ANTIMYCOBACTERIAL ACTIVITY AND IMMUNOGENIC PROFILES OF SELECTED MANGROVE PLANTS FROM SULAMAN WETLAND IN SABAH AGAINST *MYCOBACTERIUM TUBERCULOSIS*

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BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
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I hereby declare that the content in this thesis is my own except for quotations, equations, summaries and references, which have been duly acknowledged.

8 September 2023

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DEGREE	:	**DOCTOR OF PHILOSOPHY IN APPLIED SCIENCE**
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ABSTRACT

The high tuberculosis mortality rate has piqued the interest of researchers and prompted efforts to address this public health issues. Social deprivation, contributing to the high incidence of tuberculosis (TB) cases, remains a significant threat in the state of Sabah, particularly considering the emergence of *Mycobacterium tuberculosis* drug resistance, which is not well understood. On the other hand, mangrove species have exhibited remarkable compounds with medicinal significance, including antimicrobial activity that has the potential to induce immunomodulating effects. This study aimed to assess the potential antimycobacterial activity of plant extracts in both treatment and pre-treatment approaches. The study analyzed the effects of these extracts on the expression profiles of immunogenic genes in mice infected with *M. tuberculosis* strain SBH257, which is a clinical strain from Sabah. Initially, 26 samples were collected from different parts of six mangrove plant species (*Avicennia marina*, *Bruguiera gymnorrhiza*, *Ceriops tagal*, *Rhizophora apiculata*, *Rhizophora mucronata* and *Xylocarpus granatum*) in Sulaman Lake Mangrove Forest, Tuaran, Sabah. Hexane and ethyl acetate extracts were obtained and analyzed for their total phenolic content (TPC) and their ability to scavenge DPPH free radicals via Folin-Ciocalteu's and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays, respectively. The antimycobacterial activity of the extracts was then evaluated using broth microdilutions and the microtiter resazurin assay at concentration ranges from 0.02 mg/mL to 10 mg/mL. The extract with the highest inhibition strength and the lowest MIC value was selected for the *in vivo* study. In the animal study, acute and sub-acute toxicity tests were conducted following the OECD guidelines 423 using male and female BALB/c mice aged 8 – 10 weeks to assess the safety of the extracts. In the acute oral toxicity test, a single dose of 2000 mg/kg body weight of the tested extract was administered orally to the mice. For the sub-acute toxicity test, the extract was administered daily at doses of 200 mg/kg and 300 mg/kg body weight for up to 28 days in different treatment groups. The mice were closely observed for any signs of toxicity. Vital organs and blood samples were collected from the mice to analyze relative organ weight, kidney function, and liver function. To further evaluate the immunomodulatory effects of the extract, the expression of different immune-

related genes involved in the innate and adaptive responses of TB-infected mice against plant extracts was analyzed using RT-qPCR. Tuberculosis infection was induced in the mice through intranasal challenge with a *M. tuberculosis* suspension. The extracts were administered orally for two weeks, starting 14 days post-infection at a selected dose of 200 mg/kg. Moreover, the mice were pre-treated with the extract for 14 days prior to the MTB infection. Mice were divided into five groups, each consisting of 6 animals. Lung tissues were harvested from the mice, and total RNA was extracted for gene expression analysis. Furthermore, the gene expression of the MTB virulence genes, *IS6110* and *MPB64*, was quantified in each group. Interestingly, the results revealed that all the tested extracts demonstrated antimycobacterial activity via resazurin assay. The ethyl acetate extract of *Ceriops tagal* leaf exhibited the highest antimycobacterial activity, with a minimum inhibitory concentration (MIC) value of 0.039 mg/ml. On the other hand, the hexane extracts of *R. apiculata* root and *R. mucronata* stem displayed the lowest antimycobacterial activity, with a MIC of 2.5 mg/ml. Among the extracts, the ethyl acetate extracts of *Ceriops tagal* leaf were among the higher total phenolic content (129.59 mg GAE/g) and antioxidant activity (778.89 µg/mL). These findings suggest that the phenolic compounds present in the extracts may be responsible for the antimycobacterial activity observed. Furthermore, the acute and sub-acute *in vivo* toxicity tests showed no observable symptoms of toxicity present with 100 % survival rate among the mice. Additionally, there were no significant changes in body weight compared to the control group. It is noteworthy that the interferon gamma (*IFN-γ*) gene, which plays a crucial role in protective immunity against MTB, showed a significant upregulation in the lungs of MTB-infected mice compared to healthy mice. However, the ethyl acetate extract of *Ceriops tagal* leaf remarkably suppressed *IFN-γ* production in both the treatment and protective groups, comparable to the rifampicin group. This demonstrates a potent immunomodulatory potential at a dosage of 200 mg/kg. The results align with the expression levels of other innate immune genes (*IL2, CLEC4E, CLEC7A)* and adaptive immune genes (*LY6I, IFN-γ,* and *TNF-α*) in the treatment group, which exhibited significant upregulation. Furthermore, the innate immune genes (*IL2, CLEC4E, CLEC7A*, and *MMP9*) and adaptive immune genes (*LY6I, IFN-γ, TNF-α,* and *FCRG1*) displayed significant downregulation in the protective group. These findings suggest that the ethyl acetate extract of *Ceriops tagal* leaf exerted a

