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Flavonoids as Antidiabetic Agents, Challenges and Future Directions

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Diabetes is a global health concern, affecting hundreds of millions of people worldwide. The number of adults living with diabetes has risen dramatically, from approximately 200 million in 1990 to nearly 589 million in 2024. Type 2 diabetes (T2DM) accounts for 90% of all cases. The incidence is increasing more rapidly in low- and middle-income countries than in high-income countries. By 2050, the number of people with diabetes is likely to reach over 853 million. Diabetes was the ninth leading cause of death globally in 2020, contributing to over 2 million deaths annually. In 2021, diabetes and kidney disease due to diabetes were responsible for more than 2 million deaths, and approximately 11% of cardiovascular deaths were associated with high blood glucose (International Diabetes Federation, 2025).

Antidiabetic drugs play a vital role in managing blood glucose levels in individuals with diabetes mellitus, particularly Type 1 and Type 2 diabetes. These medications act through various mechanisms including enhancing insulin secretion, improving insulin sensitivity, reducing glucose absorption, and promoting glucose excretion. Insulin therapy is essential for individuals with Type 1 diabetes and those with advanced Type 2 diabetes. It is typically administered through injections, insulin pumps, or inhalers, using formulations such as rapid-acting, short-acting, intermediate-acting, and long-acting insulin. For oral medications, different drug classes offer distinct mechanisms and effects: Biguanides (Metformin) improve insulin sensitivity but may cause gastrointestinal issues; Sulfonylureas stimulate insulin secretion but can lead to hypoglycemia and weight gain; Dipeptidyl peptidase-4 (DPP-4) inhibitors enhance insulin release while posing risks of joint pain and respiratory infections; Sodium-glucose cotransporter-2 (SGLT2) protein inhibitors work by inhibiting the SGLT2 protein in the kidneys, which prevents the reabsorption of glucose back into the blood, allowing excess glucose to be excreted in urine but may result in dehydration and urinary infections; Thiazolidinediones increase insulin sensitivity but carry risks of fluid retention and heart failure; and Glucagon-like peptide-1 (GLP-1) receptor agonists slow digestion, assisting with appetite control, though they may trigger nausea and pancreatitis. Given the potential side effects of current treatments, research is ongoing to find safer and more effective drug alternatives (Lai et al., 2019).

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Flavonoids are a diverse group of polyphenolic compounds found abundantly in plants. These compounds are known for their antioxidant, anti-inflammatory, antidiabetic, and antimicrobial properties. Structurally, flavonoids share a C6-C3-C6 backbone, comprising two aromatic rings (A and B) connected through a three-carbon bridge (C-ring). Based on structural differences, they are typically classified into several subgroups, including flavonols (e.g., quercetin, kaempferol), flavones (apigenin, luteolin), flavanones (hesperidin, naringenin), flavanols (catechins) (epicatechin, epigallocatechin), anthocyanins (cyanidin, pelargonidin), and isoflavones (genistein, daidzein). These flavonoids play essential roles in both plant defense mechanisms and human health, with regular consumption contributing to disease prevention (Ahmed et al., 2020; Nur Farisya et al., 2022).

Flavonoids are abundantly found in medicinal plants and a wide variety of foods, including berries, citrus fruits, apples, grapes, onions, parsley, broccoli, green and black tea, red wine, soybeans, and cocoa beans. Their absorption and bioavailability vary depending on various factors such as gut microbiota composition, food processing techniques, and individual metabolism. Upon ingestion, flavonoids undergo metabolism in the liver, where they are modified into active metabolites that exert beneficial health effects (Martin and Ramos 2021).

Flavonoids are increasingly recognized as potential antidiabetic agents, with a growing body of preclinical evidence demonstrating their efficacy through multiple mechanisms, including modulation of glucose transporters, enhancement of insulin secretion, and protection of pancreatic β cells from damage caused by oxidative stress and inflammation (Al-Ishaq et al., 2019). By influencing metabolic pathways, flavonoids contribute to better glucose homeostasis while decreasing complications associated with diabetes. Studies on various flavonoids such as quercetin, kaempferol, luteolin**,** rutin, naringenin, fisetin and epicatechin (Fig. 1,

Table 1) have demonstrated significant antidiabetic effects in animal models and *in vitro* experiments, often showing better outcomes than conventional drugs like metformin (Ansari et al., 2022; Yang et al., 2022; Ke et al., 2023). Among different flavonoids possessing antidiabetic properties, the quercetin has been reported to demonstrate antidiabetic activity through at least

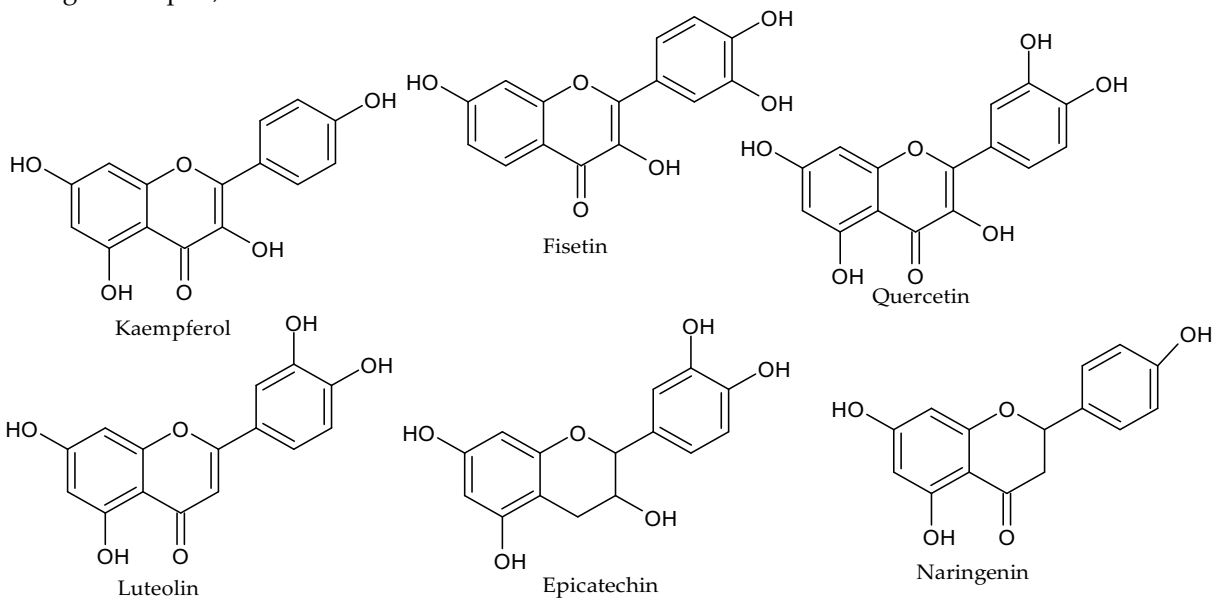

Fig. 1: Structure of key flavonoids demonstrating antidiabetic activities

8 distinct mechanisms, making it a multifaceted candidate for diabetes management. For instance, quercetin inhibits α-amylase and α-glucosidase, slowing starch breakdown and reducing postprandial glucose spikes (Günal-Köroğlu et al., 2025). It stimulates pancreatic β-cell function, promoting insulin release and improving glucose tolerance (Dhanya & Kartha, 2021). Quercetin activates the AMPK pathway, enhancing glucose uptake in muscle cells and reducing insulin resistance (Dhanya et al., 2017). By interacting with intestinal transporters, it limits glucose entry into the bloodstream (Spínola et al., 2020). Quercetin reduces oxidative stress, which is a major contributor to β-cell dysfunction and insulin resistance. It suppresses pro-inflammatory cytokines like IL-1β, IL-6, TNF-α, and blocks NF-κB signaling, helping mitigate diabetic complications. By inhibiting DPP-IV enzyme, it prolongs the half-life of GLP-1 and GIP, enhancing insulin secretion and glucose regulation. Lastly, quercetin inhibits advanced glycation end products (AGEs) formation, which contributes to vascular and tissue damage in diabetes. These mechanisms work synergistically to regulate blood glucose levels, protect pancreatic cells, and reduce complications associated with diabetes (Ansari 2022). Though human studies remain limited, existing evidence suggests flavonoids can serve as effective, natural alternatives for blood sugar regulation, offering potential benefits without the adverse effects linked to synthetic medications. Furthermore, flavonoids may complement conventional diabetes treatments, acting as adjunct therapies to improve patient outcomes and lower long-term health risks (Caro-Ordieres et al., 2020). With continued research, flavonoids hold promise as an essential component in diabetes management and prevention.

Despite their promising pharmacological properties, flavonoids face significant challenges in becoming effective therapeutic agents. A key obstacle is their low bioavailability and limited absorption, primarily due to poor water solubility and rapid metabolic degradation. These factors significantly diminish their systemic availability and therapeutic efficacy (Hu et al., 2025). Innovative solutions such as nanoparticle formulations, liposomal delivery, and prodrug modifications are being explored to improve their bioavailability (Stevens Barrón et al., 2023). Moreover, flavonoids face metabolic instability and rapid clearance, with extensive liver metabolism changing their structure and weakening their therapeutic efficacy (Kozłowska 2025). Researchers associated with medicinal chemistry are investigating structural or molecular modifications and enzyme inhibitors to improve stability. Another stern challenge is their poor target specificity, as broad-spectrum activity can lead to unintended biological interactions, demanding computational approaches like molecular docking and targeted drug design for precision (Fan et al., 2019; Shamsudin et al., 2022; Nur Farisya et al., 2022). Drug formulation and delivery present further hindrances, as conventional methods may not ensure sustained release or efficient tissue penetration, prompting advancements in encapsulation techniques using micelles, liposomes, and polymer-based carriers (Qian et al., 2023). In spite of promising *in vitro* and animal studies, flavonoids lack sufficient clinical validation, posing regulatory obstacles that necessitate standardized formulations, toxicity evaluations, and pharmacokinetic studies before approval (Davies and Yáñez, 2012).

