Research title: Elucidating the Antimalarial Efficacy of Curcumin Derivatives on Immunomodulatory Protein using in silico Quantum Computational Studies, Synthesis and *In vitro* Assessment

Project team members: Siti Nur Hidayah Jamil[1,2], Su Datt Lam[3], Shevin Rizal Feroz[4], Rani Maharani[5], Jalifah Latip[1,*]

[1]Department of Chemical Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.
[2]International Joint Department of Materials Science and Engineering Between National University of Malaysia and Gifu University, Graduate School of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[3]Department of Applied Physics, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.
[4]Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
[5]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor 45363, West Java, Indonesia

Final Report

Acknowledgement: This study was supported by the South Asia Research Hub, Foreign, Commonwealth & Development Office (FCDO), Government of United Kingdom. The views expressed in this report, however, do not necessarily reflect the official policies of the Government of United Kingdom.

Executive summary

The central aim of this research project is to validate the antimalarial and anti-inflammatory activities of curcumin derivatives through their effects as potential antimalarial and on the human GSK-3β, a kinase implicated in the regulation of human inflammatory response in malaria infection. This is particularly critical in the context of malaria, a pervasive global health concern, where resistance to existing treatments continues to escalate. Our research is centred

on the hypothesis that by inhibiting GSK-3β, the derivatives are expected to not only impede the proliferation of *Plasmodium falciparum*, the parasite responsible for the most lethal form of malaria but also modulate inflammatory pathways mediated by GSK-3β in human cells, thus offering an effective dual therapeutic strategy.

Our ongoing research also aims to overcome the constrained bioavailability (ADMET) of curcumin, which limits its antimalarial activity as a potent malarial medicine, by developing derivative compounds with enhanced bioavailability. The main planned activities of this project comprise molecular dynamics simulations, chemical synthesis, and biological assessments, which provide insights into curcumin derivatives' interactions with the key inflammatory-related protein, GSK-3β, involved in malarial infection. This approach aims to establish curcumin derivatives as highly potent drug candidates with dual antiparasitic and anti-inflammation for malaria treatment, by incorporating two different biological assessments; isothermal titration calorimetry (ITC) and antiplasmodial pLDH *in vitro* study.

A comprehensive ITC is employed to quantitatively analyse the binding interaction between the curcumin derivatives and haemin, an iron-containing compound utilised by malaria parasites to detoxify free heme, which is toxic to them. This interaction is crucial as it could reveal a novel mechanism of action for the derivatives, potentially disrupting the parasite's heme detoxification process and contributing to its antimalarial activity. Further, the antiplasmodial assays against two strains of *P. falciparum*, K1 and 3D7, represent the spectrum of drug-resistant and drug-sensitive malaria parasites, respectively. These assays provide initial insights into the compound's efficacy.

From a non-technical standpoint, the significance of this project lies in its pursuit of a non-toxic, dual-purpose therapeutic avenue. Curcumin, a compound with a long history of safe use in traditional medicine, presents an opportunity to develop a treatment that is effective against malaria parasites and gentle on the patient's body. This could revolutionise the way we approach malaria treatment, offering a safer alternative to current drugs that often come with harsh side effects.

The impact of this research extends beyond the scientific community, offering tangible benefits to global health. By leveraging the inherent safety profile of curcumin, we are developing a treatment strategy that minimises the risk of adverse reactions, making it a particularly appealing option for vulnerable populations such as children and pregnant women who are most affected by malaria. Furthermore, the non-toxic nature of curcumin derivatives also

means that they could be used as a long-term prophylactic treatment, which is crucial in malaria-endemic regions where continuous exposure to the parasite is a persistent threat.

The results of our research are expected to illuminate a path forward for the scalable production of curcumin-based antimalarial treatments. By demonstrating the effectiveness of these derivatives in laboratory settings, we lay the groundwork for future clinical trials and eventual mass production, which could significantly reduce the global burden of malaria. The potential for a cost-effective, easily distributable, and culturally accepted treatment could be a game-changer, particularly in resource-limited settings where malaria is most prevalent.

1. Background

The global infection rate of malaria was estimated to have reached 247 million in 2021, covering 84 malaria-endemic countries, with a fatality rate of 14.8 per 100,000 population and an estimated 619,000 deaths [1]. Caused by *Plasmodium* parasites infecting humans, mainly *P. falciparum* and *P. vivax*, these parasites are transmitted by female *Anopheles* mosquitoes and can enter the circulatory system within 30 minutes after a bite [2, 3]. The parasites first target liver cells and erythrocytes, and if the affected individual remains untreated, the infection could develop into deadly haemorrhagic conditions including severe and cerebral malaria which involve parasite invasion into the brain and immune system.

Although much progress has been achieved towards eliminating malaria, the recent rise in malaria cases, particularly in Southeast Asia, is alarming [1, 4]. For example, approximately 17,125 cases have been reported in Malaysia over a period of only four years (2017 – 2021). In addition to the growing number of malaria cases, the resistance of *Plasmodium* parasites against current antimalarial drugs such as artemisinin and chloroquine is highly concerning challenge.

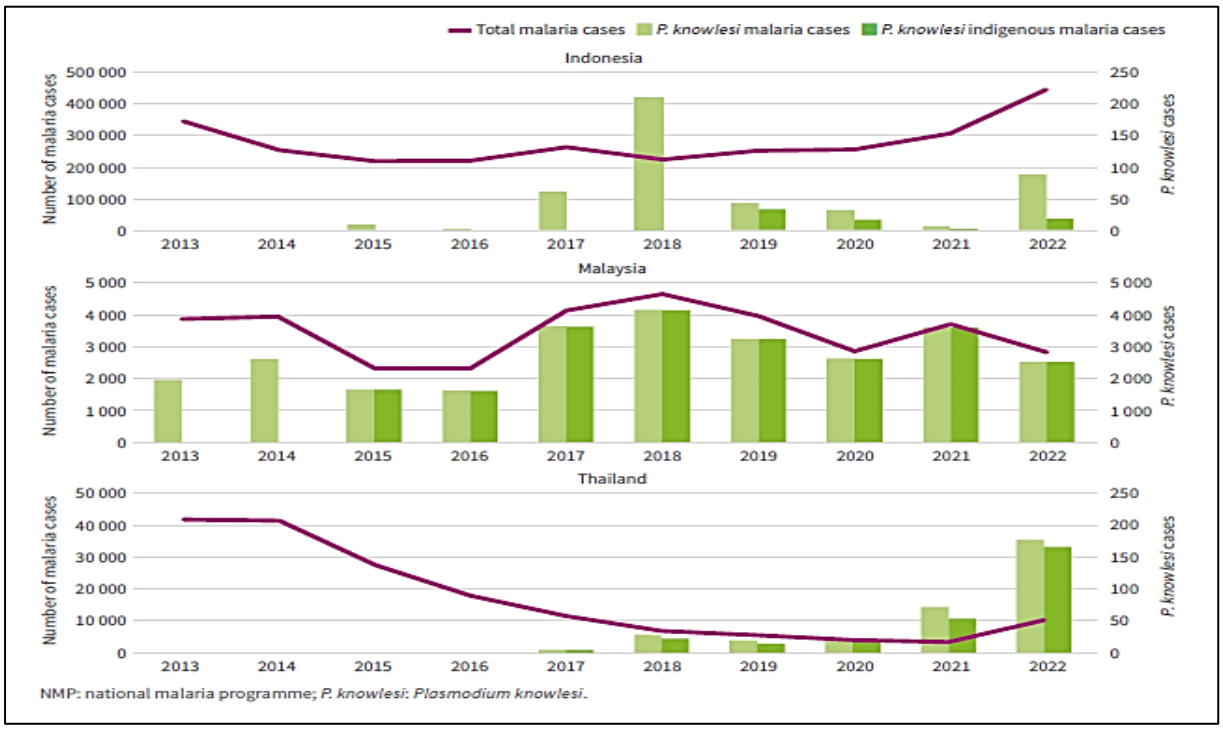


Figure 1: Number of total *P. knowlesi* and malaria cases in Malaysia, Indonesia, and Thailand from 2013-2022.