substantial immunomodulatory activity, enhancing protection in the lungs of MTB-infected mice. Additionally, the downregulation of MTB virulence genes (*IS6110 and MPB64*) further supports these results, indicating that the daily treatment and pre-treatment of mice with the extract led to bactericidal action against MTB comparable to the effect of rifampicin. In summary, these findings provide a platform for future identification of phytoconstituents that contribute to the antimycobacterial activity of the ethyl acetate extracts of *Ceriops tagal* leaf, as well as for conducting pre-clinical studies. The discovery of potential compounds with anti-tubercular activity from these extracts could facilitate the development of novel drugs to combat tuberculosis.

ABSTRAK

AKTIVITI ANTIMIKOBAKTERIA DAN PROFIL IMUNOGENIK TUMBUHAN BAKAU TERPILIH DARI SULAMAN WETLAND DI SABAH TERHADAP MYCOBACTERIUM TUBERCULOSIS

Kadar kematian TB yang tinggi telah menarik perhatian para penyelidik dan menggesa usaha untuk menghentikan isu kesihatan awam ini. Kekurangan sosial yang menyumbang kepada insiden kes tuberkulosis (TB) yang tinggi kekal sebagai salah satu daripada ancaman terbesar di negeri Sabah memandangkan kemunculan kebolehkebalan ubat tuberkulosis yang tidak difahami dengan baik. Sebaliknya, spesies bakau telah mendedahkan sebatian yang luar biasa berkaitan dengan kepentingan perubatan termasuklah aktiviti antimikrob yang mempunyai potensi kesan imunomodulasi yang efektif. Kajian ini bertujuan untuk menilai potensi aktiviti antimikobakteria ekstrak tumbuhan dalam pendekatan rawatan dan pra-rawatan. Kajian ini menganalisis kesan ekstrak ini terhadap profil ekspresi gen imunogenik pada tikus yang dijangkiti dengan strain M. tuberculosis SBH257, yang merupakan strain klinikal dari Sabah. Pada mulanya, dua puluh enam sampel daripada bahagian berlainan daripada enam spesies tumbuhan bakau ((Avicennia marina, Bruguiera gymnorrhiza, Ceriops tagal, Rhizophora apiculata, Rhizophora mucronata and Xylocarpus granatum) telah diperolehi dari Sulaman Lake Mangrove Forest, Tuaran, Sabah. Ekstrak heksana dan etil asetat telah diperoleh dan dianalisis untuk jumlah kandungan fenolik (TPC) dan keupayaan untuk menghilangkan radikal bebas 'DPPH' melalui ujian 'Folin-Ciocalteu' dan '1,1 – diphenyl-2-picrylhydrazyl (DPPH)' masing-masing. Selain itu, aktiviti antimikobakteria ekstrak tersebut dinilai dengan menggunakan kaedah ujian 'broth microdilutions' dan 'microtiter resazurin' dalam julat kepekatan dari 0.02 mg/mL hingga 10 mg/mL. Kekuatan perencatan tertinggi dengan nilai konsentrasi perencatan minimum (MIC) terendah telah dipilih dan digunakan dalam kajian in vivo. Untuk kajian ke atas haiwan, ujian ketoksikan akut dan sub-akut dinilai menggunakan tikus 'Balb/c' jantan dan betina yang berumur antara 8 hingga 10 minggu, dengan tujuan menilai keselamatan ekstrak mengikut panduan 'OECD' 423. Dos ekstrak pada kepekatan 2000 mg/kg berat tubuh telah diberikan sekali secara oral kepada tikus dalam ujian ketoksikan oral akut. Sementara