Table 1: Key Flavonoids with Antidiabetic Properties (Shamsudin et al., 2022)

Flavonoid	Mechanisms of Action	Sources
Quercetin	Enhances insulin sensitivity, reduces oxidative stress	Onions, apples, berries
Kaempferol	Stimulates insulin secretion, protects pancreatic β-cells	Kale, spinach, tea
Rutin	Inhibits α-glucosidase, reduces blood glucose spikes post-meal	Buckwheat, citrus fruits
Naringenin	Improves insulin signaling, reduces inflammation	Grapefruit, oranges
Fisetin	Modulates glucose metabolism, protects against diabetic complications	Strawberries, cucumbers

Their structural intricacy further complicates synthetic modifications and chemical stability under physiological conditions, driving researchers toward bioengineering and semi-synthetic derivatives to optimize their pharmacological potential. Future strategies will focus on nanotechnology-based drug delivery, structural modifications for stability, targeted drug design, and rigorous clinical trials to confirm their efficacy and safety, paving the way for their integration into modern therapeutic applications (Wang et al., 2025).

In conclusion, the flavonoids hold promise as future antidiabetic agents, owing to their multifaceted mechanisms of action and potential benefits in managing diabetes and its complications. However, their successful application as therapeutic compounds is hindered by challenges such as low bioavailability, poor absorption, and rapid metabolism. These limitations necessitate innovative formulation strategies, advanced drug delivery systems, and rigorous clinical validation. Furthermore, identifying new flavonoids with antidiabetic properties and exploring their synergistic and antagonistic effects represent vital directions for future research. Continued investigation through well-designed clinical trials will be crucial to fully harness their therapeutic potential and overcome current barriers to their effective use.

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Pharmacy Students' Views on the Inclusion of Immunisation Training in the Pharmacy Curriculum: Focus Group Discussions

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Abstract

Introduction: Immunisation services are usually provided by healthcare workers who receive training in the field of immunisation. In Malaysia, limited exposure of pharmacy students in receiving immunisation training is partly due to lack involvement of pharmacists in immunisation programme. This study aimed to explore the insights of pharmacy students who received introductory training on the role of pharmacist in immunisation and the feasibility of introducing immunisation modules in the pharmacy curriculum. **Methods:** Five focus group discussions (FGDs) were conducted with undergraduate final year pharmacy students who participated in an immunisation workshop. A guide was used to explore students' experiences, challenges faced, learning perceived, perception, and suggestions for improvement from participants. Data were extracted from interview transcripts, sorted, and coded using Atlas.ti® version 9 and subjected to thematic analysis. **Results:** There were four themes emerged from the FGDs: 1) Acquisition of new learning and skills, 2) Challenges in competency development, 3) Applicability of knowledge in practice, and 4) University initiatives on immunisation training. Students appreciated the exposure to the immunisation workshop with practical skills training on injection techniques. There were challenges as an individual and as a team in building the competencies, but knowledge and skills acquired from the training were important to improve students' confidence and learning. **Conclusion:** Pharmacy students who received introductory training on the role of pharmacist in immunisation perceived its importance and suggested introducing immunisation modules into the undergraduate pharmacy curriculum.

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Introduction

Immunisation is a process to protect people from diseases through vaccination. Vaccines help in building protection to reduce the risk of getting a disease by working with the human body's natural defence. The first vaccine presented in history was in the late 18th century, which proved to eradicate smallpox disease that was responsible for 300 million deaths (Plotkin, 2014). Following this, many vaccines were developed to prevent more than 20 life-threatening diseases, including tetanus, diphtheria, influenza, pertussis, and measles (Safadi, 2023). There are also several research lines on therapeutic vaccines for chronic noncommunicable diseases such as hypertension, diabetes mellitus type 1, amyotrophic lateral sclerosis, Alzheimer's disease, cancer, and dyslipidemia (Tian et al., 2022). The advancements in vaccine technology for wider coverage of diseases require the enhanced role of health care professionals to ensure their effectiveness and utilisation.

Suboptimal immunisation rates worldwide and in Malaysia are attributed to multiple factors. These include vaccine hesitancy, misinformation, financial barriers, accessibility, the spread of anti-vaccination messages, and vaccine hesitancy (Wong, Wong & Abu Bakar, 2020). Challenges such as vaccine costs, lack of insurance, and limited healthcare access hinder vaccination efforts and have been prevalent in various regions (Kolobova et al, 2020). Additionally, the shortage of skilled healthcare professionals can impact immunisation rates by limiting vaccine availability and outreach (Gibson et al, 2023). The complex interaction of various factors has been well reported and requires a combination of strategies to address the issues effectively.

The World Health Organisation (WHO) has stated that immunisation against a wide variety of diseases prevents millions of deaths every year (Bustreo et al., 2015). Some countries, including Australia, Canada, the United Kingdom and the United States, have increased the number of healthcare professionals, including pharmacists permitted to administer vaccines. However, in some countries, pharmacists are more involved in other roles in immunisation programmes such as vaccination education, vaccine storage, vaccine advocacy, vaccine administration and vaccine adverse event reporting (Yemeke et al., 2021). Pharmacists can act as immunisers, improve vaccine-related health literacy, increase vaccination coverage rates, and remove barriers to healthcare access. By involving pharmacists in immunisation programmes, the healthcare system can enhance the effectiveness of vaccination campaigns and address vaccine hesitancy (Bragazzi 2019). Pharmacy services serve at the frontline of the health system effectively in combating suboptimal immunisation in the population.

Several studies have examined the inclusion of immunisation courses in pharmacy school curricula and its impact on student competence. For example, among the 80 accredited U.S. pharmacy schools, there were 91.3% offered the American Pharmacists Association (APhA) Pharmacy-Based Immunisation Delivery Programme, and 86.3% have integrated immunisation topics into their required core curriculum. This is in alignment with the Accreditation Council for Pharmacy Education (ACPE) standards, as well as recommendations from the American Association of Colleges of Pharmacy (AACP) and the American College of Clinical Pharmacy (ACCP), which suggested a comprehensive approach to immunisation education (Prescott et al, 2019). In Australia, a study reported significant improvements in students' confidence, self-perceived knowledge, and skills related to immunisation post-training following the integration of immunisation training into the final year of Bachelor of Pharmacy (BPharm) and Master of Pharmacy (MPharm) programmes (Mills et al., 2021). Such curricular integration effectively enhances students' competencies and readiness to provide immunisation services.

Currently, pharmacists in Malaysia are not authorised to administer vaccinations. However, pharmacist-administered vaccination programmes in other countries have been shown to increase vaccination rates, and thus it is reasonable to assume that pharmacist-administered vaccination programmes will be introduced in Malaysia (Ang et

al., 2022; Le et al., 2022). Accordingly, it is reasonable for Malaysian pharmacy schools to develop programmes to teach immunisation skills to pharmacy students. Nonetheless, the public expects pharmacists to be knowledgeable about all medications as well as vaccination services (Al-Lela et al., 2012). Therefore, it is important for Malaysian pharmacy schools that have not already done so to introduce immunisation theory into their curricula and, as with planning any new coursework, to take their students' perspectives into account (Constantino et al, 2016). This study sought to explore the thoughts and opinions of pharmacy students in the Kulliyyah of Pharmacy, International Islamic University Malaysia (IIUM), regarding their experiences after receiving training on immunisation.

Materials and methods

Study Design and Setting

A semi-structured focus group discussion (FGD) guide, comprised of open-ended questions aligned with study objectives, was developed by the researchers as summarised in Table 1. The specific study objectives were 1) to explore perceptions of pharmacy students on the feasibility of incorporating an immunisation module into the curriculum, 2) to explore the important components of immunisation training for undergraduate pharmacy students, 3) to investigate the challenges and obstacles associated with undergraduate pharmacy curriculum related to the immunisation programme, 4) to identify the impact of immunisation training provided to undergraduate pharmacy students towards their readiness in immunisation services, 5) to investigate the changes in knowledge and skills among pharmacy students. The primary investigator, who was an undergraduate student, conducted the online focus group discussions and was supervised by two other researchers. The student had been taught and trained before conducting the FGD.

Participant Recruitment

One hundred and thirteen final-year IIUM pharmacy students were invited through an email invitation to participate in the study. These students had previously participated in a one-and-a-half-day immunisation training workshop conducted by the IIUM Kulliyyah/Faculty of Pharmacy, the IIUM Kulliyyah/Faculty of Nursing, and a faculty member from the University of Colorado Skaggs School of Pharmacy and Pharmaceutical Sciences experienced in certifying pharmacy students in the United States to administer vaccinations. The one-and-a-half-day programme consisted of didactic teaching followed by hands-on training on injecting manikins (Model: Adult Manikin Medical Training, Brayden CPR Manikin, Practi-man Advance Adult Manikin, Company: TheLifeCare & VitalFour Medical Sdn Bhd). The participants were divided into 10 groups and were given one manikin each. The were instructed to practice injecting normal saline solution to the manikin and follow the step-by-step method as demonstrated by the nursing clinical instructors.

