



UNIVERSITI PUTRA MALAYSIA

**CHEMICAL PROFILING AND ASSESSMENT OF
IMMUNOMODULATORY PROPERTIES OF *Terminalia catappa* LINN
AND *Terminalia subspatulata* KING ON RED HYBRID TILAPIA
(*Oreochromis sp.*)**

YAHAYA YAKUBU

IB 2021 20



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By

YAHAYA YAKUBU

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

May 2020

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DEDICATION

This thesis is dedicated to Allah the most gracious, most merciful whom all praise befits the glory of his countenance and the greatness of his might and to my amiable late father, Mallam Yahaya Baba Yakubu and amiable mother, hajiya Zainab Yakubu who raised me and gave me their all. Also, to my understanding and loving wife, Aisha Zakari Othman and daughter, Zafeera Yahaya, for their limitless support and understanding.



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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

CHEMICAL PROFILING AND ASSESSMENT OF IMMUNOMODULATORY PROPERTIES OF *Terminalia catappa* LINN AND *Terminalia subspathulata* KING ON RED HYBRID TILAPIA (*Oreochromis* sp.)

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May 2020

Chairman : Professor Khozirah binti Shaari, PhD
Institute : Bioscience

Fish farming is a rapidly growing venture throughout the globe. This is due to the revenue it generates and the lesser health risk it poses to humans upon consumption. However, they are faced with pathogens and disease outbreaks. Although treatment with antibiotics and prevention with vaccines are still effective but have their attendant challenges, as such, they are gradually giving way to natural plant-based therapy, which is believed to have lesser to negligible side effects for preventing these diseases outbreaks. One of such plant whose leaf is traditionally used by fish farmers to prevent disease outbreak or boost the immunity of fish is the *Terminalia catappa* (Ketapang). *Terminalia catappa* (TC) belongs to the *Terminalia* species (TS), which are phytotherapeutic plants with over 200 known species but having only very few explored. The traditional uses of some of the explored species like *Terminalia catappa* have not been scientifically proven to leave them as a myth. On the other hand, *Terminalia subspathulata* (TST) (Jelawi Jaha), to the best of our knowledge, is one of the species of this family that is unexplored for any medicinal purpose. This research investigates the chemical profiles of the leaves, barks, nut and fruit of *Terminalia catappa* and *Terminalia subspathulata* crude extracts using ultra high performance liquid chromatography electrospray tandem mass spectrometry (UHPLC-ESI-MS/MS) and gas chromatography (GCMS) techniques, in addition, to their antioxidant potential. Furthermore, the acute toxicity, immunostimulant potential and disease resistance on red hybrid tilapia (*Oreochromis* sp.) model were also assayed. The UHPLC-ESI-MS/MS metabolite profiling of the leaves, bark, nut and fruit of *Terminalia catappa* and *Terminalia subspathulata* resulted in the identification of 195 metabolites, mostly phenolics and few organic acids. While the GCMS profiles of the nut and fruit of *Terminalia catappa* and *Terminalia subspathulata* revealed majorly saturated and unsaturated fatty acids. The total phenolic content (TPC) and total flavonoid content (TFC) attested to the fact that *Terminalia catappa* and *Terminalia subspathulata* have phenolic medicinal metabolites which manifested in their