Recent data revealed 1,485 indigenous malaria cases in Malaysia between 2022 and May 2023, ending the country's streak of zero recorded human malaria infections from 2018 to 2021 (Ministry of Health, 2023). Malaysia, Indonesia, Thailand, Singapore, and Cambodia have emerged as pivotal hubs for *P. knowlesi* transmission worldwide. Malaysia has been the hotspot with the highest number of *P. knowlesi* cases in Southeast Asia, accounting for 90.5%, followed by Thailand with 3.1% and Indonesia with 0.1% in 2022. Despite the primary mode of transmission for *P. knowlesi* malaria being from monkeys to humans, there exists the potential for human-to-human transmission, especially with increasing prevalence rates. These statistics underscore malaria's persistent status as one of the most critical global health challenges, impacting millions of people.

Globally, the burden of malaria remains the highest within the African region. The most recent prevention of malaria recommended by the World Health Organisation (WHO) is the RTS,S/AS01 vaccine, which was issued prequalification approval by WHO in July 2022. In regard to treatment for malaria patients, the present strategy primarily relies on several drugs, including artemisinin, chloroquine, and Artemisinin-based Combination Therapies (ACTs)

(WHO, 2023). Artemisinin is highly effective against malaria, particularly when used in combination with other antimalarial drugs in ACTs.

However, due to the emergence of resistance to artemisinin and chloroquine in some regions, ACTs have become the primary alternative for treating malaria in areas where these drugs are no longer effective. According to the most recent report from WHO (World Malaria Report 2023), there are some worrying signals of resistance to the ACT partner drugs currently in that need to be investigated and action must be taken before ACTs start to fail. Drug resistance and the limited availability of novel and potent antimalarial drugs pose significant hurdles, necessitating the exploration of alternative treatment options.

The present search for novel compounds for antimalarial medicine has revolved around natural compounds. Specifically, in this research, we have chosen curcumin as our reference compound. Curcumin was reported for its antimalarial activity for the first time by Reddy et al. [5]. This prior research employed both *in vitro* and *in vivo* assessments which were undertaken to substantially underline and evidence the antimalarial effect of curcumin [5]. The study showed potent growth inhibition of chloroquine-resistant *P.falciparum* assay (IC_{50} ~5) and reduction of blood parasitaemia level by 80-90% upon treatment of *P.berghei*-infected mice with a dose of 100mg/kg over 3 weeks. Nevertheless, the main drawback of curcumin as an antimalarial and anti-inflammatory agent is its low bioavailability, limiting its clinical application and being recognised as an effective potential drug. This includes rapid metabolism, poor oral absorbability, and low aqueous solubility. Therefore, this project specifically explores the potential of compounds derived from curcumin, to address the pressing need for alternative antimalaria treatments.

Additionally, our research also features the molecular target of the specific and main anti-inflammatory-related target in malarial infection, the human glycogen synthase kinase 3 (GSK-3β) protein. GSK-3β has been identified as a key evolutionarily conserved eukaryotic Ser/Thr kinase protein involved in regulating parasite growth and host immune responses, which is essential for managing advanced malarial infections including cerebral and severe malaria [6, 7]. The evidence presenting the GSK-3β inhibition by curcumin has only been published for the first time recently in 2017 by Ali et. al. [8, 9] which was also supported by preliminary computational simulation [10].

Aligned with the E-2025 initiative aimed to eliminate malaria by 2025 launched by the World Health Organisation (WHO) in 2021 [1], this research seeks the objective of exploring the potential curcumin derivative compounds as dual antimalarial and anti-inflammatory agents by targeting the specific immunomodulatory protein GSK-3β. As part of the present research, the team has incorporated computational exploration of potential inhibition of specific GSK-3β protein by curcumin derivatives which include molecular docking, density function theory (DFT) calculations, and MD simulation (presented in detailed within the attached draft manuscript to be published).

2. Research questions / Objectives

The research project focuses on exploring the potential antimalarial effects of curcumin derivatives. The preliminary results, based on molecular docking, ADMET assessments, and molecular docking simulations, have identified promising compounds with a strong binding profile to the GSK-3β, a key protein in the immunomodulatory pathway in malaria infection. The escalation of severe malaria is also attributed to complications arising from dysregulated immune responses in humans, which heightened mortality rates. This immune response primarily involves the modulation of pro- and anti-inflammatory cytokines levels, which regulate parasite growth and host immune responses through the key protein GSK-3β within the immunomodulatory pathway.

In addition, since malaria cases showed a heavy reliance on ACTs in low-income countries, predominantly within the African region, this leads to a perceived lack of profitability for commercial entities. High development costs and lower potential returns may discourage investment in antimalarial drug development compared to diseases prevalent in wealthier regions. Curcumin, abundantly found in Asian countries, offers a cost-effective avenue for research and drug development compared to other bioactive compounds with limited concentrations in source plants.

Curcumin is a bioactive compound that is extracted from the rhizomes of the turmeric plant (Curcuma longa), which is native to the Southeast Asia region, particularly countries like India, Indonesia, and Malaysia. In terms of abundance, curcumin is quite plentiful in Southeast Asia due to the extensive cultivation of turmeric. India is the largest producer, consumer, and exporter of turmeric in the world. The cultural significance of turmeric, which is used not only

as a spice but also for its medicinal properties, contributes to its widespread cultivation and use in the region. Curcumin's accessibility potentially lowers development costs, attracting interest and investment from research organisations and commercial entities, and fostering prospects for future antimalarial drug development. Its availability could drive more comprehensive and cost-efficient endeavours in combating malaria through drug innovations.

When it comes to the development of malaria treatment, curcumin has been studied for its potential antimalarial properties. Research has indicated that curcumin can interfere with the life cycle of the malaria parasite, Plasmodium falciparum, and may enhance the efficacy of existing antimalarial drugs. However, the use of curcumin in mainstream malaria treatment is not yet a reality. This is due to several factors, including its poor bioavailability, which means that when taken orally, only a small percentage of curcumin is absorbed into the bloodstream. While curcumin is abundant in Southeast Asia due to the widespread cultivation of turmeric, its application in malaria treatment is still under development and faces challenges, particularly in terms of improving its bioavailability. The abundance of curcumin itself is not a constraint; rather, the challenge lies in the translation of its medicinal properties into effective clinical treatments for malaria.