All participants who provided written informed consent were assured that their data would be kept anonymous and confidential. They were arranged into small groups and given the link to access the specific date and time of the online FGD. The FGDs were conducted online, and all participants were informed that the sessions would be recorded for research purposes. To ensure that the participants' identities remained anonymous, each participant was given an alphanumeric code.

Data Analysis

The FGDs were recorded and fully transcribed. The duration of each FGD was between half an hour and one hour per session, and the data saturation was obtained and discussed. A computer software programme (Atlas.ti® Version 9.0) was used for data analysis. Thematic analysis was done using an inductive approach. The software facilitated the systematic process of themes to emerge naturally from the data without being influenced by any theoretical frameworks. It facilitated efficient

coding, retrieval, and categorisation of data, as well as the development of patterns within the data to improve the rigor of the analytical process. Two researchers also checked the transcripts for accuracy and independently coded and analysed the data. All themes and sub-themes identified were reviewed, discussed, and agreed on by the researchers until a consensus was reached.

Results and discussion

This study adhered to the Consolidated Criteria for Reporting Qualitative Research (COREQ) checklist (Tong et al., 2007) in reporting a qualitative study. Seventeen pharmacy students consented to the study and were divided into five groups (three to five students per FGD). Demographic characteristics of participants are presented in Table 2. Data saturation, where no new themes were identified during data analysis, was reached by the fourth FGD. The focus group discussions were completed for all five groups, and the findings were analysed. Four themes were identified: 1) Acquisition of new learning and skills, 2) Challenges in competency development, 3) Applicability of knowledge in practice, and 4) University initiatives on immunisation training.

1. Acquisition of new learning and skills

During the workshop, students learned the correct techniques for administering intramuscular and subcutaneous injections. Practical application of the skills was facilitated using manikins, allowing participants to practice injection techniques. Students also attended a one-and-a-half-day programme to revisit and reinforce immunology topics learned in previous years. They were also taught about the direct involvement of pharmacists in immunisation practices in other countries, which inspired them to consider immunisation as part of their future roles as pharmacists. The new learning and skills obtained from the training were described as both from the technical aspects of preparing and handling the injection technique, as well as the insights into the role and practice of pharmacists in immunisation.

"We learnt two ways which are intramuscular and subcutaneous. Intramuscular injections must be administered at 90 degrees (to the skin surface)" (Participant 6, Male 4)

This quote reflects the acquisition of technical knowledge and procedural skills by identifying two distinct injection techniques and the correct angle for intramuscular injection, indicating new clinical competence relevant to immunisation practices.

"We also learnt how to avoid finger-stick injuries opening, to always wear gloves, and the correct techniques to administer injections" (Participant 4, Female 2)

This quote highlights the injection technique, proper protective practices, and infection control as new hands-on skill development.

"This is a new exposure for me as a pharmacy student. Because as we already know, pharmacists in other countries are directly involved with immunisation and vaccination. So, this opens our eyes to focus more on immunisation to become a pharmacist since in the future this will be one of our jobs" (Participant 3, Female 1)

It demonstrates that the students have been exposed to the new scope of practice, recognition of professional roles, and professional development.

These findings are consistent with the previously published literature. For example, immunisation workshops in Quebec, Canada, provided pharmacists with the necessary knowledge, skills, and attitudes to seamlessly incorporate vaccination services into their daily routines (Srirangan & Lavenue, 2021). Nevertheless, our study did not investigate the differences in confidence and skill levels pre- and post-intervention that could be determined using a quantitative study. In Australia, pharmacists who completed a vaccination training course reported a notable enhancement in confidence, skills, and understanding of influenza vaccination (Carroll, et.al, 2020).

Other studies have reported similar results (Lau et al.,2017, Lin et al.,2018, Poudel et al., 2019). In Australia, pharmacy students' vaccination knowledge increased significantly post-vaccination training ($p<0.001$), with competency in the skill of

injection that also increased their confidence to practice (Bushell et al., 2020). Different forms of injection simulation could also improve confidence and reduce anxiety after a vaccination skills training. (Skoy et al., 2013). These components are useful to improve competencies in new learning.

2. Challenges in competency development

The participants faced multiple barriers in the practical aspect of the workshop, particularly with the new experience of administering injections, which was not covered in their syllabus. Nervousness was evident as it was their first time practicing injection, although using manikins. The limited time allocated for the practical session (afternoon session) was thought to be insufficient. This short timeframe and the pressure of being observed by the trainer contributed to mistakes. The participants expressed dissatisfaction with the brief practical session, highlighting the need for more time and opportunities to improve their skills. Pharmacy students, in general, lack hands-on practice in injections, and the workshop provided a valuable opportunity to address this deficiency.

"The practical part of this workshop was very short; we can only try once per person then that makes us unsatisfied with our skill. This is because we are nervous because the trainer or lecturer looks at us when we try to inject a manikin. Due to this nervous feeling, we are shaking and make mistakes with only once try" (Participant 9, Female 3)

"Maybe we also lack a pharmacy lecturer who is an expert in immunisation or vaccination injection, so that we lack a trainer to do the workshop to train students" (Participant 3, Female 1)

"Pharmacy students are not exposed to learning injection techniques. We only learn about injections regarding the insulin pen injection, so when we have this workshop, it is advantageous for us since we lack hands-on practice" (Participant 5, Male 3)

The findings demonstrated that vaccination training programmes must allow students sufficient practice opportunities for them to become comfortable and skilled in administering injections. In addition, training programmes must be flexible to meet the needs of individual students. The experience of one author (CJT) is that training for students fearful at the thought of administering injections and being embarrassed in front of their classmates is more efficient if undertaken in a quiet area separated from other students. According to Esther et al. (2017), a significant obstacle to pharmacists delivering immunisation services in Australia was the lack of competency, particularly in administering injections. Researchers in Saudi Arabia also noted the lack of relevant training courses as a significant barrier to offering vaccinations in pharmacies. Notably, pharmacists who did not attend the pharmacist's immunisation workshops identified barriers to a much greater extent (Balkhi et al., 2018). Our findings indicate that challenges span across practical exposure, instructional support, and emotional readiness. Appropriate training on competency addressing these components must be emphasised to prepare pharmacists to be well-equipped with the skills.

3. Applicability of knowledge in practice

The workshop served as a valuable platform for students to realise the potential for pharmacists to administer vaccines directly. Their reflections demonstrate a growing awareness of how academic knowledge translates into real-world responsibilities, public health impact, and accessible healthcare delivery. Participants noted that pharmacists are often sought after to clarify vaccine-related myths and provide information, especially given the prevalence of misinformation on social media.

"After COVID spread around the world, Malaysia undertook a large-scale immunisation programme. So, this situation makes it more significant to learn because who knows in the future if something similar will happen again. We as student pharmacists can play a key public health role by participating in immunisation campaigns" (Participant 2, Male 2)

"Actually, because of myths and misinformation that people spread through social media regarding vaccines and immunity, many people ask pharmacists to clarify facts about vaccines and immunity. Also, people with medical backgrounds refer members of the public to pharmacists since pharmacist are expected to have

expertise in medication-related matters" (Participant 10, Female 4)

Students also stated about the broader professional trajectory and national policy direction. For example, the Malaysian Pharmaceutical Society (MPS) has advocated for pharmacists to be included in national immunisation efforts, aligning with global trends where pharmacists play a greater role in public health delivery. They expressed the belief that exposure to such programmes is beneficial for pharmacy students. The sentiment was that pharmacists should play a more active role in immunisation, including administering vaccines, due to their expertise in drug-related matters. In community settings, pharmacists were seen as the initial point of contact for patients, where they could recommend suitable vaccines and provide information. The participants envisioned a future where community pharmacists could expand their services to offer immunisations beyond clinics, allowing patients to conveniently walk in for their shots.

"MPS Malaysia has stepped forward to involve pharmacists in immunisation programmes in Malaysia. If pharmacists are involved in immunisation programmes, then it is beneficial for pharmacy students to be exposed to immunisation programmes" (Participant 1, Male 1)

"Pharmacists must know how to communicate with individual patients as well as communities of patients and other healthcare professionals. For example, pharmacists are aware of myths regarding vaccines and that there are individuals who advocate against the use of vaccines. So, the role of a pharmacist in debunking vaccine-related myths and providing factual information to individuals and communities is important. We must provide information using layman terms instead of using medical terms unfamiliar to the public." (Participant 10, Female 4).

"For example, people want to get a vaccine to go to Makkah. So, they need to make an appointment or meet the doctor to get a shot. So, we can just make this vaccine available at community pharmacies for people just to walk in anytime they need the shot" (Participant 5, Male 3)

These indicate the role of pharmacists in health literacy and patient-centered communication for addressing vaccine hesitancy and misinformation. Pharmacists are increasingly recognised as accessible healthcare providers who can influence vaccine acceptance (Isenor & Bowles, 2019), as has been demonstrated elsewhere (Dalgado et al., 2023), that pharmacists have increases vaccination rates. The students recognised that pharmacists must be able to recommend appropriate vaccinations to patients and to address any concerns that patients might have regarding vaccinations. Patients were generally comfortable with pharmacists administering vaccinations, but their concerns include doubts about the pharmacists' skills (Al Aloola et al., 2020). The absence of recognition regarding pharmacists as adequately trained and professionally capable immunisation providers might be a reason for restrictions on pharmacist immunisation authorities (Bach & Goad, 2015). Hence, formal training and certification are deemed necessary for such integration.

