CHEMICAL CONSTITUENTS, COMPUTATIONAL STUDIES AND BIOACTIVITIES OF FOUR *Knema* SPECIES (MYRISTICACEAE)

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ABSTRACT

The purpose of this study was to investigate essential oils content, phytochemicals, computational modelling and bioactivities of four *Knema* species (Myristicaceae). Essential oils were extracted *via* hydrodistillation and were analyzed using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). Phytochemicals were isolated using silica gel column chromatography and their chemical structures were elucidated *via* spectroscopic methods including nuclear magnetic resonance, infrared spectroscopy, mass spectrometry, and ultraviolet-visible spectroscopy. Computational studies were performed to assess the binding of isolated compounds to acetylcholinesterase (AChE) and 5-lipoxygenase (5-LOX), and their antibiofilm activities. The major components of the four *Knema* essential oils were as following: *K. intermedia* (τ-muurolol, 20.1%), *K. malayana* (δ-cadinene, 20.2%), *K. hookeriana* (β-caryophyllene, 26.2%), and *K. furfuracea* (bicyclogermacrene, 23.1%). Phytochemical investigation led to isolation of a new compound (intermedianin) from *K. intermedia* and 22 known compounds including five from *K. intermedia* (cubebin, cubebin acetate, bicubebin B, apigenin, and kaempferol), six from *K. malayana* (3′,4′,5,7-tetramethoxyflavone, 3′,4′,5′,5,7-pentamethoxyflavone, 4′-hydroxy-3′,5′,5,7-tetramethoxyflavone, 3-hydroxy-3′,4′,5′,5,7-pentamethoxyflavone, 3,3′,5′,5,7-pentamethoxyflavone, and stigmasterol), seven from *K. hookeriana* (aptosimon, cubebin acetate, bicubebin A, 5,7-dimethoxyflavone, 4′,5,7-trimethoxyflavone, luteolin, and epicatechin), and four from *K. furfuracea* (cubebin, cubebin acetate, bicubebin A, and stigmasterol). Molecular docking studies revealed that intermedianin and aptosimon strongly bind AChE (–11.7 and –12.0 kcal/mol). Aptosimon showed strong binding affinity to 5-LOX (–10.1 kcal/mol) and intermedianin displayed binding to fungal cytochrome enzyme (CYP51B) (–11.0 kcal/mol). Bioactivity studies showed that 3′,4′,5′,5,7-pentamethoxyflavone gave strongest AChE inhibition (IC_{50} 10.2 μM), while bicubebin A was the most active against 5-LOX (IC_{50} 15.9 μM), and intermedianin was the strongest inhibitor of *Candida albicans* biofilm (22.8%). In conclusion, the current study demonstrated that the essential oils and isolated compounds of the four *Knema* species exhibited strong AChE, 5-LOX, and fungal biofilm inhibitory activities. These findings suggest that the *Knema* species have potential as alternative treatments for brain disorders, inflammation, and fungal infections.

KANDUNGAN KIMIA, KAJIAN KOMPUTASIONAL DAN BIOAKTIVITI EMPAT SPESIES *Knema* (MYRISTICACEAE)