3. Methodology

The present research is a continuous work of our team which comprised structure-activity relationship (SAR) analysis, molecular docking simulation, *in silico* ADMET assessments and DFT geometry optimisation. From these works, curcumin derivatives with higher binding affinity, better inhibition constant, K_i, and better molecular orbital energy profiles than curcumin have been screened and selected for further Molecular Dynamics (MD) simulations.

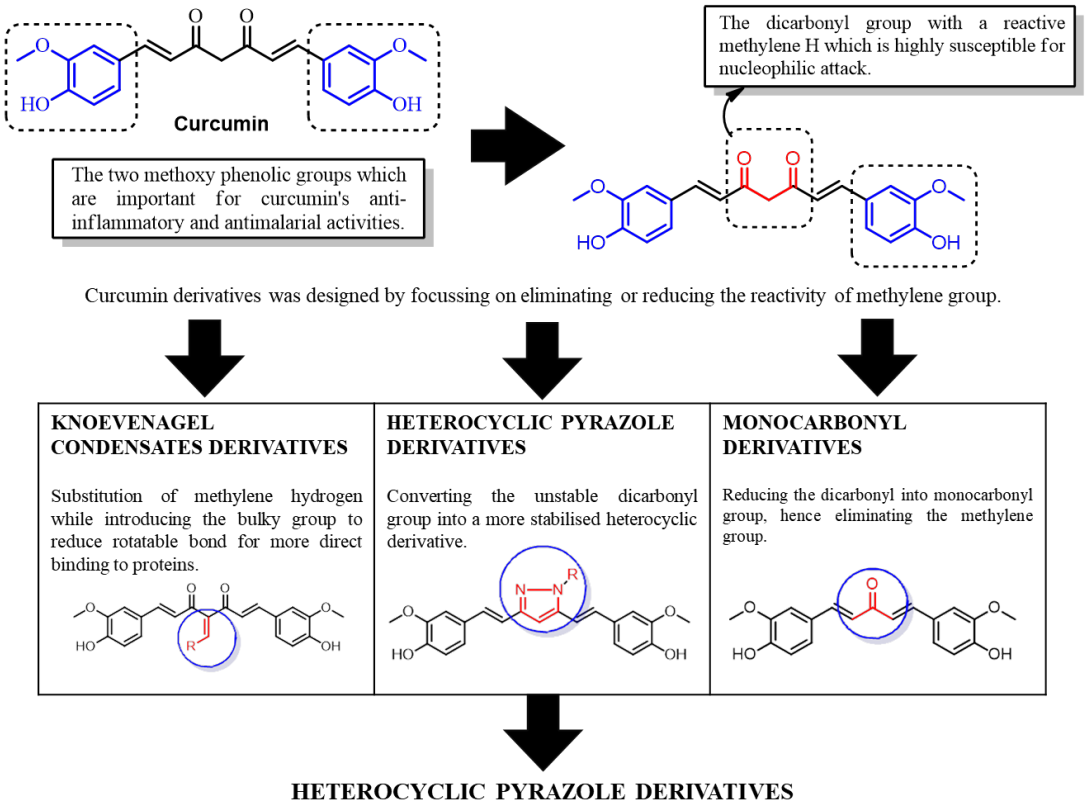
3.1 Molecular Dynamic Simulations

Department of Chemical Science, Faculty of Science and Technology, UKM, Malaysia

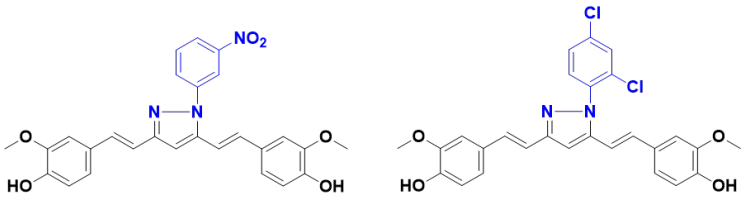
The present research embarked on MD simulations which allow the understanding of the binding stability, structural and conformation fluctuation, conformational changes, and kinetic behaviour of the compounds to the GSK-3β protein. The simulation creates a dynamic environment that is very close to the actual and mimics the interactions as

would happen in the cellular milieu. The conformational changes of the ligand-protein complexes can be observed and analysed for the time scale set for the whole simulation, to compare the stability of the different complexes.

This present study employed MD simulation using the Ubuntu 20.04 operating system employing the Gromacs throughout 100 ns [11]. The CHARMM 27 all-atom force field was used for the generation of protein topology and all the systems were set to solvated using the SPC (Simple Point Charge) water model in a cubic box. The temperature and pressure for the experimental conditions were set at 300 K and 1 atm, respectively, to imitate the actual environment as accurately as possible [12]. A complete MD simulation generate information on root mean square deviation (RMSD), root mean square fluctuation (RMSF), number of hydrogen bond interactions between the ligand and protein, and radius of gyration (R_g) along the 100 ns simulation time. The trajectories and complexes visualisation from .xtc file were observed and analysed using UCSF Chimera, in which the graphs were generated for output visualisations [13].



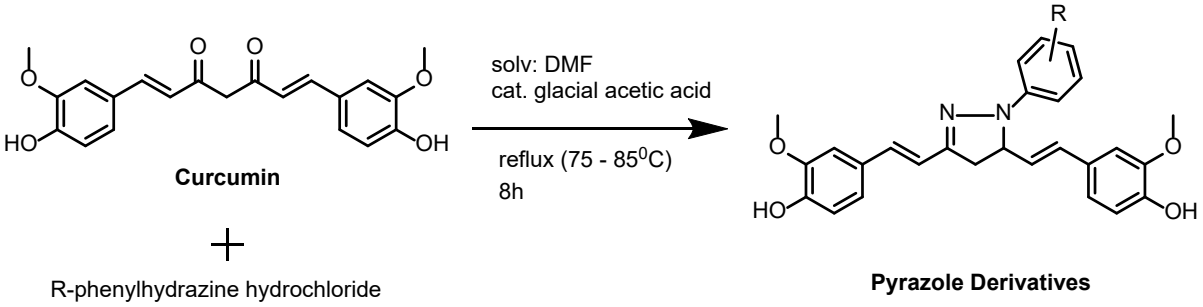
Based on experimental works, the synthesis of heterocyclic pyrazole derivatives was identified as the most achievable and feasible among the three proposed approaches. This is concluded based on the rate of forward reaction and the practicality of optimising the reaction condition to obtain a significant yield of the derivative compounds.



(10) (17)

3.2 Synthesis of curcumin derivatives

Laboratorium Sentral, Universitas Padjadjaran, Indonesia and Graduate School of Engineering, Gifu University, Japan



Scheme 1: Reaction for the synthesis of pyrazole derivatives **10** and **17**.