4. University initiatives on immunisation training

Students offered varied opinions on incorporating immunisation module into the pharmacy curriculum. Some suggested making it optional, citing Malaysia's current low demand for immunisation services. They emphasised that pharmacists primarily need knowledge to address vaccine-related myths, while the practical skills may not be immediately necessary. Others suggested making immunisation an elective rather than a core subject.

They argued that, as the demand for vaccination services is not widespread, those interested in the field should have the option to delve deeper into it. Making it an elective course would allow individuals in community pharmacy or industry, who may not be directly involved in vaccine administration, to explore the topic leisurely without pressure.

Some students proposed integrating immunisation as subtopics within existing courses, such as immunology. This approach would allow for a gradual introduction of the subject without making it a heavy core requirement. Some participants also noted that direct immunisation administration

Table 1: Domain in Focus Group Discussion Guide

No.	Domain
1.	Demographic data
2.	Student's experience, challenges and learning perceived during workshop and training on immunisation.
3.	Students' gaps and barriers in the interaction and communication with lecturers, patients, preceptor and among team members during training.
4.	Students' perceptions on the effectiveness of workshops on immunisation provided by the university.
5.	Students' opinion to improve the strategies to provide immunisation knowledge and skills for pharmacy students.

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Table 2: Demographic characteristics of the participants (n=17)

Characteristics		N (%)
Gender	Male Female	6 (35.3%) 11 (64.7%)
Age (years)	22 23	13 (76.5%) 4 (23.5%)
Ethnicity	Malay	17 (100%)
Level of education	B.Sc. Pharmacy	17 (100%)
Marital status	Single	17 (100%)

might be applicable mainly in community pharmacy settings, as hospitals typically involve doctors and nurses, and the industry may not directly administer vaccines.

"Maybe we can make it optional because the demand for this is still low in Malaysia. For now, pharmacists just need the knowledge of immunisation to counter the vaccine-related myth – vaccination skills are still not needed" (Participant 17, Female 11)

"I think we should include immunisation in the curriculum as an elective course. If we start with an elective course we can see if people are interested or not" (Participant 9, Female 3)

"We learn about viruses and vaccines in our immunology course so all that's needed is to address the practical aspects of administering vaccines. If pharmacists were allowed to administer immunisations, I think it would only apply only to community pharmacists since doctors and nurses administer vaccines in hospitals and the pharmaceutical industry focuses on the manufacture rather than the administration of medicines" (Participant 2, Male 2).

Some students suggested having lecturers teach the administration process, followed by assessments where students practice vaccine administration on a manikin (similar to Objective Structured Clinical Examination (OSCE) assessments). The idea was to emphasise the importance of evaluation in fostering understanding and skills development as well as countering the perception that a lack of traditional written exams implies a lack of seriousness.

To enhance the credibility of pharmacists in immunisation programmes, participants recommended the introduction of a special certificate, akin to the smoking cessation certificate, accredited by relevant authorities. This certificate could be obtained by pharmacy students or pharmacists who have completed an immunisation course, enabling them to actively participate in immunisation programmes. Collaboration with other disciplines, such as nursing and medicine, was proposed to enhance the learning experience, to improve pharmacy students' injection skills.

"Maybe the lecturer can demonstrate how to administer vaccines using a manikin and students can learn by practicing vaccine administration using a manikin. An OSCE exam employing a manikin could be used to formally assess students' competence to administer vaccines. If there is no formal exam, people may say we have not proven our competence to administer vaccines. A formal assessment process will ensure students are motivated to acquire vaccine-related knowledge and the skills necessary to administer vaccines" (Participant 17, Female 11)

"If Malaysia wants to recognise that pharmacists have credibility to be involved in immunisation programmes, certification could be introduced similar to the smoking cessation certificate accredited by KPT (Ministry of Higher Education) or KKM (Ministry of Health)" (Participant 4, Female 2)

"My suggestion for better improvement is to collaborate with other medical students, such as nurses and medics, because they are more exposed in practical ways on how to inject people and deal with needle things". (Participant 2, male 2)

Addressing this concern, it is notable that vaccination training in some Australian pharmacy schools was introduced before pharmacists were authorised to administer vaccinations (Bushell et al., 2020). The anticipation of regulatory changes to expand the scope of practice for pharmacist-administered vaccinations motivated both the profession and pharmacy schools to include such training (Bushell et al., 2020). Therefore, all stakeholders should play an active role to support the advanced practice in the pharmacy profession.

Additionally, providing immunisation training programmes to pharmacy students earlier in their curriculum benefits students with superior experience and develops confidence (Doyle-Campbell, C. et al., 2022). It is also worth noting that the first-year pharmacy students could complete an immunisation-training course (Kubli et al., 2017) and that immunisation training is an accreditation standard for pharmacy colleges and schools in the United States (Church et al., 2016).

Our findings are also similar to a multi-centric observational study in Germany that has highlighted that an immunisation course was highly accepted by pharmacy students, as they have also recommended the training to their colleagues (Sayyed et al., 2024). It is imperative to acknowledge and recognise the role of the university, particularly to advance the pharmacy curriculum, in this context, through integrating the immunisation training and preparing competent pharmacists meeting the local and global health care needs.

The limitation of this study could be the strategic group bias, as commonly occurred in focus group discussion sessions (Nyumba et al., 2018). It could be influenced by perceived group norms, viewpoints, or expectations, as well as the presence

of any dominant participants influencing the group opinions. For example, there were positive insights on immunisation training among pharmacy students, it is possible that other participants had a negative perspective but did not admit due to strategic group bias, and they were being recorded. To minimise this bias, the moderator has arranged the groups carefully based on age and experience to reduce power dynamics, encouraged open dialogue, and proactively and neutrally prompted on the issues to gain positive and negative insights. The groups were kept small, without hierarchical relationships among students, which could inhibit honest participation, and the moderator facilitated each session to ensure balanced participation and inclusive communication. Participants were also assured of their confidentiality. Future studies may explore data triangulation to ensure the accuracy and dependability of data.

Conclusion

Exposure to international practices where pharmacists are actively involved in immunisation inspired pharmacy students to envision a broader role for themselves in public health. Despite barriers and challenges, workshops combining theoretical review and practical application were effective in preparing pharmacy students for the evolving role of pharmacists in immunisation services. It reflected on students' professional identity and career aspirations. Therefore, equipping students with immunisation skills and knowledge is a timely and strategic educational priority.

Authors contributions

The researcher (NAAMN) designed the study, collected data, and analysed data. Other researchers (NHMT, NSAR & CJT) supervised, reviewed, and edited the writing. All authors have read and reviewed the manuscript.

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Ethical approval statement (if applicable)

This study was approved by the Ethics Committee of International Islamic University Malaysia (IIUM) (ID No.: IREC 2023-166).

Informed consent statement (If applicable)

Informed consent was obtained from all subjects involved in the study.

Conflict of interest

NAAMN, NHMT, NSAR and CJT declared no conflicts of interest in the conduct of this study and the publication of this manuscript.

Declaration of generative AI and AI-assisted technologies in the writing process

Grammarly was utilised to assess the grammar and enhance the readability of the manuscript. Additionally, ChatGPT was used to aid in formulating responses to reviewer feedback. It is essential to remember that, despite the support of AI technologies, all outputs were rigorously examined by humans to guarantee their accuracy, appropriateness, and alignment with the authors' aims.

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Appendix A

Not applicable.

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Predictors Associated with Delayed Methotrexate Clearance among Patients with Haematological Malignancies

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Abstract

Introduction: High dose methotrexate is commonly utilised in haematological malignancies; however, the prevalence of delayed clearance is not well-defined. The study aimed to determine the prevalence of delayed clearance of methotrexate, to analyse correlation between rate of methotrexate infusions and its concentrations, and to identify the predictors associated with delayed clearance. **Method:** A cross-sectional study was conducted among adult patients with haematological malignancies who received high-dose methotrexate. Spearman's correlation was utilised to analyse correlation between the rates of methotrexate infusions with its concentrations at 48 and 72 hours. Multiple logistic regression was used to identify factors associated with delayed clearance. **Results:** A total of 63 patients with 159 methotrexate infusions were included, with a mean age of 42.2 years (±18.06) and a median body mass index of 23.36 kg/m^2 (19.91-26.14). Delayed methotrexate clearance was observed in 29 (46%) patients, which affected 41 (25.6%) methotrexate infusions. A poor negative correlation was found between the rate of methotrexate infusion and 48-hour concentration (r=-0.206, p=0.009). Older age (odds ratio (OR) 1.06, 95% confidence interval (CI) 1.03-1.10, p<0.001) and dose of methotrexate >3000 mg/m^2 (OR 3.33, 95% CI 6.45-120.88, p<0.001) were identified as the predictors of delayed methotrexate clearance. **Conclusion:** Almost half of the patients experienced delayed methotrexate clearance. A slower rate of infusion was found to correlate with higher 48-hour concentrations. Older age and higher doses of methotrexate were identified as predictors for delayed clearance. Prospective study is needed with larger sample size to ensure generalisability of the outcomes.

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Introduction

High dose methotrexate (HD-MTX) is widely utilised for various types of cancers, including osteosarcoma, lymphoma, and leukaemia. It is defined differently in several works of literature, some reports defined it as 1000 mg/m^2 and above (Kowalski et al., 2021; Li et al., 2019; May et al., 2014), while other defined HD-MTX as 500 mg/m^2 and above (Dhanushkodi, 2021; Howard et al., 2016; Kawakatsu et al., 2019; Shi et al., 2020; Valade et al., 2020). In this study, HD-MTX is defined as 500 mg/m^2 and above. For lymphoma, HD-MTX is utilised in natural killer (NK)/T cell lymphoma, primary central nervous system (CNS) lymphoma and T or B cell lymphoma with CNS involvement (Allen & Lechowicz, 2019; Grommes & DeAngelis, 2017; Li et al., 2016).