scavenging activities in the assessment of their antioxidant potential (AOP), using 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant potential (FRAP) and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays. The acute toxicity study was conducted using the doses; 31.125 mg/kg, 62.5 mg/kg, 125 mg/kg, 250 mg/kg and 500 mg/kg. Fish were sacrificed after 96 hours, blood samples collected for analysis (complete blood haemogram and biochemistry) and five organs (gill, heart, kidney, liver and spleen) were also collected for histological assessment. From the results, toxic signs were observed from 125 mg/kg to 500 mg/kg and as such, were not used for the immunostimulant assessment. The innate immune parameters (phagocytosis, respiratory burst, lysozyme activity, and complement assay) studied using 31.125 mg /kg, 62.5 mg/kg leaf crude extracts of *Terminalia catappa* and *Terminalia subspathulata* showed an increase in the immunity of fish after two weeks in the phagocytotic, respiratory burst, lysozyme activities. This led to the partitioning of the leaf crude extracts of *Terminalia catappa* and *Terminalia subspathulata* with n-hexane, chloroform, ethyl acetate and methanol to determine the most effective fraction on the aforementioned immune parameters leading to isolation of compound(s). From the immune study of the partitioned extracts, the ethyl acetate and chloroform fractions showed a more improved response in the immune parameters. Consequently, leading to the isolation of β -sitosterol, stigmasterol-3-O- β -D-glucopyranoside, gallic acid and quercetin-3-O-rhamnoside from the ethyl acetate fractions of *Terminalia subspathulata* leaf, which happens to be an unexplored species in the genus of *Terminalia* species. The results prove the claim scientifically from different quarters, saying that *Terminalia catappa* leaf boosts the immunity of fish and has demonstrated that other species of *Terminalia* can also perform the same function. Furthermore, the result also reveals medicinal metabolite in *Terminalia subspathulata* and its biological potentials. We believe these findings will help strengthen the use of these readily available plants (*Terminalia catappa* and *Terminalia subspathulata*) as a cheaper way of boosting the health of fish against pathogens in aquaculture.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PEMROFILAN KIMIA DAN PENILAIAN CIRI-CIRI MEMODULET
IMUN *Terminalia catappa* DAN *Terminalia subspatulata* PADA TILAPIA
HYBRID MERAH (*Oreochromis sp.*)**

Oleh

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Penternakan ikan merupakan satu bidang yang kian berkembang pesat di seluruh dunia. Hal ini disebabkan oleh pendapatan yang diperoleh daripadanya adalah lumayan dan risiko kesihatan pada konsumen adalah lebih rendah. Walaubagaimanapun, penternak ikan berhadapan dengan masalah seperti patogen dan wabak penyakit. Selain itu, penggunaan antibiotik dan vaksin sebagai langkah pencegahan adalah semakin mencabar. Oleh sebab itu, penyelidikan terhadap penggunaan terapi berasaskan tumbuhan semulajadi untuk mencegah wabak penyakit semakin diceburi dan dipercayai mempunyai kurang kesan sampingan. Daun ketapang (*Terminalia catappa*, TC) ialah salah satu tumbuhan yang digunakan oleh penternak ikan secara tradisional untuk mencegah wabak penyakit dan menguatkan imuniti ikan. *T. catappa* tergolong dalam genus *Terminalia*, satu genus yang terdiri lebih daripada 200 spesies tetapi hanya sebahagian kecil daripadanya telah dikaji. Penggunaan *T. catappa* secara tradisional masih belum dibuktikan secara saintifik. Jelawi Jaha (*Terminalia subspatulata*, TST) juga merupakan satu spesies dalam genus ini dan ia masih belum dikaji untuk sebarang tujuan perubatan. Penyelidikan ini bertujuan mengkaji potensi antioksidan dan profil kimia ekstrak mentah daun, kulit pohon, kacang dan buah TC dan TST menggunakan teknik kromatografi cecair berprestasi ultra tinggi-semburan elektro-spektrometri jisim seiring (UHPLC-ESI-MS/MS) dan teknik kromatografi gas- spektrometri jisim (GC-MS). Ketoksikan akut, potensi memodulet imun dan rintangan penyakit pada tilapia hibrid merah (*Oreochromis sp.*) turut diuji. Pemprofilan metabolit daun, kulit, kacang dan buah TC dan TST menggunakan UHPLC-ESI-MS/MS telah mengenalpasti 195 metabolit, dimana kebanyakannya ialah sebatian fenolik dan beberapa asid organik. Profil metabolit GC-MS kacang dan buah TC dan TST menunjukkan kebanyakan sebatian adalah terdiri daripada asid lemak tepu dan tidak tepu. Jumlah kandungan fenolik (TPC) dan jumlah kandungan flavonoid (TFC) telah membuktikan bahawa TC dan TST mempunyai sebatian fenolik yang menunjukkan aktiviti ‘memakan’ dalam penilaian potensi