Pyrazole derivatives of curcumin were synthesised by heating curcumin with hydrazine compound in acetic acid for 8 hours. 0.136 mmol of curcumin (2 eq.) along with the desired 0.203 mmol phenyl hydrazine compound (1.5 eq.) were dissolved in dry DMF 3.0 mL (solvent), with the presence of 1.0 mL glacial acetic acid as the catalyst. The reaction was refluxed for 8 hours, and monitored by TLC (n-hexane/ethyl acetate) to completion. The precipitate product was filtered and then the solvent was removed in vacuo. Yield (**10** – 52%, yellow crystalline, **17** – 68%, brownish yellow crystalline) was collected and analysed using ^{1}H NMR (400 MHz) for characterisation and structural confirmation. (Refer to the Appendix – Supporting Information)

3.3 Profiling the binding of curcumin derivative to heme using Isothermal Titration Calorimetry (ITC)

Malaysia Genome and Vaccine Institute (MGVI), Bangi, Malaysia and Universiti Malaya (UM), Malaysia

Out of the many techniques available for studying biomolecular interactions, isothermal titration calorimetry (ITC) is currently considered as the gold standard. This activity was employed to study the interaction of derivative compounds with haemin and elucidate the potential of the derivatives to interfere with the heme detoxification process employed by *Plasmodium* parasites, thereby disrupting their survival. It is a fast, accurate and label-free method for measuring the thermodynamics and binding affinities of molecular associations in solution. In comparison to other methods to monitor binding, ITC is the only method that can directly measure binding energetics including Gibbs free energy, enthalpy, entropy, and heat capacity changes. Since ITC measures any reaction that results in heat change, it can be used to measure binding events between essentially any type of biological or chemical ligand.

The interaction of the curcumin derivatives to haemin was studied using a TA Instruments NanoITC microcalorimeter, based on the experimental design described by Feroz et al. 2016 [14]. Solutions of curcumin derivatives and haemin were prepared in the same buffer (10 mM sodium phosphate buffer, pH 7.4) and degassed under vacuum for 20 min prior to the titration experiments. After loading the sample cell with hemin,

a syringe (250 µL) containing the curcumin derivatives was placed into the microcalorimeter. The titration experiment involved 25 consecutive injections of 10 µL of titrant added intermittently every 400 s, with a stirring speed of 250 rpm to ensure homogeneous mixing of the solutions. Appropriate blank experiments involving injections of the ligand into the buffer solution was also performed.

The experiments were performed individually for each derivative. The amount of heat liberated with each injection was calculated by integrating the heat rate peaks using the NanoAnalyze software provided by the manufacturer. The corrected calorimetric data was analysed based on a suitable binding model to determine the values of the association constant, binding stoichiometry, and the thermodynamic parameters (changes in free energy, enthalpy, and entropy) that accompany the reaction.

3.4 Evaluation of the antiplasmodial effect of curcumin derivatives using pLDH assay

Department of Biological Sciences and Biotechnology, Faculty of Science & Technology, Universiti Kebangsaan Malaysia (UKM) and Department of Parasitology, Faculty of Medicine, Universiti Malaya (UM)

P. falciparum 3D7 (chloroquine-sensitive) and K1 (chloroquine-resistant) strains were be obtained from the Malaria Research and Reference Reagent Resource Centre (MR4), Manassas, Virginia. The parasite was revived from cryo-preservation and maintained in culture at 1% haematocrit of purified O^+ human erythrocytes in RPMI 1640 media supplemented with 0.5% Albumax I (GIBCO, Life Technologies, USA), 25 mM HEPES, 100 µM hypoxanthine, 12.5 µg/mL gentamicin and 1.77 mM sodium bicarbonate at 37°C, 5% CO_2. The parasitaemia level was evaluated by microscopic examination of field-stained thin smears. The susceptibility of the parasites to curcumin was measured using the parasite lactate dehydrogenase (pLDH) assay. pLDH assay was be performed according to Makler et al. (1993) in flat-bottomed 96-well microtiter plates. The parasite cultures, prior to experimentation, were synchronised by treatment with 5% D-sorbitol. Parasites were plated in the 90% ring phase at 2% haematocrit and 2% parasitaemia in culture media containing curcumin at defined concentrations. Colour development in the plates was monitored at 655 nm using a microplate reader

(Fluostar Optima) after an hour of incubation at room temperature in the dark. Data was analysed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA) by non-linear regression to calculate 50% inhibitory concentrations (IC_{50}).

4. Results

The culmination of our project on synthesising curcumin derivatives and exploring their potential antimalarial effects has yielded compelling final results. Our key findings not only demonstrate the feasibility of novel technologies but also underscore the scalability of existing methodologies, presenting a robust pathway to impact aligned with Sustainable Development Goals (SDGs). To date, the present research has progressed to the stage of the molecular docking study validation through MD simulation and further establishment of the antimalarial activity using Isothermal Calorimetry (ITC) and antiplasmodial pLDH *in vitro* assessments. Prior to this, an integrated *in silico* approach, encompassing SAR analysis, ADMET assessment, molecular docking, and DFT calculations has been completed.

4.1 MD Simulations

MD simulation is performed as a fundamental *in silico* tool for analysing the dynamics of ligand-protein complexes, which also validates the binding established from the docking analysis. Throughout the 100 ns simulation time, the environment of the system mimics the actual biological system for the behaviour and stability of bounded ligand and protein, while allowing conformational changes of the ligand within the active site of the protein [15]. In this study, the MD simulations for curcumin and the eleven derivatives have been accomplished to evaluate the binding potential as proposed based on the docking analysis, as well as the dynamics of the ligand-protein complexes.

The simulation over 100 ns generated information on the RMSD, RMSF, $R_{g,}$ and hydrogen bond interactions. To highlight and validate the docking results, which converged to derivative **10** as the top hit ligand with the highest binding affinity, the plots of the generated information are obtained as depicted in Figure 5.

RMSD information (Figure 4 – 1 and Figure 5 – 1a and 1b) present the stability of a compound to stay within its docked confirmation and maintain its binding throughout the simulation time [15]. Curcumin started at 0.1 nm and stabilised within the range 0.4 – 0.7 nm after 10 ns, while **10** started at 0.1 nm and stabilised within 0.3 nm – 0.4 nm after 25 ns through the whole simulation period, in which the conformational change is less deviated than curcumin.

The RMSF plots (Figure 4 – 2 and Figure 5 – 2a and 2b), which express the flexibility and fluctuations of particular residues during the simulations [16], show that curcumin, with RMSF values ranging between 0.05 and 0.40 nm, fluctuates more than **10** and **17**, with RMSF 0.05 - 0.35 nm for both. These fluctuations could be influenced by the conformational changes adopted by the residues, to facilitate and involve in binding with the ligand [16]. Notably, there are fluctuations observed involving the Ile62, Val70, and Ala83 which signifies the hydrophobic interactions, (Figure 3) and residues Asp133, Tyr134, and Val135 which reasoned the hydrogen bonding interactions. Additionally, the highest fluctuation at 0.35 nm RMSF is observed at residue Ser219, which is one of the catalytic residues that might also influence significant dynamics of the protein.