In acute lymphoblastic leukaemia (ALL), HD-MTX has been used in adult and paediatric protocols, with the latter utilising higher doses of MTX (Moricke et al., 2008). Some clinicians have incorporated paediatric-inspired protocols to be used in adolescent and young adults (AYA), due to better outcomes in achieving minimal residual disease (MRD) as well as survival outcomes in these population (Ribera et al., 2014; Stock et al., 2019). The Adolescent and Young Adult Oncology Progress Review Group (AYAO PRG) defined the AYA population as comprising individuals aged 15 through 39 years at cancer diagnosis (National Cancer Institute (U.S.), 2006).

Delayed MTX clearance is defined as MTX concentration >1 μmol/L at 48 hours or >0.1 μmol/L at 72 hours from starting of MTX (Kowalski et al., 2021; Nakano et al., 2021; Young et al., 2020). The rationale behind these cutoff points are based on studies demonstrated that these cutoff points were predictive of renal toxicity (Widemann & Adamson, 2006). Furthermore, previous pharmacokinetic study revealed that the toxicity is expected to be present in delayed MTX clearance cases (Stoller et al., 1977).

The prevalence of delayed MTX clearance has been reported by a few literatures in paediatric and adult patients with osteosarcoma, but not well-defined in patients with haematological malignancies. A study among osteosarcoma patients reported that 48.5% of their patients had delayed MTX clearance, in which the dose was 12 g/m^2, higher than those utilised in lymphoma patients (Young et al., 2020). Despite that, a study reported higher incidence in lymphoma patients as compared to osteosarcoma (May et al., 2014). In a recent study, the prevalence of delayed MTX clearance was high, which was reported as 79.9% of the infusions, for osteosarcoma and leukaemia (Mosleh et al., 2023).

Delayed clearance of MTX may increase the risk of adverse effects, including acute kidney injury, myelosuppression, megaloblastic anaemia, oral mucositis, and liver impairment. The incidence of acute kidney injury occurred in 2 to 12% of the patients (Widemann & Adamson, 2006). In terms of hepatotoxicity, elevation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) most of the time are transient and does not have any clinical significance (Weber et al., 1987).

The factors associated with delayed MTX clearance have been reported in several papers for osteosarcoma and paediatric population, however there was insufficient data available among adult patients with haematological malignancies. A study reported that the prevalence of delayed MTX clearance was significantly associated with male gender and white ethnicity in patients with osteosarcoma (Young et al., 2020). This is further supported by another study that reported the association of delayed clearance with male gender, older age, and higher serum concentration at 24 hours of MTX administration (Zhang et al., 2016). In contrast, a recent paper did not find male gender as a predictive factor; instead, the study reported the diagnosis of leukaemia and reduced urine output on day 1 were associated with delayed MTX clearance (Mosleh et al., 2023). In addition, several studies investigated associations between pharmacogenetics with MTX toxicity, such as ABCC2, MTHFR C677T, and A1298C (Campbell et al., 2016; Razali et al., 2020).

The goal of supportive care during MTX

administration is to increase the solubility of MTX in the urine, promote timely excretion and protection against lethal toxicity by administration of leucovorin rescue therapy. Prior to starting HD-MTX, medication reconciliation needs to be performed in order to prevent potential drug-drug interactions that will interfere with MTX clearance. Drugs that possibly elicit highest risk of adverse drug-drug interaction mechanism are drugs that are competing with MTX for renal tubular secretion, such as non-steroidal anti-inflammatory drugs (NSAIDs), penicillin antibiotics, probenecid, proton-pump inhibitors (PPI), and sulphonamides (Widemann et al., 2006). Another consideration prior HD-MTX administration is the presence of third spacing; this condition may cause delayed elimination of MTX and subsequently severe neutropenia and thrombocytopenia (Goh et al., 1979). Generally in practice, HD-MTX will not commenced in patients presented with third spacing, an alternative regimens will be discussed further.

The next step is hyperhydration and urinary alkalinisation. MTX primarily excreted through kidney, hence the purpose of hyperhydration is to increase urinary flow rates during MTX administration. Urine alkalinisation is defined as a treatment regimen that increases drug elimination by administration of intravenous sodium bicarbonate to produce urine with pH of ≥7.5 (Proudfoot et al. 2004). However, other studies quoted urine pH target as 7 or greater for MTX infusions (Perazella et al., 2010; Widemann et al., 2006). The purpose of alkalinising the urine is to increase MTX solubility and preventing crystals nephropathy. Hyperhydration with 100-150 mL/m^2 per hour starting from 12 hours before commencement of HD-MTX is recommended in many paediatric protocols. Administration of 40 mEq/L of sodium bicarbonate is recommended during and after HD-MTX administration, until MTX concentration is proven to be non-toxic. Urine pH should be monitored to ensure pH is 7 or greater to reduce risk of crystal formation (Perazella et al., 2010; Widemann et al., 2006). Another study suggested to start urine alkalinisation with sodium bicarbonate 150 mEq/mL in 1 litre of dextrose 5% or sterile water, run at 125 mL/hour with target urine pH ≥7.5 (Kowalski et al., 2021).

Another important supportive measure is leucovorin rescue dose. Leucovorin has been used for more than 30 years in HD-MTX treatment, and it is effective in prevention or reduction of adverse effects during HD-MTX treatment, such as myelotoxicity, gastrointestinal toxicity, and neurotoxicity (Ackland et al., 1987; Widemann et al., 2006). The mechanism of action of leucovorin is through bypassing DHFR inhibition by MTX. Leucovorin (5-formyl tetrahydrofolate) enters the cell via the folate carrier and readily converted into a 5,10-methylene tetrahydrofolate without requiring the involvement of DHFR, thus bypassing DHF blockade. Various dosing regimens have been used as rescue dose after HD-MTX administration; however, the usual doses are 10-15 mg/m^2 every 6 hourly until plasma MTX concentration below toxic range. Leucovorin should not be started too early during administration of HD-MTX, as it will nullify the activity of MTX, hence reducing the anti-cancer effects (Howard et al., 2016).

There is an increasing trend on the use of HD-MTX in AYA and adult patients with haematological malignancies. This is due to several studies that showed improved survival outcome in patients who were given paediatric-inspired protocols (Gupta et al., 2019; Hanbali et al., 2021; Stock et al., 2019; Winter et al., 2018). Most of paediatric protocols in ALL utilise a higher dose range of MTX compared to adults (Gökbuget et al., 2000; Moricke et al., 2008). This will render adult population to higher dose of MTX and increase the risk of toxicity. Currently, there is limited data on delayed MTX clearance among AYA and adult haematology patients, hence the risk of delayed MTX excretion each time patients were given HD-MTX is not known. The established data is currently more towards osteosarcoma, which utilise MTX in a different manner; the regimen commonly utilises higher dose of MTX with shorter infusion time, i.e. 4 hours (Young et al., 2020). Furthermore, the dosing approach used in haematological malignancies, particularly paediatric-inspired protocols for leukaemia, utilise doses of 5 g/m^2 over longer infusion time, i.e. 24 hours (Moricke et al., 2008). A study by Mosleh et al.

reported on the delayed clearance in leukaemia patients, however the oldest age was not specified, and the demographic data only presented the proportion of patients aged 15 years and older. Therefore, the inclusion of adults aged 40 years and above remains unclear.

There is a need to identify patients who are at risk of delayed MTX clearance to effectively monitor the patients and carefully decide on the suitable HD-MTX regimen specifically for the patient. In this study, we want to identify the prevalence of delayed MTX clearance and MTX toxicity among patients with haematological malignancies, to analyse correlation between dose and duration of administration with MTX concentrations, and to identify predictors associated with delayed MTX clearance.

Materials and methods

Study Design

This is a single-centre cross-sectional study which was conducted in Hospital Tengku Ampuan Afzan (HTAA) Kuantan. Patients who received HD-MTX were either traced through the system or manual. Demographic data, chemotherapy regimen and infusion data, supportive measures, pharmacokinetic parameters, concomitant drugs, and toxicity profile were collected using data collection form. Purposive sampling method was used in this study. All MTX blood samples of patients with haematological malignancies were included. The calculated sample size was 335 MTX infusions based on single proportion sample size calculation, with 31.9% prevalence reported from previous study (Dhand et al., 2014; May et al., 2014).

Study Population

The target population for this study was all AYA and adult patients diagnosed with haematological malignancies and received HD-MTX. Types of haematological malignancies included were lymphoma and leukaemia, in which the diagnosis were obtained from tumour and bone marrow biopsy, respectively. Inclusion criteria were patients receiving HD-MTX 500 mg/m^2 and above from year 2016 until 2021, comprising of all patients aged more than 15 years old. Renal functions were normal at baseline for all patients, as MTX is contraindicated to be given in patients with renal impairment. Generally, patients presented with third spacing are contraindicated for HD-MTX administration at the included facility; therefore, no patients meeting this criterion were given HD-MTX . All planned on HD-MTX received urine alkalinisation with hydration and sodium bicarbonate, as per recommendation (Howard et al., 2016).