antioksidan (AOP) mereka, menggunakan 2,2-difenil-1-pikrilhidrazil (DPPH), kuasa antioksidan pengurangan ferik (FRAP) dan 2,2'-azino-bis(3-etilbenzotiazolin-6-asid sulfonik) (ABTS). Kajian ketoksikan akut dijalankan menggunakan dos 31.125 mg/kg, 62.5 mg/kg, 125 mg/kg, 250 mg/kg dan 500 mg/kg. Ikan dikorbankan selepas 96 jam dan sampel darah digunakan untuk analisis (hemogram darah lengkap dan biokimia) dan lima organ (insang, jantung, buah pinggang, hati dan limpa) dikumpulkan untuk penilaian histologi. Keputusannya, 125 mg/kg hingga 500 mg/kg menunjukkan tanda-tanda toksik dan konsentrasi ini tidak digunakan untuk penilaian ciri-ciri memodulet imun. Parameter imun semulajadi (fagositosis, 'ledakan respirasi', aktiviti lisozim dan ujikaji pelengkap) menggunakan 31.125 mg/kg dan 62.5 mg/kg ekstrak mentah daun TC dan TST menunjukkan peningkatan imuniti ikan selepas dua minggu dalam fagositosis, 'ledakan respirasi' dan aktiviti lisozim. Rentetan dapatan ini, ekstrak mentah daun TC dan TST dipecahkan secara berperingkat dengan menggunakan *n*-heksana, klorofom, etil asetat dan metanol untuk menentukan pecahan mana yang paling berkesan pada parameter imun untuk tujuan pengasingan sebatian asli. Dari kajian parameter imun menggunakan pecahan-pecahan yang telah diperolehi, pecahan etil asetat dan klorofom menunjukkan tindakbalas yang lebih baik. Dapatan ini membawa kepada pengasingan beta-sitosterol, stigmasterol-3-*O*- β -D-glukopiranosid, asid galik dan kuersetin-3-*O*-ramnosid daripada pecahan etil asetat daun TST. Keputusan kajian membuktikan secara saintifik bahawa daun ketapang meningkatkan imuniti ikan dan spesies ketapang yang lain juga dapat melakukan fungsi yang sama. Tambahan pula, kajian juga menjumpai metabolit bersifat ubatan dalam Jelawi Jaha dan potensi biologinya. Hasil kajian ini dapat memperkasa penggunaan kedua-dua tumbuhan yang mudah didapati ini (Ketapang dan Jelawi Jaha) sebagai kaedah yang lebih menjimatkan dalam meningkatkan kesihatan ikan daripada jangkitan patogen akuakultur.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
APCI	Atmospheric Pressure Chemical Ionization
AOP	Antioxidant potential
CID	Collision-Induced Dissociation
DPPH	2,2-Diphenylpicryl-1-hydrazyl
EC ₅₀	Half maximum concentration
ESI	Electrospray ionisation
FFBS	Flavour and Fragrance Natural and Synthetic Compounds
FRAP	Ferric Reducing Antioxidant Potential
GCMS	Gas Chromatography Mass Spectrometry
HHDP	Hexahydroxydiphenoyl
HPLC	High Performance Liquid Chromatography
LC-ESI-MS/MS	Liquid chromatography electrospray ionisation mass spectrometry
LCMS	Liquid Chromatography Mass Spectrometry
MS	Mass Spectrometry
NIST	National Institute of Science and Technology
NMR	Nuclear Magnetic Resonance
PDA	Photodiode array
UHPLC	Ultra High Performance Liquid Chromatography
UPM	University Putra, Malaysia
ROS	Reactive oxygen species
RP	Reverse-phase
SIM	Selective ion monitoring
TC	<i>Terminalia catappa</i>

TCL	<i>Terminalia catappa</i> leaf
TCN	<i>Terminalia catappa</i> nut
TFC	Total Flavonoid Content
TIC	Total Ion Chromatograms
TPC	Total Phenolic Content
TS	<i>Terminalia</i> species
TSB	<i>Terminalia subspathulata</i> bark
TSF	<i>Terminalia subspathulata</i> fruit
TSL	<i>Terminalia subspathulata</i> leaf
TST	<i>Terminalia subspathulata</i>
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Research background