ABSTRAK

Tujuan kajian ini adalah untuk menyiasat kandungan minyak pati, fitokimia, model komputasional dan bioaktiviti empat spesies *Knema* (Myristicaceae). Minyak pati diekstrak melalui penyulingan hidro dan dianalisis menggunakan kromatografi gas (GC) dan kromatografi gas-spektrometri jisim (GC-MS). Fitokimia telah dipencilkan menggunakan teknik kromatografi turus silika gel dan struktur kimianya ditentukan melalui kaedah spektroskopi termasuk resonans magnetik nuklear, spektroskopi inframerah, spektrometri jisim dan spektroskopi ultra-lembayung nampak. Kajian komputasional dijalankan untuk mengenalpasti ikatan sebatian yang dipencilkan kepada asetilkolinesteras dan anti-radang, dan aktiviti antibiofilemnya. Komponen utama dalam minyak pati oleh empat spesies *Knema* adalah seperti berikut: *K. intermedia* (τ-murolol, 20.1%), *K. malayana* (δ-kadinen, 20.2%), *K. hookeriana* (β-karyofailin, 26.2%), dan *K. furfuracea* (bisiklogermakrena, 23.1%). Penyiasatan fitokimia membawa kepada pemencilan satu sebatian baharu daripada *K. intermedia* (intermedianin) dan 22 sebatian termasuk lima daripada *K. intermedia* (kubebin, kubebin asetat, bikubebin B, apigenin, dan kemferol), enam daripada *K. malayana* (3′,4′,5,7-tetrametoksiflavon, 3′,4′,5′,5,7-pentametoksiflavon, 4′-hidroksi-3′,5′,5,7-tetrametoksiflavon, 3-hidroksi-3′,4′,5′,5,7-pentametoksiflavon, 3,3′,5′,5,7-pentametoksiflavon, dan stigmasterol), tujuh daripada *K. hookeriana* (aptosimon, kubebin asetat, bikubebin A, 5,7-dimetoksiflavon, 4′,5,7-trimetoksiflavon, luteolin, dan epikatekin), dan empat daripada *K. furfuracea* (kubebin, kubebin asetat, bikubebin A, dan stigmasterol). Kajian padanan molekul mendedahkan bahawa intermedianin dan aptosimon mengikat kuat AChE (–11.7 dan –12.0 kcal/mol). Aptosimon menunjukkan kecenderungan ikatan kuat terhadap 5-LOX (–10.1 kcal/mol) dan intermedianin mempamerkan ikatan terhadap enzim sitokrom fungi (–11.0 kcal/mol). Kajian bioaktiviti menunjukkan 3′,4′,5′,5,7-pentametoksiflavon memberikan perencatan terkuat AChE (IC_{50} 10.2 μM), sementara bikubebin A paling aktif terhadap 5-LOX (IC_{50} 15.9 μM), dan intermedianin adalah perencat terkuat terhadap biofilem *Candida albicans* (22.8%). Kesimpulannya, kajian terkini menunjukkan minyak pati dan sebatian yang dipencilkan oleh empat spesies *Knema* mempamerkan aktiviti kuat AChE, 5-LOX, dan perencatan biofilem fungi. Penemuan ini mencadangkan bahawa spesies *Knema* berpotensi sebagai rawatan alternatif untuk gangguan otak, keradangan, dan jangkitan fungi.

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LIST OF ABBREVIATIONS

α	Alpha
Abs	Absorbance
AChE	Acetylcholinesterase
β	Beta
^{13}C	Carbon-13
CC	Column Chromatography
$CDCl_3$	Deuterated chloroform
$CHCl_3$	Chloroform
cm^{-1}	Reciprocal centimetre
COSY	Correlation spectroscopy
CYP51	Cytochrome P450 51
1D	1 Dimension
2D	2 Dimension
δ	Chemical shift
d	doublet
DCM	Dichloromethane
dd	doublet of doublets
DEPT	Distortionless Enhancement by Polarization Transfer
EIMS	Electron Impact Mass Spectrometry
Et_2O	Diethyl ether
EtOAc	Ethyl acetate

GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GC-FID	Gas Chromatography-Flame Ionisation Detector
^{1}H	Proton
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
HR-ESIMS	High Resolution Electron Spry Ionisation Mass Spectrometry
Hz	Hertz
IR	Infrared
J	Coupling constant
KBr	Potassium bromide
KI	Kovats Index
L	Liter
LOX	Lipoxygenase
m	multiplet
M^{+}	Molecular ion
MeOH	Methanol
MHz	Megahertz
min	Minute(s)
m/z	Mass to charge ion
mg	milligram
$MgSO_4$	Magnesium sulphate
mL	milliliter

mm	millimeter
MS	Mass Spectrometer
NaCl	Sodium chloride
NMR	Nuclear Magnetic Resonance
nm	nanometer
s	singlet
SiO_2	Silica gel
t	triplet
TLC	Thin Layer Chromatography
PTLC	Preparative Thin Layer Chromatography
VOR	Voriconazole

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Natural products are bioactive compounds derived from plants, animals, or microbes. They have long been integral to medicine, food, and cosmetics. Iconic examples such as aspirin (from willow bark) and cannabidiol (from cannabis) underscore their medical importance (Li et al., 2022; O'Reilly et al., 2022). These compounds are often perceived as safer and more sustainable than synthetic drugs, although they are not without risks (Custodio et al., 2022). Despite occasional toxicity or allergenicity, natural products remain in high demand due to their renewability and lower environmental impact. In recent years, technological advances in high-throughput screening, metabolomics, and molecular docking have revolutionized natural product research (Pasdaran et al., 2024), accelerating drug discovery from natural sources, particularly in biodiversity-rich regions such as Malaysia.

The study of natural products is particularly significant in biodiversity-rich regions like Malaysia, where the tropical rainforests and marine ecosystems harbor untapped resources. These environments are a treasure trove of chemical diversity, yet many potential compounds remain unexplored. Advances in technology, including high-throughput screening, metabolomics, and molecular docking, are accelerating the discovery and development of natural product-based drugs. However, challenges such as sustainable sourcing, complex compound isolation, and the need for extensive clinical trials must be addressed to fully harness their potential (Pasdaran et al., 2024).