There was no specific in-house urine alkalinisation protocol that was utilised in the hospital; thus, hydration protocol was ordered as per physician's discretion. Generally, patients were started on maintenance hydration with sodium chloride 0.9% with rate of infusion ranging from 125 to 167 mL/H, with intravenous sodium bicarbonate starting from 60 mEq/L. The dose of sodium bicarbonate was adjusted based on urine pH; in some instances, the dose was higher than the general recommendation, which can be titrated up to 160 mEq/L, however, the decision was based on the physicians' discretion (Howard t al., 2016). HD-MTX was administered only when urine pH ≥7.5. Pharmacokinetic monitoring was taken at 48 and 72 hours of starting HD-MTX. Serum MTX concentrations were outsourced to other facilities, Cobas Integra 400+ and Architek assays were used to determine serum MTX concentration. Patients whose data were untraceable or MTX concentration not available were excluded.

Data Collection

The data collection was conducted over 5 months, from March to July 2022. In HTAA, all patients who received HD-MTX from January 2016 until December 2021 were traced from Pharmacy Information System (PhIS) and Laboratory Information System (LIS), whereas for HCTM, data were traced by using Integrated Laboratory Management System. Each patient was assigned a patient identification number (ID), and each MTX infusion was assigned a sample ID.

Statistical Analysis

Statistical Software for Social Sciences (SPSS) version 25 was used for data analysis. All statistical analysis was considered significant if p value <0.05.

Descriptive statistics were used for demographic data. Normality testing was done using skewness and kurtosis. All categorical data were presented as frequencies and percentages, whereas continuous data were presented as mean (standard deviation (SD)) for normally distributed data and median (interquartile range (IQR)) for skewed data. The prevalence of delayed MTX clearance and MTX toxicity were presented as descriptive statistics. Delayed MTX clearance is defined as above. Point of clearance was defined when MTX concentration <0.1 μmol/L. Prevalence of delayed MTX clearance was calculated as:

$$\frac{Cycle\ with\ delayed\ MTX\ clearance}{Total\ chemotherapy\ cycles} \times 100$$

Univariate analyses were performed using independent t-test, Chi-square test, and Mann-Whitney U test, as appropriate. Spearman's rank correlation was utilised to analyse correlation between dose and duration of HD-MTX with MTX concentrations. Degree of correlation is defined by the value of correlation coefficient (r); very strong (≥0.8), moderate (0.6 to 0.8), fair (0.3 to 0.5), and poor (<0.3) (Chan, 2003). Univariate logistic regression was used to identify relationship between the demographic data and prevalence of delayed MTX clearance. Age, gender, race, BMI, dose of MTX, duration of infusion, and concomitant intrathecal (IT) MTX were included for analysis. Concomitant IT MTX is defined as a presence of IT MTX in the chemotherapy regimen alongside with HD-MTX. A p-value cut-off point of 0.25 was used to select variables for inclusion in the multivariate logistic regression analysis, for determination of the predictors (Bursac et al., 2008). All p-values were two sided, and differences were statistically significant when $p<0.05$.

Results

A total of 67 patients with 184 infusions were identified. 4 patients with 25 infusions were excluded from study due to incomplete data on MTX concentrations. Finally, a total of 63 patients with 159 MTX infusions were included and analysed. Mean age was 42.2 years (±18.06), with median BMI of 23.36 kg/m^2 (IQR 19.91 to 26.14) and median body surface area (BSA) of 1.7 m^2 (IQR 1.51 to 1.82). Each patient received median MTX dose of 2000 mg/m^2 (IQR 1500 to 2500) and mean infusion duration of 15.7 hours (±9.94). The details on the chemotherapy regimens received were shown in Figure A1 (Appendix). Delayed MTX clearance occurred in 29 (46%) of the patient, which accounted for 41 (25.6%) of MTX-containing infusion. The difference in median number of MTX cycles was not statistically significant between the groups (3 versus 1.5 cycles, U=363, z=-1.87, p=.061). There was a significant difference between age and delayed MTX clearance (t=2.257, 95% confidence interval (CI) 0.92 to 13.83, p=0.025). Details on the demographic data and associations with delayed MTX clearance were shown in Table 1.

Table 1: Demographic data of patients and associations with delayed MTX clearance. AYA, adolescent and young adults; IQR, interquartile range; IT, intrathecal; MTX, methotrexate; SD, standard deviation. [a]Chi-square test, [b]Fisher's Exact test, [c]Mann-Whitney U test, [d]independent t-test.

Variables	**Delayed MTX clearance**		**Test**	**p Value**
	Yes	**No**		
(n= patients)	**(n = 29)**	**(n = 34)**		
Gender, n (%)				
Male	24 (82.8)	22 (64.7)	2.589[a]	0.108
Female	5 (17.2)	12 (35.3)		
Race, n (%)				
Malay	25 (86.2)	33 (97.1)	2.523[b]	0.171
Non-Malay	4 (13.8)	1 (2.9)		
Age group, n (%)				
AYA	14 (48.3)	17 (50)	0.019[b]	1.000
Adult	15 (51.7)	17 (50)		
Diagnosis, n (%)				
Lymphoma	16 (55.2)	18 (52.9)	0.031[a]	0.859
Leukaemia	13 (44.8)	16 (47.1)		
Median number of MTX cycles (IQR)	3 (1 to 4)	1.5 (1 to 3)	-1.87[c]	0.061

Variables	Delayed MTX clearance		Test	p Value
	Yes	No		
n = infusions)	(n = 41)	(n = 118)		
Mean age, years (SD)	47.73 (19.15)	40.35 (17.43)	2.257[d]	0.025
Median BMI, kg/m² (IQR)	23.52 (18.86 to 26.54)	22.99 (19.93 to 25.78)	-0.353[c]	0.724
Median BSA, m² (IQR)	1.70 (1.52 to 1.85)	1.69 (1.51 to 1.81)	-0.540[c]	0.589
Median dose of MTX, mg/m² (IQR)	2500 (1000 to 4500)	1500 (1500 to 2500)	-1.534[a]	0.125
Mean duration of infusion, hours (±SD)	16.28 (±10.12)	15.48 (±9.93)	-0.434[d]	0.665
IT MTX, n (%)				
Yes	25 (61)	69 (58.5)	0.253[a]	0.615
No	15 (39)	50 (41.5)		

Correlation analysis showed that there was a poor positive correlation between dose of MTX and its concentration at 72 hours (r 0.192, p=.016). Fair positive correlation was found between duration of MTX infusion and the concentration at 48 hours (r 0.301, p<.001). However, when rate of infusion was used, it was found that there was a poor negative correlation between rate of MTX infusion and its concentration at 48 hours (r -0.206, p=.009) (Table 2).

Table 2: Correlations between dose and duration of MTX with its concentrations. MTX, methotrexate. [a]Spearman's rank correlation, *significance at p<0.05.

Variables	MTX concentrations			
	At 48 hours		At 72 hours	
	r[a]	p Value	r[a]	p Value
Dose of MTX	0.043	0.596	0.194	0.015*
Duration of MTX infusion	0.301	<0.001*	0.068	0.393
Rate of MTX infusion	-0.206	0.009*	-0.001	0.991

Figure 1: Correlation between dose of MTX and MTX concentration at 72 hours. MTX, methotrexate.

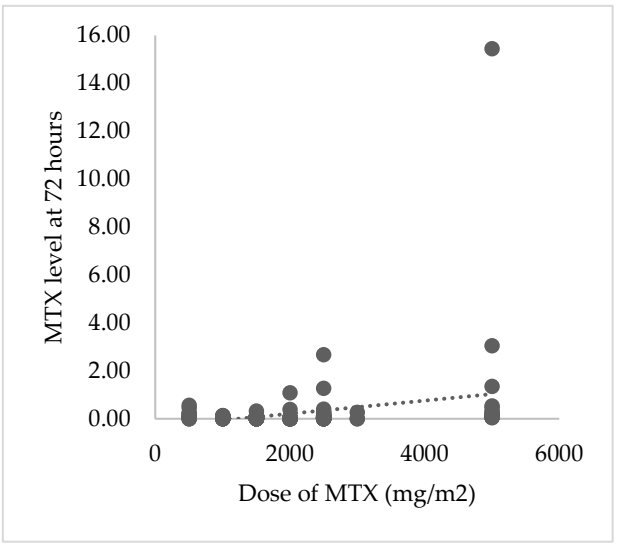
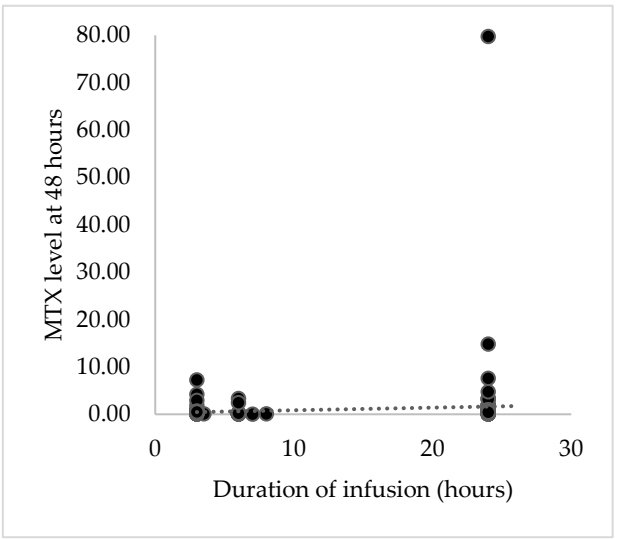


Figure 2: Correlation between duration of MTX infusion and MTX concentration at 48 hours. MTX, methotrexate.