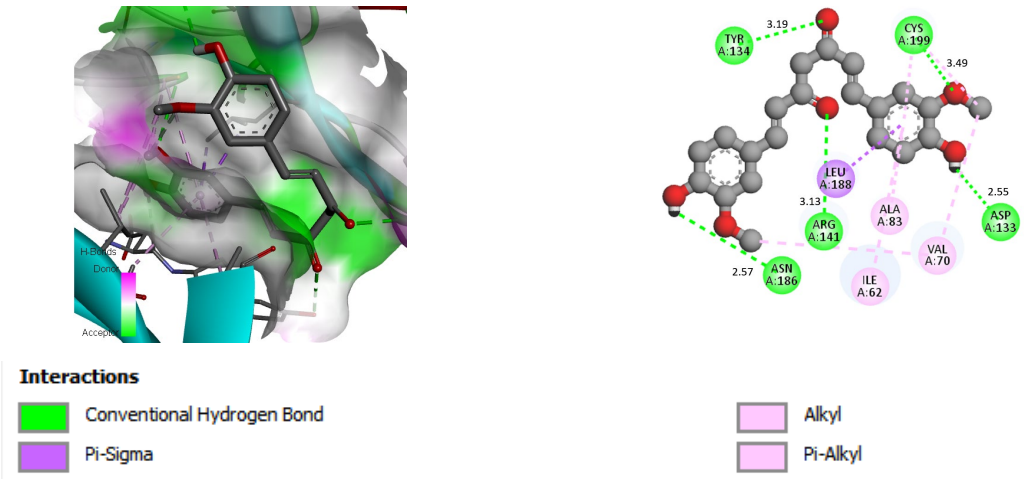


Figure 2: Visualised binding between curcumin and GSK-3β

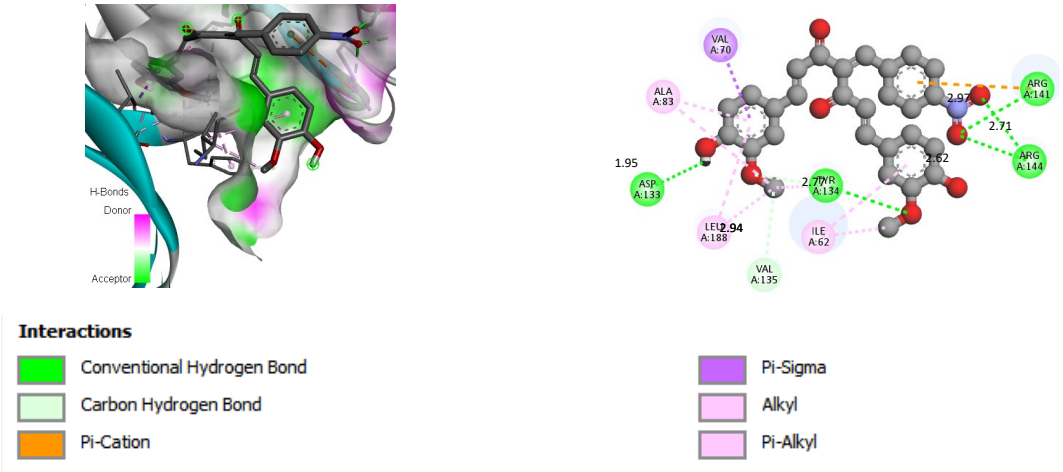


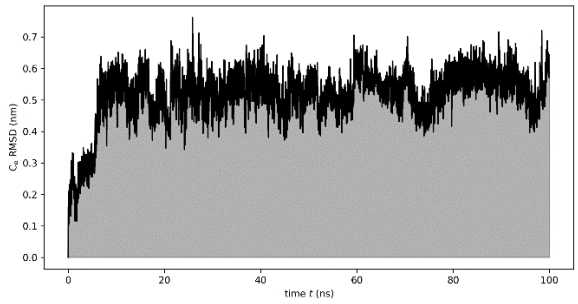
Figure 3: Visualised binding between derivative **10** and GSK-3β

Further, the hydrogen bonds involving Asp133, Tyr134, Val135, Arg141, Arg144 residues are also maintained (Figure 5 – 3b) as the observed number of hydrogen bonds were retained between two to four throughout the MD simulation for the **10**-GSK-3β complex, which is more than that observed for the curcumin-GSK-3β complex. This suggests that derivative **10** is more stable while maintaining the hydrogen bonds and, hence holds stronger interactions with GSK-3β, compared to derivative **17** and curcumin.

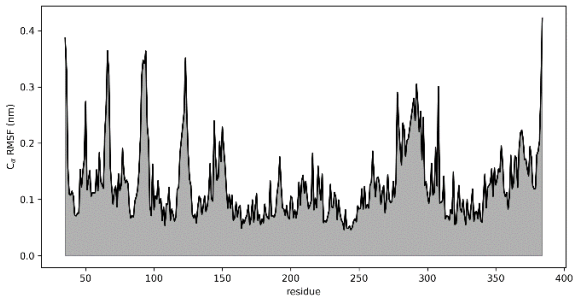
The gyration plots (Figure 4 – 4 and Figure 5 – 4a and 4b) explain the spatial distribution or compactness of the complexes. The gyration for curcumin projected highly variable and fluctuating trends on the plots with values ranging from 2.112 to 2.185 gyration, compared to the **10** and **17**, which maintain a consistent and stable range of gyration from 2.115 to 2.180 and 2.140 to 2.200, respectively. Hence, this could explain why the structure of the **10**-GSK-3β and **17**-GSK-3β complexes is more compact than curcumin.

Based on these, it was computationally evidenced that both of the derivatives show better binding profiles, while derivative **10** is the top-hit compound with the highest probability for binding and dynamically stable in maintaining its ligand-protein structure, hence potentially inhibiting GSK-3β, better than curcumin.