Medicinal plants are utilized in traditional medicine to treat or prevent illnesses and diseases. Cultures worldwide have relied on these plants for thousands of years, and many modern medicines continue to be derived from plant compounds. Medicinal plants contain active compounds with specific properties, such as anti-inflammatory, antioxidant, or antimicrobial effects, which make them effective for treating various conditions. In recent years, the popularity of medicinal plants has increased as people seek natural remedies and alternatives to pharmaceutical drugs. Notably, many pharmaceutical medications are derived from plant compounds, including morphine from the opium poppy and taxol from the Pacific yew tree. The use of medicinal plants and their applications is likely to continue gaining traction in the future (Hamedi et al., 2022).

Malaysia is renowned for its rich biodiversity and traditional use of medicinal plants, which play a significant role in the healthcare practices of its multicultural society. The country's rainforests are home to numerous endemic species that have been used for centuries in traditional Malay, Chinese, and Indian medicine. These

plants are integral to practices like Malay jamu, Traditional Chinese Medicine (TCM), and Ayurveda, addressing ailments ranging from minor infections to chronic diseases. Many medicinal plants from Malaysia have been scientifically studied, revealing promising pharmacological properties and potential for modern drug development (Tan et al., 2023).

Among the most notable medicinal plants is *Eurycoma longifolia*, commonly known as Tongkat Ali, traditionally used as an energy enhancer and aphrodisiac. It contains bioactive compounds like quassinoids and alkaloids, exhibiting antioxidant and anti-malarial properties. Another widely recognized plant is *Labisia pumila*, or Kacip Fatimah, valued for its role in women's health, particularly in postpartum care and hormonal balance, due to its rich content of flavonoids and saponins. *Orthosiphon stamineus*, or Misai Kucing, is another popular herb used to promote urinary tract health, thanks to its diuretic and anti-inflammatory effects. Similarly, *Centella asiatica*, locally known as Pegaga, is prized for its wound-healing and neuroprotective properties, attributed to its triterpenoids like asiaticoside. Other examples include the Bitter Gourd (*Momordica charantia*), known for its hypoglycemic effects in managing diabetes, and Temu Lawak (*Curcuma xanthorrhiza*), which supports digestion and liver health (Nasir et al., 2015).

Despite the immense potential of these plants, challenges such as overharvesting, lack of standardization in herbal products, and limited clinical studies persist. Sustainable harvesting practices, preservation of traditional knowledge, and rigorous research are crucial to unlocking the full potential of Malaysia's medicinal plants. With proper conservation and scientific validation, these natural resources

could significantly contribute to global healthcare and pharmaceutical development while preserving Malaysia's cultural heritage.

Plants from the Myristicaceae family hold a prominent place in traditional medicine due to their extensive therapeutic applications. The significance of Myristicaceae in natural products research and traditional medicine is well-documented, showcasing its dual role as a source of both traditional remedies and novel drug discovery leads. Indigenous knowledge about these plants reflects a deep understanding of their medicinal potential, passed down through generations. This traditional use underscores the broader importance of natural products in healthcare, serving as the foundation for many traditional healing systems globally, including Malaysia's (Barman et al., 2021).

Moreover, the therapeutic benefits of Myristicaceae plants highlight the essential role of natural products in bridging traditional practices with modern pharmacology. Shahidan et al. (2022) emphasize the need for further exploration and preservation of this indigenous knowledge, as it offers insights into sustainable healthcare solutions and new opportunities for developing plant-based pharmaceuticals. This integration of traditional wisdom and scientific validation continues to demonstrate the enduring relevance of natural products in addressing both historical and contemporary health challenges.

1.2 Problem Statement

The discovery of an alternative treatment method using plant-based traditional medicine has emerged due to the various adverse effects associated with modern drug-based therapies commonly used today. Several *Knema* species are used medicinally, with the seeds and bark traditionally employed to treat skin disorders, mouth and throat sores, cancer, wounds, acne, and rheumatism. Additionally, some *Knema* species are utilized to address conditions such as jaundice, chronic fever, inflammation, spleen disorders, respiratory issues, and impaired taste sensation. Moreover, these plants are reportedly used in traditional Thai medicine as a whole-body tonic, blood tonic, and anticancer agent (Chuakul et al., 2004; Salleh & Ahmad, 2017; Supriya & Sreekanth, 2021).