Medicinal plants are regarded as the bedrock for drug discovery (Singh, 2015) owing to the fact that they contain several metabolites that have biological functions (Gostner et al., 2012; Thongam et al., 2016). Despite the knowledge that there are abundant important medicinal metabolites in plants, only an infinitesimal percentage of these medicinal plants have been studied in the past. This is so due to the complexity of the metabolite matrix (Srivastava et al., 2014). However, modern analytical techniques such as liquid chromatography mass spectrometry (LCMS), gas chromatography mass spectrometry (GCMS) and nuclear magnetic resonance (NMR) spectroscopy have made the identification and characterisation of secondary metabolites easier, allowing a more holistic picture of the metabolites present in a plant extract. Knowledge of a plant chemical profile is vital for its pharmacological activity and may not be linked to a specific metabolite but the synergistic effect of the metabolites present.

Among the array of medicinal plants used in alternative medicine that are yet to be extensively studied for their metabolites and biological activities are the *Terminalia species*. They are located mainly in the subtropical regions of Asia, Africa and Australia (Cock, 2015). An example of these species includes *Terminalia arjuna*, the stem bark of which is used to treat cardiovascular diseases (Meghwani et al., 2017). *Terminalia chebula* has been used for treating gastrointestinal problems and afflictions of the central nervous system (Kumar et al., 2018). Meanwhile, *Terminalia bellerica* is useful in the treatment of hepatitis, bronchitis, asthma, dyspepsia, piles, diarrhoea, coughs and eye diseases (Jayesh et al., 2017). The leaves, bark and roots of *Terminalia catappa*, regarded as one of the wonder plants of this genus, have also been used in phytotherapeutic medicine (Oyeleye et al., 2018). Its leaves are used traditionally to treat dermatitis and hepatitis in many parts of India and the Philippines. Research has shown that the plant has anticancer, antidiabetic, anti-inflammatory, immunomodulatory, analgesic, hepatoprotective and wound healing activities (Chandrasekhar et al., 2017; Nugroho et al., 2016). Furthermore, the leaf is also used in Asia and around the world to improve the health of fish (Syahidah et al., 2015).

Fish health management has gained attention in recent years due to the rapidly growing aquaculture practice throughout the world, especially the inland fish farming (Froehlich et al., 2017). However, it is faced with a major setback of disease outbreaks, pesticides in aqua farms, chemotherapeutants, drug resistant microbes, and non-adherence to scientific management and above all, accumulation of antibiotics and pesticide residue in fish. As a result, scientists are forced to seek safer alternatives to manage the health of fish. A potentially cheaper alternative way of improving the health of fish is to boost their immunity against pathogens using natural plant extracts (Kumar et al., 2014; Miriam et al., 2017).

The present study is aimed at profiling the metabolites present in the leaves, bark, nut and fruit of *Terminalia catappa* (TC) and *Terminalia subspathulata* (TST) and determining their antioxidant potential. Besides, the immunostimulant potential of *Terminalia catappa* leaf and *Terminalia subspathulata* leaf extracts on red hybrid tilapia (*Oreochromis* sp.) fish model was also ascertained. We believe this will give scientific insight into the traditional claim that *Terminalia catappa* leaves improve the health of fish and at the same time, establish the metabolite profile and biological potential of *T. subspathulata*, which is an unexplored species from the genus.

1.2 Problem statement

The leaf of *Terminalia catappa* has been used in traditional medicine to improve the health of fish (Nugroho et al., 2016). In addition, other studies have shown that *Terminalia catappa* leaf fights bacteria, parasites and fungi in fish (Chitmanat, 2005; Chitmanat et al., 2005; Ikhwanuddin et al., 2014). However, there is little or no scientific evidence to prove how *T. catappa* leaf affects the innate immune parameters, which happen to be the first line of defence in fish and other animals, including humans.

In addition, the genus *Terminalia* with over 200 species is known to have medicinal values, but very few have been scientifically explored and proven for the properties that they are traditionally used for (Cock, 2015; Fahmy et al., 2015). Hence, there is a need to know the phytochemicals present in the unexplored species and check if there is another species of *Terminalia* genus that have the same potential of improving the health of fish.