Univariate logistic regression was performed to identify relationship between the selected variables with delayed MTX clearance. Age, gender, and dose of MTX had p value of less than 0.25, hence multivariate logistic regression was performed on these variables. Results showed that for every 1-year increase in age, the odds of having delayed MTX clearance was 6% higher (odds ratio (OR) 1.06, 95% CI 1.03 to 1.07, p<0.001). Dose of MTX >3000 mg/m² has 28 times higher odds of delayed MTX clearance compared to ≤3000 mg/m² (OR 27.91, 95% CI 6.45 to 120.88, p<0.001). Table 3 summarizes the logistic

regression analysis and the significant variables, controlling for other factors. The model equation is as follow:

$$logit(odds) = -4.275 + 0.06(\text{Age}) + 3.329(\text{Dose})$$

whereby age is a continuous data, dose ≤3000 mg/m²=0, and dose >3000 mg/m²=1. The model has been checked for goodness-of-fit, multicollinearity, interaction, and outliers. The multicollinearity was assessed using tolerance and variance inflation factor (VIF). Both variables included had the same tolerance value and VIF (0.85 and 1.177, respectively), which indicates no multicollinearity. The final model met all the assumptions, and demonstrated a good model, whereby 76.7% of the cases can be predicted correctly.

Discussions

We present an analysis of AYA and adult patients who received HD-MTX. The prevalence of delayed MTX clearance was 25% in this study. This prevalence was slightly lower compared to other published studies, whereby delayed MTX clearance was reported to be 31.9% of the lymphoma cases (May et al., 2014). Another study reported prevalence of 29% delayed clearance among total MTX infusions given (Young et al., 2020). Furthermore, another study found a higher occurrence of delayed MTX clearance, which was 41.4% (Ng et al, 2016). The reason for this difference may be due to single centre study, which may not be representative of the whole population; some of the cases might not be counted. The results across studies suggest that delayed MTX clearance is prevalent across all cancer diagnosis, and prompt intervention is needed.

A weak positive correlation was found between dose of MTX and 72-hour concentration (Figure 1) and a fair positive correlation between duration of MTX infusion and 48-hour concentration (Figure 2). When both data combined into rate of infusion it showed poor negative correlation (Table 2). These poor correlations might be due to low concentrations of MTX in most of the samples, especially concentration at 72 hours. Furthermore, there were outliers, which may drive towards the significance. These outliers were the toxic cases encountered during the data collection.

On the other hand, there was no correlation found between dose and 48-hour concentrations. Based on the two pharmacokinetic studies, the relationship between dose and peak concentration (C_{max}) was seen in HD-MTX infused over 6 hours (Bacci et al., 2006; Lin et al., 2009). However, this association was not observed in 24 and 48-hour concentration (Lin et al., 2009). The lack of correlation between the dose and 48-hour concentration may be due to interindividual variability in renal clearance. This may be due to a higher drug clearance when higher C_{max} was reached, as suggested by previous study (Cano et al., 1981).

In contrast, previous pharmacokinetic study revealed that the toxicity of MTX was related with the infusion time, in this case haematological toxicity and liver derangement (Goldie et al., 1972). This may be due to the longer exposure of MTX when it is being administered over longer period, rendering patient to elevated risk of MTX toxicity. However, our data showed inconsistent results, whereby the dose was related to delayed MTX clearance instead. It was noted that Goldie et al. did not report any statistical analysis to back up the findings; it was more of a trend observed descriptively rather that a true significance.

Multivariate logistic regression showed that age and dose MTX of >3000 mg/m² were independent predictors associated with delayed MTX clearance. Similar results have been demonstrated in a study which identified dose as a predictor (Ng et al., 2016). Increasing the dose of MTX will ultimately increase the drug exposure, hence elevating the risk of delayed MTX clearance. A study by Nakano et al reported that age could not be identified as risk factor although there was a significant association with delayed MTX clearance (Nakano et al., 2021). MTX was primarily excreted through urine, hence renal function plays an important role in the drug clearance. Older age may have a decline in renal function compared to young age, which may explain this situation even though patients had a

Table 3: Multivariate logistic regression on the predictors associated with delayed MTX clearance. CI, confidence interval, IT, intrathecal; MTX, methotrexate; OR, odds ratio. [a]Method: Forward Stepwise (likelihood ratio). *Variables that have p<0.25 were included for multivariate logistic regression.

Variables	Univariate Logistic Regression				Multivariate Logistic Regression			
	B	OR	95% CI	p Value	B[a]	Adjusted OR	95% CI	p Value
Age	0.023	1.02	1.00 to 1.05	0.028*	0.060	1.06	1.03 to 1.10	<0.001
Gender								
Male	0.743	2.10	0.89 to 4.98	0.091*	-	-	-	-
Female		1.00						
Race								
Non-Malay	0.28	0.76	0.27 to 2.12	0.594	-	-	-	-
Malay		1.00						
BMI	-0.017	0.98	0.93 to 1.04	0.565	-	-	-	-
Dose of MTX								
>3000 mg/m^2	1.674	5.33	1.87 to 15.19	0.002*	3.329	27.91	6.45 to 120.88	<0.001
≤3000 mg/m^2		1.00				1.00		
Duration of MTX infusion	0.008	1.01	0.97 to 1.05	0.662	-	-	-	-
Concomitant IT MTX								
Yes	0.189	1.21	0.58 to 2.52	0.615	-	-	-	-
No		1.00						

normal renal function at baseline.

We could not find gender as a significant predictor for delayed MTX excretion. This finding was supported by other studies which did not find any relationship between gender and delayed MTX clearance (Barreto et al., 2021; Nakano et al., 2021). A study among osteosarcoma patients reported that female gender was associated with delayed MTX excretion (Misaka et al., 2020; Zhang et al., 2016). In contrast, two studies found an association between male gender and delayed MTX clearance (May et al., 2014; Young et al., 2020). These inconsistent results showed that gender may not be a clear predictor, it may be explained by genetic polymorphism which may varies among gender.

Overall, the understanding of the predictors may be beneficial for individualizing therapy among patients and provide the best supportive care measures. Further exploration of pharmacogenetics and its relationship with specific types of toxicity may further improve predictions and patient outcome, and a betterment towards personalized medicine.

There are some limitations in this study that may restrict the application of the outcome. First, this is an underpowered study, with post-hoc power calculation of 38.4% due to insufficient samples. A larger sample size is required to address the generalisability of the outcomes in this study. There are a few potential biases in this study. The first one is selection bias, as this study was a single centre study. In addition, the use of purposive sampling may introduce bias, as it may not represent a broader population. Secondly, information bias also may be present; there was no standardised urine alkalinisation protocol throughout Malaysia which may lead to variability in patient management. Furthermore, the use of different laboratory assays might introduce the inconsistency in the reported MTX concentrations. Thirdly, confounding bias also could not be excluded totally, as there may be other variables which could not affect the outcome, such as genetic polymorphism and variations in chemotherapy regimen. Standardisation of urine alkalinisation regimen among facilities is recommended to reduce variability among patients.

Conclusion

In conclusion, the prevalence of delayed MTX clearance is prominent among adult patients with haematological malignancies in our study population. A weak positive correlation was found between the rate of MTX infusion with 48 hours' concentrations. Older age and higher dose of MTX were identified as the predictors for delayed clearance. Further prospective study is needed with larger sample size to ensure generalisability of the outcomes.

Authors contributions

M. N. Y. designed and conducted the research, performed data collection and analysis, and wrote the manuscript; N. M. S. and N. A. M. T. supervised the whole research, provided ideas, verified the analytical methods, and reviewed the write-up; S. N. N. A. J. conceived the idea for research method, assisted in data collection; A. N. K. provided conceptualization of ideas. A. N. K. and N. R. T. provided expert opinions in the field of haematology. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Ethical approval statement

Ethical approvals were obtained from National Medical Research and Ethics Committee (MREC) of the Ministry of Health (MOH) Malaysia (Ref No: NMRR ID-22-00022-1TH (IIR)) and Research Ethics Committee UKM (REC UKM) (Ref No: UKM PPI/111/8/JEP-2022-238).

Informed consent statement

Patient consent was waived due to retrospective nature of the study.

Conflict of interest

The authors declare that there are no conflicts of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that no generative artificial intelligence (AI) or AI-assisted technologies was used for the preparation of this manuscript.

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Appendix A

Figure A1: Chemotherapy regimens used for HD-MTX, total of 159 HD-MTX infusions. HD-MTX, high dose methotrexate; R, rituximab.