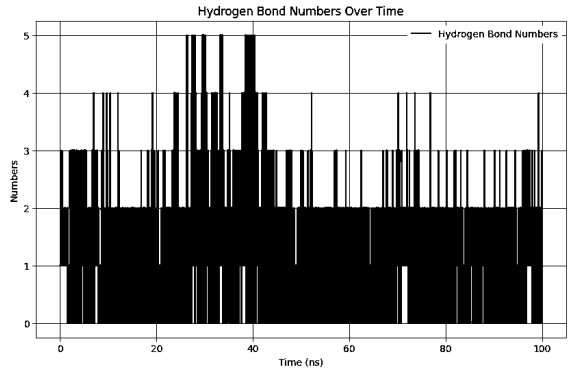
1: RMSD for curcumin-GSK-3β complex



2: RMSF for curcumin-GSK-3β complex



3: Hydrogen bond interaction for curcumin-GSK-3β complex



4: Gyration for curcumin-GSK-3β complex

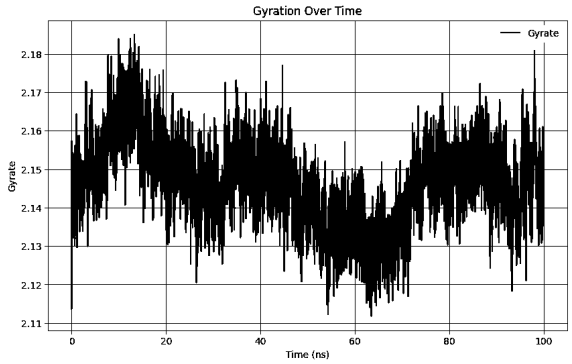
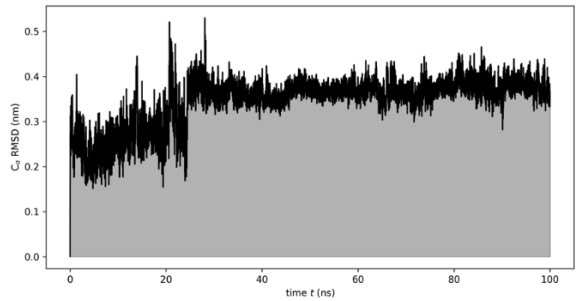
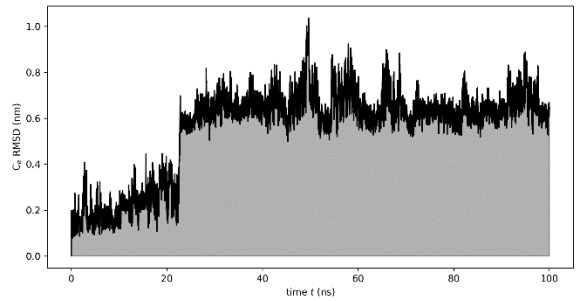


Figure 4: Results generated from MD simulations for curcumin-GSK-3β complex

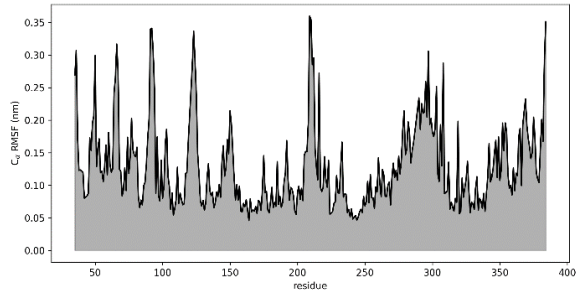
1a: RMSD for **10**-GSK-3β complex



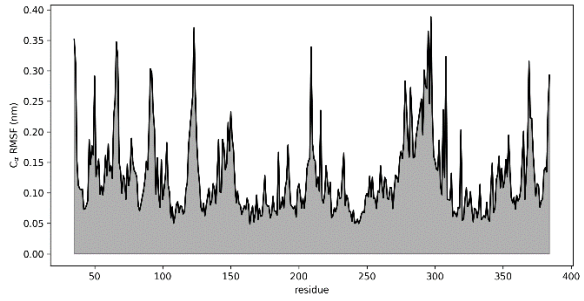
1b: RMSD for **17**-GSK-3β complex



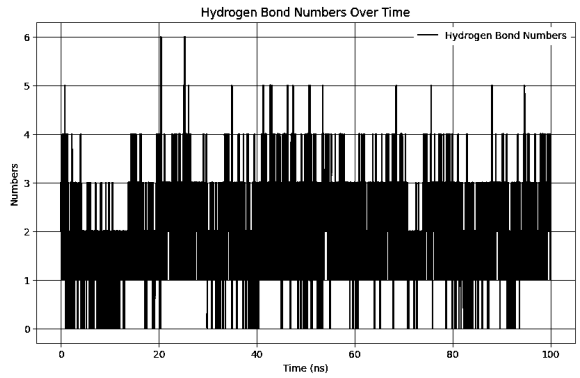
2a: RMSF for **10**-GSK-3β complex



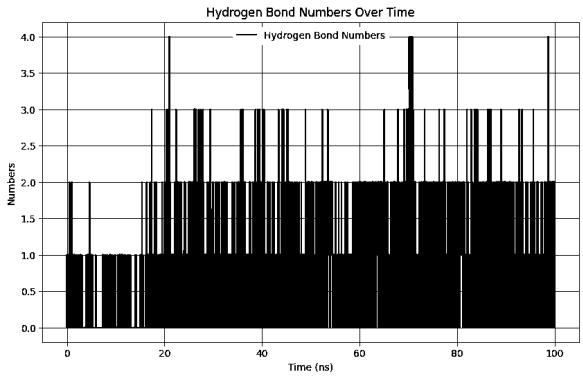
2b: RMSF for **17**-GSK-3β complex



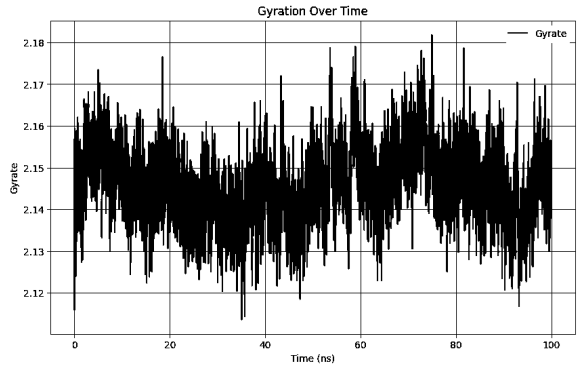
3a: Hydrogen bond interaction for **10**-GSK-3β complex



3b: Hydrogen bond interaction for **17**-GSK-3β complex



4a: Gyration for **10**-GSK-3β complex



4b: Gyration for **17**-GSK-3β complex

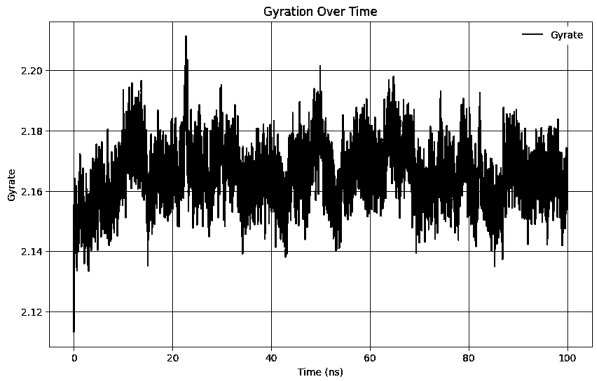


Figure 5: Results generated from MD simulations, including ligand-GSK-3β complexes (derivatives **10** and **17**)

This *in silico* of early-stage drug discovery has also been a faster and cost-effective approach, that would establish and justify preclinical drug production, allowing this research to delve into the computational background of curcumin derivatives, prior to further clinical tests. The molecular docking studies and MD simulations demonstrated the intermolecular interactions of curcumin derivatives.

