.7. Structures of quassinoids isolated from *Eurycoma longifolia* (1970 – 2020).

1.8 Problem statement and study aims

Previous studies have shown the quassinoids of *Eurycoma longifolia* to significantly improve spermatogenesis, reverse oligospermia and improve male fertility in rats, thus giving credence to the potential reproductive health benefits of the plant (Low et al., 2010; Low et al. 2013a; Ebrahimi et al., 2016; Chung, Chan & Lee, 2021).

The quassinoids enhanced sperm count of the rats via a positive effect on testosterone production (Low et al., 2013b). Recent clinical trials also found an increase in testosterone levels in both young and aging men given *Eurycoma longifolia* extracts (Leitão et al., 2021; Chan et al., 2021). This testosterone increase has created concern that frequent intake of Tongkat Ali supplements may induce BPH or even bring about prostate cancer in the long term (Jarvis, Chughtai & Kaplan, 2015). On the contrary, a quassinoid-enriched root fraction of *Eurycoma longifolia*, TAF2 displayed inhibitory activity against LNCap human prostatic cancer cells implanted in athymic mice (Tong et al., 2015). Treatment of TAF2 in rat Leydig cells resulted in a decrease in the basal testosterone release, whereas the human chorionic gonadotropin (hCG)-induced testosterone synthesis by the cells was increased (Low et al., 2013b) and the rat aromatase activity was inhibited (Low et al., 2013b), leading to an increase in testosterone production. The promising results shown by the quassinoids and quassinoid-enriched extract (Tong et al., 2015) on reducing the proliferation of prostate cancer cells led to the question of whether these compounds may have the same effect in non-cancerous (benign) prostate cells associated with BPH and thus possess potential anti-BPH property.

The second research question that motivates this study relates to the excess mass of the quassinoid-enriched fractions obtained from the plant. Chromatographic separation of a crude root extract (4 to 5 kg) typically yielded a mass between 450 to 500 grams of quassinoid-enriched fractions. Despite high content of quassinoids present within the fractions, the quantities of compounds isolated were far lower than the mass of the fractions leading to the question of what these unaccounted-for constituents could be. From the previous review (Section 1.7), over sixty quassinoids have been discovered from *Eurycoma longifolia*, some as recently as 2020. There seems a possibility that novel, structurally interesting compounds may yet be uncovered by re-examining the roots of the plant especially in the polar fractions of the extract.

With these two research questions as thrusts, the specific objectives of this thesis are:

1. To perform a systematic chromatographic separation of *Eurycoma longifolia*, isolate and identify quassinoids by structure elucidation with a specific aim to discover novel, hitherto unreported structures.

2. To evaluate the anti-proliferative properties of the isolated quassinoids against a benign prostate hyperplasia cell line BPH-1 *in vitro*, identify active compounds, and discern structure-activity relationships.

3. To evaluate the anti-BPH properties of a quassinoid-enriched fraction and a selected quassinoid *in vivo* in a testosterone-induced rat model of BPH.

CHAPTER 2

ISOLATION AND CHARACTERISATION OF QUASSINOIDS FROM *EURYCOMA LONGIFOLIA*

2.1 Introduction

This chapter details the isolation of twelve quassinoids from the roots of *Eurycoma longifolia* by means of a systematic fractionation of the crude ethanolic extract with an aim towards uncovering new compounds. The isolated compounds were extensively characterized by spectroscopic methods. Of the twelve quassinoids, three compounds, one C_{19} and two C_{20} quassinoids were novel structures hitherto unreported.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

Analytical grade solvents (methanol, ethanol, chloroform acetone and dimethylsulfoxide), sulfuric acid and liquid chromatography grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Deionised water for chromatography was prepared using the Maxima ultrapure water system (ELGA LabWater, Buckinghamshire, UK). Macroporous resin Diaion HP20 and dextran-based resin Sephadex LH-20 were supplied by Mitsubishi Chemicals Corporation (Tokyo, Japan). NMR grade pyridine-d_5 was purchased from Acros Organics. (Geel, Belgium). Silica gel 60 (0.040 - 0.063 mm) for open column chromatography, thin layer chromatography aluminium plates pre-coated with Kieselgel 60-F_{254} (0.2 mm thickness, No. 5544) and Whatman 1 filter paper (11 µm pore size) were purchased from Merck (Darmstadt, Germany).