To achieve this, we decided to explore *Terminalia subspathulata*, which is one of the species placed on red alert of extinction (National Board Parks, 2015). As such, in the present study, we aim to profile *T. catappa* and *T. subspathulata* and evaluate the immune boosting potential of *T. catappa* leaf on red hybrid tilapia (*Oreochromis* sp.) and equally study the same using *T. subspathulata* leaf.

Furthermore, the health of tilapia fish is particularly of importance because it is the second most cultured economic fish in the world and account for most countries income but is plagued with diseases due to high stocking density (Steckert et al., 2018). At present, *S. agalactiae* that is said to have no vaccine (Lin set al., 2018) is considered as one of the pathogens that are responsible for more deaths in tilapia fish (Li et al., 2014; Suhermanto et al., 2019). In addition, there exist limited know-how on the type of extract, concentration, and the knowledge of the constituent of the herbs administered to improve their wellbeing (Kuebutornye & Abarike, 2020; Ndakalimwe, 2019). As such, more research needs to be conducted in this light.

1.3 Hypotheses

1.3.1 Null hypothesis (H_0)

Terminalia species do not contain compound(s) that have immune boosting potency in fish, and also doesn't have antioxidant activity.

1.3.2 Alternate hypothesis (H_A)

Terminalia species contain compound(s) that have immune boosting potency in fish, and also have antioxidant activity.

1.4 Objectives of the study

The specific objectives are:

1. To establish the metabolite profile of *Terminalia catappa* and *Terminalia subspathulata* species.
2. To evaluate the antioxidant activity of *Terminalia catappa* and *Terminalia subspathulata* species.
3. To evaluate the toxicity level of *Terminalia catappa* and *Terminalia subspathulata* in tilapia fish.
4. To evaluate the immunostimulant potential of *Terminalia catappa* and *Terminalia subspathulata* leaves extract on tilapia.
5. To isolate and characterise active compound(s) from the most active fraction of *Terminalia catappa* and *Terminalia subspathulata* leaves.

1.5 Justification

There have been reports of the use of *T. catappa* leaf to improve the health of fish throughout the world. *T. catappa* also belongs to the genus of plants with a lot of species that are far less explored or not explored at all. Reports on how the extract of *T. catappa* leaf fares on the immune parameter of fish is not known and also is less extensively profiled for its chemical constituent. As such, a better understanding of it on an economically and globally well-cultured fish like the tilapia that is plagued with diseases is a right step in the right direction because researchers widely recommend the plant extract as a cheaper and safer alternative for fish health management. Furthermore, getting another species among the listed endangered species marked for extinction from the *Terminalia* genus that can serve the same purpose will equally show the scientific importance of the unexplored species and by extension lead to the exploration of the endangered species. Hence, the use of *T. catappa* and *T. subspathulata* for this research work.

1.6 Thesis Outline

This thesis is presented in six chapters. Chapter 1 briefly described the thesis in general. Chapter 2 highlighted the review of *Terminalia* species, their morphology, ethnopharmacology and phytochemical. Metabolite profiling and some metabolite profiling techniques were briefly discussed. Finally, a brief overview of the fish model (*Oreochromis* sp.) was also presented. Chapter 3 examined the antioxidant properties of TC (leaf, bark and nut) and TST (leaf, bark and fruit) 70% methanol crude extracts assayed and correlate their scavenging properties to their amount of total phenolic content (TPC) and total flavonoid content (TFC) quantified using person's correlation. Furthermore, the TC and TST extracts were profiled using liquid chromatography electrospray ionisation mass spectrometry (LC-ESI-MS/MS) and GCMS. Chapter 4 discussed the in vivo toxicity of TC and TST leaves and barks crude extracts on blood parameters, blood biochemistry and histological evaluation of red hybrid tilapia (*Oreochromis* sp.) fish. Also, an innate immune study of the crude extracts of TC and TST leaves was conducted on red hybrid tilapia using the safe dose determined from the toxicity study.

Furthermore, the crude extracts of TC and TST leaves were partitioned using solvents of increasing polarity and were used to study the immune parameters. Chapter 5 focused on the isolation and characterisation of compound(s) from TST leaf extract of the partition that showed the highest immunostimulant property from the results of chapter 4. This is so since it is less explored compared to TC. Finally, chapter 6 is the overall conclusion and summary of the entire work, along with a future perspective of the work.

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