Up to these findings, our team is in the process of final reviewing a manuscript draft for publication:

Jamil, S.N.H., Lam, S.D., Feroz, S.R., Ali, A.H., Maharani, R., Oka, N. & Latip, J. 2024. Computational Exploration of Curcumin Derivatives: Molecular Docking, DFT and Molecular Dynamics Analysis for Potential Antimalarial and Anti-Inflammatory Agents Targeting GSK-3β. To be submitted to a Special Issue on "Phytobioinformatics: Advancing In Silico Research for Transforming Modern Healthcare", *Current Plant Biology*. (Refer to the Appendix – Draft Manuscript)

4.2 Haemin binding assessment of curcumin and the derivatives

Antimalarial drugs bind to heme, preventing the malaria parasite from synthesising hemozoin. As a result, the lethal heme accumulates in the digestive vacuole. The *Plasmodium* parasite is killed by this excess heme, which also causes oxidative damage and interferes with crucial metabolic processes. This crucial step provides insights into the potential of the derivatives to interfere with the heme detoxification process employed by *Plasmodium* parasites, thereby disrupting their survival. The results from ITC are part of the support and proof of concept explored from the computational insights of the research.

Table 1: Thermodynamic parameters and c-values resulted from the ITC analysis for the binding of curcumin and the derivatives with haemin. (Refer to the Appendix – Supporting Information)

Complex	Haemin-curcumin	Haemin-**17**	Haemin-**10**
K_d (M)	6.33×10^{-7}	7.99×10^{-7}	1.84×10^{-7}
n	0.657	0.824	0.775
ΔH(kJ/mol)	-100	−100	-100
K_a (M^{-1})	2.50×10^{6}	1.25×10^{6}	$9.89\ 10^{6}$
-TΔS (kJ/mol)	64.32	63.80	59.20
ΔG (kJ/mol)	-35.58	−36.2	-40.80
ΔS (J/mol.K)	-207.4	−205.7	-190.9
c-value	32.9	21.05	153

Overall, the result converges to validate the predicted antimalarial potential of the derivatives, based on the favourable and better inhibition constant (K_d) and Gibb's free energy (ΔG) of binding for derivatives **10** and **17**, than curcumin. This validates the *in silico* findings whereby the derivatives could potentially exhibit better antiplasmodial activity against malarial infection, specifically in inhibiting the haemin binding, hence disrupt the parasite survivability.

4.3 Antiplasmodial activities

Further antiplasmodial assessment through pLDH *in vitro* assays was established by our team. The anti-plasmodial activity of curcumin and its two derivatives was evaluated using the pLDH 3D7 and K1 assays, which are representative of

chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum* strains, respectively. The compounds were tested at varying concentrations to determine their half-maximal effective response (EC_{50}) values.

The findings from the antimalarial assessment showed that the curcumin derivative **10** had potent antimalarial activity against the CQ-sensitive *Plasmodium falciparum* 3D7 strain, and exhibited a good inhibitory effect on the multi-drug-resistant *P. falciparum* K1 strain. The curcumin derivative **17** exhibited good inhibitory effects on both *P. falciparum* 3D7 and *P. falciparum* K1 strains. When comparing **10** and pOH, pNO_2 demonstrated a stronger parasite inhibitory effect on both the 3D7 and K1 strains compared to the curcumin derivative **17**.

Table 2: Anti-plasmodial activities of curcumin derivatives against *Plasmodium falciparum* 3D7 (CQ-sensitive strain) and *Plasmodium falciparum* K1 (multi-drug resistant strain). (Refer to the Appendix – Supporting Information)

Compounds	EC_{50} ± SD (µM) against Pf3D7	EC_{50} ± SD (µM) against PfK1
Chloroquine	0.008 ± 0.003	0.53 ± 0.12
Curcumin (Parent compound)	8.32 ± 2.62	30.66 ± 5.44
10	0.82 ± 0.37	16.70 ± 5.76
17	3.29 ± 0.70	25.90 ± 0.79

Threshold level: <1 µM: Potent; 1-20 µM: Active/Good; 21-100 µM : Moderately Active; 101-200 µM: Weak; >201 : Inactive µM

The superior efficacy of the curcumin derivatives can be attributed to structural modifications that enhance their interaction for malarial inhibition. The modifications likely result in increased binding affinity and stability of the enzyme-inhibitor complex, leading to more effective inhibition of the pLDH enzyme, which is crucial for the survival of the *Plasmodium* parasite.

These results indicate that both derivatives are more potent than curcumin against the tested *Plasmodium falciparum* strains. The observed antiplasmodial potency of the curcumin derivatives also aligns with the predictions from our previous *in silico* studies. The molecular docking and dynamics simulation data have suggested higher binding affinities for derivatives **10** and **17** compared to curcumin. Along with the pLDH antiplasmodial validation, this theoretical affinity was also substantiated by

ITC assessments, which provided thermodynamic evidence of stronger interactions of derivatives **10** and **17** with the haemin protein.

While the results are promising, it is also important to acknowledge the limitations of the study. The *in vitro* nature of the assays does not account for the pharmacokinetic and pharmacodynamic properties of the compounds, which can influence their efficacy *in vivo*. Additionally, the potential toxicity of the derivatives to human cells was not addressed in this study and warrants further investigation. Future research should focus on evaluating the *in vivo* efficacy and safety profiles of the curcumin derivatives. Studies exploring the mechanism of action at the molecular level would also provide valuable insights into the understanding of specific inhibition targets for malaria treatment.

5. Impact / Significance

The data obtained from these series of experiments and analyses provided a strong foundation for further experimental research and development, with the ultimate goal of improving curcumin's clinical applicability and harnessing its full therapeutic potential in antimalarial drug research and development. With proven antimalarial activity demonstrated by compounds incorporating ITC and further antiplasmodial *in vitro* assessments, our research group aims to become the leading authority in antimalarial research feasible within, but not limited to, UKM and UNPAD, setting the benchmark for future studies. We envision establishing the group as a training hub for upcoming researchers in antimalarial/antiplasmodial drug research and development, focusing primarily on Malaysia, Indonesia, and the wider ASEAN region.

The significance of this research lies in its potential to address a critical global health challenge – malaria. Derivatives sourced from curcumin with widespread availability also make it more source and cost-feasible for research and commercial investment in developing antimalarial drugs. Additionally, the project's objectives are multi-faceted, aiming to validate the efficacy of these derivatives through in-depth characterisation and analysis while also fostering collaborations with key stakeholders across academia, industry, and regulatory bodies.

The collaboration with institutions also ensures a holistic approach encompassing clinical validation, regulatory compliance, industry partnerships, and sustainability. The project also aligns with Sustainable Development Goals (SDGs), particularly those related to health,

innovation as well as the context of low-income and vulnerable communities across Southeast Asia. The potential antimalarial drugs derived from curcumin not only offer a novel therapeutic approach but also contribute to regional and global efforts in combating infectious diseases.

The robustness of the project results is evidenced by the comprehensive methodologies employed, including molecular docking, ADMET assessments, and Molecular Dynamics simulations. The successful synthesis and characterisation of curcumin derivatives, validated through NMR analysis, further reinforce the reliability of the findings. The evidence on the antimalarial activity of the derivatives is also established through collaborative engagements with leading research institutions including the Universiti Sains Malaysia (USM) and Universiti Malaya (UM). This underscores the rigorous approach taken to ensure scientific integrity, regulatory compliance, and industry relevance.