itu, ekstrak diberikan setiap hari selama 28 hari pada kepekatan 200 mg/kg dan 300 mg/kg berat tubuh dalam ujian ketoksikan sub-akut dalam kumpulan rawatan yang berbeza. Haiwan tersebut diperhatikan untuk sebarang tanda ketoksikan. Organ penting dan darah tikus telah diambil untuk analisis berat organ relatif, ujian fungsi buah pinggan dan hati. Bagi menilai lebih lanjut kesan imunomodulator ekstrak, ekspresi gen imun yang berbeza yang berkaitan dengan imunisasi 'innate' (nonspesifik) dan imunisasi adaptif (spesifik) pada tikus yang dijangkiti TB dan yang menerima ekstrak tumbuhan telah dianalisis melalui ujian RT-qPCR. Jangkitan tuberkulosis pada tikus telah dilakukan melalui kaedah intranasal dengan suspensi M. tuberculosis, dan ekstrak diberikan secara oral selama dua minggu dari hari ke-14 selepas jangkitan pada dos kepekatan yang terpilih iaitu 200 mg/kg. Selain itu, tikus telah dirawat terlebih dahulu dengan ekstrak selama 14 hari sebelum jangkitan MTB. Tikus dibahagikan kepada lima Kumpulan, di mana satu kumpulan mempunyai 6 ekor tikus. Paru-paru tikus telah diambil, dan 'total' RNA telah diekstrak untuk analisis ekspresi gen. Ekspresi gen virulan MTB, termasuk IS6110 dan MPB64, juga dianalisis dalam setiap kumpulan. Menariknya, hasil analisis menunjukkan bahawa semua ekstrak yang diuji menunjukkan aktiviti antimikobakteria melalui ujian 'resazurin', dan aktiviti antimikobakteria yang paling aktif ditemui dalam ekstrak etil asetat daun Ceriops tagal dengan nilai kepekatan perencatan minimum (MIC) sebanyak 0.039 mg/mL. Sementara itu, ekstrak heksana akar R. apiculata dan heksana batang R. mucronata menunjukkan aktiviti antimikrobakteria paling rendah, dengan MIC sebanyak 2.5 mg/mL. Antara ekstrak yang dikaji, ekstrak etil asetat daun Ceriops tagal adalah yang memiliki jumlah kandungan fenolik yang tinggi (129.59 mg GAE/g) dan aktiviti antioksidan (778.89 µg/mL). Penemuan ini menunjukkan bahawa sebatian fenolik yang terdapat dalam ekstrak mungkin bertanggungjawab terhadap aktiviti antimikobakteria yang diperhatikan. Ujian ketoksikan in vivo akut dan sub-akut menunjukkan bahawa tiada gejala ketoksikan yang boleh diperhatikan, dengan tiada kematian tikus yang dilaporkan, dan tiada perubahan ketara dalam berat badan berbanding kumpulan kawalan. Nampaknya, gen IFN-γ, yang memainkan peranan penting dalam imuniti perlindungan terhadap MTB, menunjukkan peningkatan yang ketara dalam paru-paru tikus yang dijangkiti MTB berbanding dengan tikus yang sihat. Walaubagaimanapun, ekstrak etil asetat daun Ceriops tagal telah menyekat pengeluaran IFN-γ dalam kedua-dua kumpulan rawatan dan perlindungan, setanding

dengan kumpulan rifampicin. Ini menunjukkan potensi imunomodulator yang kuat pada dos 200 mg/kg. Hasil tersebut selaras dengan tahap ekspresi gen imunisasi 'innate' (IL2, CLEC4E, dan CLEC7A) dan gen imunisasi adaptif (LY6I, IFN-γ, dan TNF-α) dalam kumpulan rawatan, yang menunjukkan peningkatan yang ketara. Tambahan pula, gen imun semula jadi (IL2, CLEC4E, CLEC7A, dan MMP9) dan gen imun adaptif (LY6I, IFN-γ, TNF-α, dan FCGR1) menunjukkan penurunan ekspresi yang ketara dalam kumpulan pelindung. Penemuan ini menunjukkan bahawa ekstrak etil asetat daun Ceriops tagal mempunyai aktiviti imunomodulator yang tinggi, seterusnya meningkatkan perlindungan dalam paru-paru tikus yang dijangkiti MTB. Data ini disokong oleh penurunan regulasi gen virulan MTB (IS6110 dan MPB64), yang menunjukkan aktiviti bakterisidal terhadap MTB yang terhasil daripada pra-rawatan dan rawatan harian dengan ekstrak yang diberikan kepada tikus, setanding dengan kesan rifampicin. Secara keseluruhannya, penemuan penyelidikan ini boleh menyediakan platform untuk mengenal pasti fitokonstituen yang dapat menghasilkan aktiviti antimikrobakteria dan kajian pra-klinikal pada masa hadapan. Sebatian yang menunjukkan potensi aktiviti anti-tuberkular daripada ekstrak etil asetat daun Ceriops tagal akan membawa kepada penemuan penghasilan ubat-ubatan baru untuk penyakit tuberkulosis.

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