Although plants in the Myristicaceae family have attracted considerable research attention for the isolation and identification of phytochemicals, the genus *Knema* remains largely unexplored in this regard. Additionally, limited research has been conducted on the essential oils of species within this genus. To the best of our knowledge, no studies have reported on the essential oils of the selected *Knema* species in this study; *K. intermedia*, *K. malayana*, *K. hookeriana*, and *K. furfuracea*. Moreover, phytochemical investigations of these species are scarce, with the exception of *K. hookeriana* (Geny et al., 2016; Alen et al., 2000). Given these gaps and the reported traditional and ethnobotanical uses, there is a clear motivation to identify the active compounds responsible for the biological activities.

Therefore, we aim to isolate and characterize the biologically active compounds present in these four species, as well as to perform computational studies to understand how these compounds exert therapeutic effects in the human body. This will include predicting interactions between drugs and proteins and analyzing their impact on biological pathways and functions

1.3 Objectives of Study

The objectives of the study are:

1. To investigate the chemical compositions of the essential oils from the leaves of four *Knema* species (*K. intermedia*, *K. malayana*, *K. hookeriana*, and *K. furfuracea*).
2. To isolate the phytochemicals from the leaves extract of four *Knema* species (*K. intermedia*, *K. malayana*, *K. hookeriana*, and *K. furfuracea*) and characterize spectroscopically (IR, NMR, UV, and MS).
3. To perform the computational studies on the isolated phytochemicals using molecular docking and drug-likeness (ADMET).
4. To determine the biological activities (acetycholinesterase, anti-inflammatory, and antibiofilm) of the essential oils, crude extracts, and selected phytochemicals.

1.4 Scopes of Study

The study was divided into four parts. The first part focused on the extraction of essential oils from the leaves of *K. intermedia*, *K. malayana*, *K. hookeriana* and *K. furfuracea* using the hydrodistillation method. The chemical compositions of the essential oils were analyzed using gas chromatography (GC-FID), gas chromatography-mass spectrometry (GC-MS), and Kovats indices.

The second part involved the extraction, isolation, purification, and characterization of phytochemicals from the dried leaves of *Knema* species. The cold extraction method was employed with solvents of varying polarities. The crude extracts were fractionated using vacuum liquid chromatography (VLC), followed by purification of the fractions through gravity column chromatography (CC) and preparative thin-layer chromatography (TLC) to obtain pure phytochemicals. Characterization of the isolated compounds was conducted using various spectroscopic methods, including infrared (IR), ultraviolet (UV), one-dimensional and two-dimensional nuclear magnetic resonance (NMR), and mass spectrometry (MS).

The third part comprised computational studies, which included molecular docking and drug-likeness (ADMET) prediction. These studies aimed to predict the most active phytochemicals and elucidate their interactions with target enzymes (proteins), providing critical insights for selecting phytochemicals for *in vitro* assays.

Finally, the biological activities, including acetylcholinesterase inhibition, anti-inflammatory effects, and fungal antibiofilm activity, were assessed for the essential

oils, crude extracts, and selected phytochemicals. Acetylcholinesterase activity was measured using the electric eel (*Electrophorus electricus*) with acetylthiocholine iodide as the substrate. Anti-inflammatory activity was evaluated against the lipoxygenase enzyme, while antibiofilm activity was tested using crystal violet assay against *Candida* species.

1.5 Significance of Study

The significance of this study lies in its pioneering and comprehensive investigation of the phytochemical composition of *K. malayana*, *K. intermedia*, *K. hookeriana* and *K. furfuracea*. This research is the first to explore the chemical constituents of *K. malayana* and *K. intermedia*, with the aim of isolating and identifying novel bioactive compounds from these species. The discovery of new compounds is crucial, as they may exhibit unique pharmacological properties with potential therapeutic applications. Despite the traditional medicinal use of these *Knema* species in Southeast Asia, scientific validation of their bioactive compounds and medicinal properties has been limited. This study addresses that gap by identifying bioactive compounds and evaluating their biological activities, thereby contributing to the growing body of knowledge on the genus *Knema*.

The discovery of new compounds from these species could significantly advance the development of treatments for inflammatory conditions, fungal infections, and microbial infections. Furthermore, this research has broader implications for drug discovery and the pharmaceutical industry's natural product-based medicines. In

addition to its pharmacological contributions, the study emphasizes the importance of preserving Malaysia's rich biodiversity by highlighting the medicinal potential of *Knema* species, which contributes to the conservation of natural resources for future generations. Through this pioneering investigation of *K. malayana* and *K. intermedia*, the study provides valuable insights into the chemical, enzymatic, and medicinal profiles of these plants.