This research project not only addresses a pressing health challenge but also exemplifies a strategic and multidisciplinary approach towards drug development. The integration of scientific rigour, collaborative partnerships, regulatory compliance, and sustainability considerations positions the project's results as robust, impactful, and poised for real-world applications in the fight against malaria.

6. Scalability at the ASEAN level

Scalability of existing technologies is a critical aspect of translating research findings into practical applications with widespread impact. In the context of our project on synthesising curcumin derivatives for potential antimalarial applications, scalability plays a pivotal role in ensuring the efficient production and distribution of these compounds for clinical use. Therefore, the feasibility of the synthesis method to produce the derivative compound is essential to ensure that the scalability in terms of the production, either for research or clinical, is possible and reproducible.

Noting that further studies on experimentally proving the ADMET properties of the derivatives and assessing its potential toxicity are required for clinical applications, however, due to the constrained timeline of the present project, the scope of validating assessments and potential further studies of other synthesis pathways are limited. While the project scope for synthesising the derivatives is based on established methodologies, exploring a broader range of structural

modifications, analogues, or combination compounds could uncover novel antimalarial candidates with enhanced potency, selectivity, and pharmacological properties. Diversifying the compound library may overcome limitations related to potential structure-activity relationships (SAR) or target specificity.

The well-established computational screening, organic synthesis methods and advanced analytical techniques such as NMR can be readily adapted in further productions, for research and clinical applications without compromising the integrity of the synthesised compounds. Additionally, this will encompass an assessment of the synthetic routes for the production of these derivatives, their yield, cost-effectiveness, and the feasibility of large-scale production.

On a side note, the reference compound, curcumin, despite its promising therapeutic potential, is hindered by bioavailability issues that limit its clinical scalability and recognition as an effective drug. The validation assessments, including pLDH and ITC, have discussed and established that the derivatives are structurally more stable than curcumin, and exhibit better therapeutic efficacy against malaria. Further explorations in research are also highly demanded to assess the toxicity, pharmacokinetic parameters for ADMET profiles and insights into the potential of these compounds, and potentially further novel derivatives, to be developed into clinically viable antimalarial agents

On top of that, the main exchange of expertise between the two main institutions involved in this project (UKM and UNPAD) has been greatly enhanced through this project. The collaboration represents a synergistic exchange of expertise and resources. UKM contributes expertise in bioinformatics, *in silico* technologies, antimalarial/antiplasmodial assessments using ITC and *in vitro*, and advanced instrumentation for compound characterisation. On the other hand, UNPAD provides expertise in organic synthesis, laboratory facilities, and hands-on laboratory work. This collaboration fosters a comprehensive research approach, encompassing computational modelling, synthesis, validation, and scalability.

While the current engagements primarily focus on specific institutions in Malaysia and Indonesia, the project plan also envisions a broader ASEAN-wide engagement strategy beyond the present project timeline. As the project progresses, planned interactions with research institutions and pharmaceutical companies in other SEA and Asian countries will be crucial. Collaborative engagements with different institutions (present and future) ensure that the

project's impact transcends scientific discovery. Among many organisations/commercial entities related to technology scalability, we established discussions on future collaborative endeavours with these bodies, which mainly revolve around expanding the drug research methodologies and technologies implemented throughout this project.

1. Center for Research and Instrumentation Management (CRIM), UKM Malaysia – Main research facility centre managed within the UKM allows accessible and continuous access to the advanced instrumentation and analytical facilities in the future scalability of the project.

2. Laboratorium Sentral, Universitas Padjadjaran (UNPAD) Indonesia – Collaborations for providing expansion for expertise and further research. While the team from UKM possess expertise in biological assessments (*in vitro* and ITC facilities) on antimalarial activities of the synthesised compounds, this bridges the expansion for expertise from UKM to UNPAD for future research endeavours.

3. Badan Riset dan Inovasi Nasional (BRIN) Indonesia – As one of the primary research collaborators of UNPAD, discussions on potential collaborations have been on the list, mainly within the analytical research as one of the crucial aspects in the present research for validating the synthesis success and feasibility.

4. Indonesian FDA (BPOM) Indonesia – BPOM plays a pivotal role in regulating pharmaceutical products and ensuring their quality and safety. Collaboration with BPOM is essential for obtaining regulatory approvals for scaled-up production and market authorisation of antimalarial drugs derived from curcumin. BPOM's involvement streamlines the pathway to commercialisation and distribution within Indonesia's healthcare system.

5. University of the Philippines (UP) Manila – A premier Health Sciences Center of the Philippines. UP Manila has renowned expertise in clinical research and healthcare. Collaborating with UP Manila could involve conducting clinical validation and trials for antimalarial candidates. The access to patient populations, clinical facilities, and

expertise in clinical trial management would be invaluable for further validating the efficacy and safety of the synthesised derivatives in real-world settings.

6. Gifu University, Japan – Specialised in synthesis, this can greatly contribute to discovering and optimising the synthesis pathways for the production of curcumin derivatives. The existing UKM-Gifu University partnership, adds a layer of international depth to the present research endeavour on the potential antimalarial effects of curcumin derivatives.

7. Universiti Malaysia Sabah – Further collaborative works are currently being planned to elucidate the mechanism of action of the derivative compounds for their potential inhibition of a specific immunomodulatory protein, GSK-3β. This is expected to enhance the understanding of specific mechanisms for malarial treatment and protein inhibition at the molecular level, hence paving ways to understand the dual antimalarial and anti-inflammatory activities of curcumin derivatives.

These collaborations are expected to be the continuation of this project beyond the funding. This aims to further identify the limitations of outcomes of the present research and to optimise the efficacy of the compounds. This collaborative work is foreseen to facilitate the exchange of knowledge and access to advanced technologies and facilities, potentially accelerating the pace of the research.

Overall, the scalability of innovation at the ASEAN level hinges on collaborative synergies, regulatory harmonisation, capacity building, and strategic partnerships, ultimately driving the adoption and impact of our research in addressing regional health challenges and fostering sustainable development. This mainly addresses the scalability of our research on curcumin derivatives for antimalarial applications so it can be realised, and ultimately benefit healthcare systems and communities across Malaysia, Indonesia, and the broader ASEAN region.

Additionally, as our research is currently in the experimental validation phase, to confirm the derivatives with the most promising profiles as drug candidates for malaria treatment, the UK can play a crucial role in supporting this endeavour at the regional level in Southeast Asia from a health regulation standpoint. We believe that future research collaboration and

exchange between UK-based institutions which are more highly accessible to advanced research technologies would be a great support for the continuity of the research to the clinical stage. This could include exchange programs for scientists, shared clinical trials, and joint publications, which would not only enhance the research but also build a foundation for regulatory alignment.

Further, pathways in translating the experimental findings into regulatory science could also be the potential support for our present works, which thereby ensuring that the novel curcumin derivatives are not only efficacious but also meet the safety and efficacy standards required for regulatory approval. Contributions to building the capacity of regulatory bodies in ASEAN countries could also be proposed through training programs, workshops, and the sharing of best practices in drug regulation and approval. These are expected to be invaluable towards providing R&D routes to ensure that the most effective treatments are made available, particularly for the fight against malaria.

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