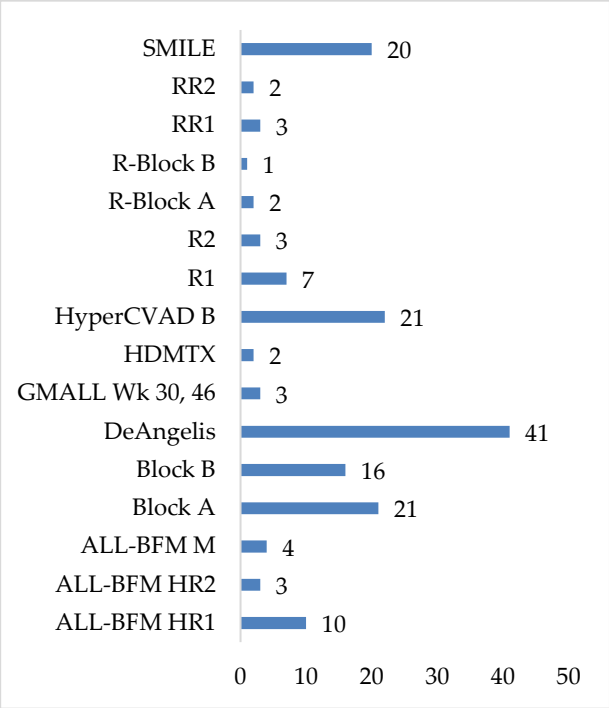
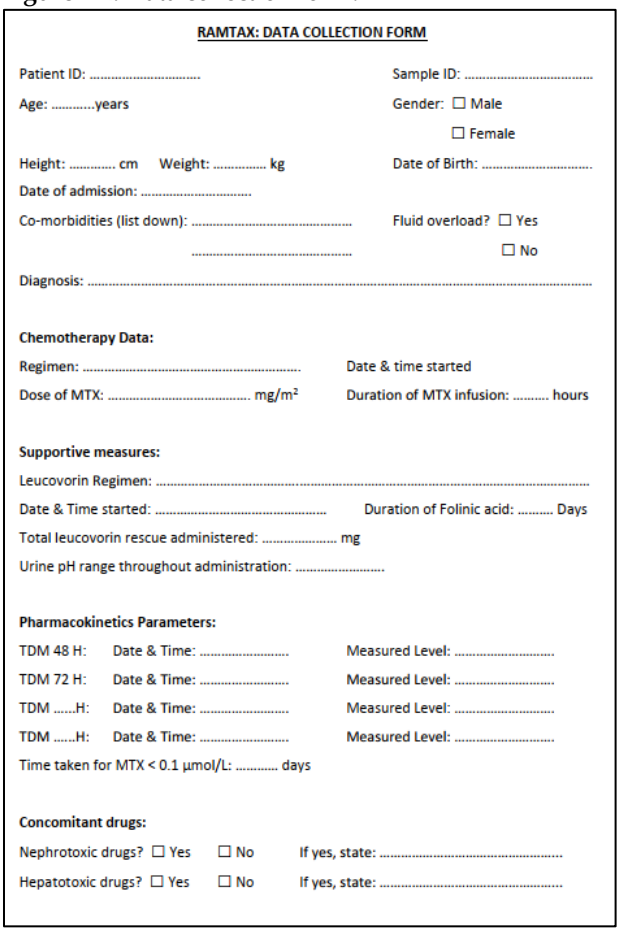


Figure A2: Data collection form.



RAMTAX: DATA COLLECTION FORM

Patient ID: ..... Sample ID: .....

Age: .....years Gender: ☐ Male ☐ Female

Height: ..... cm Weight: ..... kg Date of Birth: .....

Date of admission: .....

Co-morbidities (list down): ..... Fluid overload? ☐ Yes ☐ No

.....

Diagnosis: .....

Chemotherapy Data:

Regimen: ..... Date & time started

Dose of MTX: ..... mg/m² Duration of MTX infusion: ..... hours

Supportive measures:

Leucovorin Regimen: .....

Date & Time started: ..... Duration of Folinic acid: ..... Days

Total leucovorin rescue administered: ..... mg

Urine pH range throughout administration: .....

Pharmacokinetics Parameters:

TDM 48 H: Date & Time: ..... Measured Level: .....

TDM 72 H: Date & Time: ..... Measured Level: .....

TDM .....H: Date & Time: ..... Measured Level: .....

TDM .....H: Date & Time: ..... Measured Level: .....

Time taken for MTX < 0.1 µmol/L: ..... days

Concomitant drugs:

Nephrotoxic drugs? ☐ Yes ☐ No If yes, state: .....

Hepatotoxic drugs? ☐ Yes ☐ No If yes, state: .....

1 **Table A1:** Chemotherapy agents used in HD-MTX regimens. MTX, methotrexate; IT, intrathecal

Protocol Name	Chemotherapy Agents	Leucovorin Rescue Dose	Duration of MTX Infusion (hours)
ALL-BFM HR1	Methotrexate 5000 mg/m² D1 Vincristine 1.5 mg/m² (max 2 mg) D1,6 Cyclophosphamide 200 mg/m² BD × 5 doses D2-4 Cytarabine 2000 mg/m² bd D5 L-Asparaginase 25,000 u/m² D6 IT Methotrexate 12 mg D1 IT Cytarabine 30 mg D1	15 mg/m² QID start at 42H of MTX	24
ALL-BFM HR2	Methotrexate 5000 mg/m² D1 Vindesine 3 mg/m² (max 5 mg) D1,6 Ifosfamide 800 mg/m² × 5 doses D2-4 Daunorubicin 30mg/m² D5 L-Asparaginase 25,000 u/m² D6 IT Methotrexate 12 mg D1 IT Cytarabine 30 mg D1	15 mg/m² QID start at 42H of MTX	24
ALL-BFM M	6-Mercaptopurine 25 mg/m² D1-56 Methotrexate 5000 mg/m² D8,22,36,50 IT Methotrexate 12 mg D8,22,36,50	15 mg/m² QID start at 42H of MTX	24
Block A	Methotrexate 1500 mg/m² D1 Vincristine 2 mg D1 Ifosfamide 800 mg/m² D1-5 Cytarabine 150 mg/m² BD D4-5 Etoposide 100 mg/m² D4-5 IT Methotrexate 15 mg D1,5 IT Cytarabine 40 mg D1,5	30 mg/m² start at 42 & 48H of MTX, then 15 mg/m² QID	24

Block B	Methotrexate 1500 mg/m^2 D1 Vincristine 2 mg D1 Cyclophosphamide 200 mg/m^2 D1-5 Doxorubicin 25 mg/m^2 D4-5 Etoposide 100 mg/m^2 D4-5 IT Methotrexate 15 mg D1,5 IT Cytarabine 40 mg D1,5	30 mg/m^2 start at 42 & 48H of MTX, then 15 mg/m^2 QID	24
DeAngelis (Week 1,3,5,7,9)	Methotrexate 2500 mg/m^2 D1 Vincristine 1.4 mg/m^2 (max 2.8 mg) D1 Procarbazine 100 mg/m^2 (max 150 mg) D1-7 (Week 1,5,9)	20 mg QID start at 24H of MTX	3
GMALL Week 30, 46	Methotrexate 1500 mg/m^2 D1,15 L-Asparaginase 10,000 u/m2 D2,16 6-Mercaptopurine 60mg/m^2 OD D1-7,15-21 IT Methotrexate 15 mg D1 IT Cytarabine 40 mg D1	30 mg/m^2 start at 42 & 48H of MTX, then 15 mg/m^2 QID	24
HD-MTX	Methotrexate 3000 mg/m^2 D1	20 mg QID start at 24H of MTX	3
HyperCVAD B	Methotrexate 1000 mg/m^2 D1 Cytarabine 3000 mg/m^2 BD D2- 3 IT Methotrexate 12 mg D2 IT Cytarabine 100 mg D2	15 mg QID start at 48H of MTX	24
R1	Methotrexate 500 mg/m^2 D1 Vincristine 1.5 mg/m^2 (max 2 mg) D1 Cytarabine 300 mg/m^2 D5 Etoposide 100 mg/m^2 D5 L-Asparaginase 10,000 u/m^2 D6-8 6-Mercaptopurine 100 mg/m^2 OD D1-5 IT Methotrexate 15 mg D1	15 mg/m^2 start at 42 & 48H of MTX, then 10 mg/m^2 QID	24
R2	Methotrexate 500 mg/m^2 D1 Vincristine 1.5 mg/m^2 (max 2 mg) D1 Cytarabine 300 mg/m^2 D5 Daunorubicin 50 mg/m^2 D5 Ifosfamide 400 mg/m^2 D1-5 6-Thioguanine 50 mg/m^2 BD D1-5 IT Methotrexate 15 mg D1	15 mg/m^2 start at 42 & 48H of MTX, then 10 mg/m^2 QID	24
SMILE	Methotrexate 2000 mg/m^2 D1 Ifosfamide 1500 mg/m^2 D2-4 Etoposide 100 mg/m^2 D2-4 L-Asparaginase 6000 u/m^2 D8,10,12,14,16,18,20	30 mg/m^2 start at 30H & 36H of MTX, then 15 mg/m^2 QID	6

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Formulation and evaluation of topical gels containing *Phyllanthus muellerianus* leaf extract using various gelling agents

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Abstract

Introduction: The high expense of current pharmaceuticals used to treat wounds, as well as some of their adverse effects, has spurred the quest for alternatives, particularly those derived from natural sources that have minimum side effects, less microbial susceptibility and are less expensive. *Phyllanthus muellerianus* leaf extract incorporated in creams and ointments greatly decreased wound closure time and increased epithelialization at the wound site. This study aims to formulate and evaluate a gel made from *P. muellerianus*. **Methods**: Leaves of *P. muellerianus* were extracted using water. Phytochemical screening for tannins, flavonoids, alkaloids and reducing sugars was performed on the extract. The water extract was used to formulate twenty gels with varying gelling agents. Physicochemical analysis, toxicity, wound healing and stability studies were performed on the gels. **Results:** The extraction of *P. muellerianus* leaves yielded 13.1 %w/w. Only tannins, glycosides, saponins, sterols and triterpenoids were present. *P. muellerianus* gels (1 %w/v) were formulated with five different concentrations of each of four different gelling agents. The gels had satisfactory physicochemical properties, and the microbial load and drug content were within the acceptable range for herbal formulations. There was no indication of chemical interactions between the extract, polymer, and other excipients in Fourier transform infrared spectroscopy investigations. There were no significant changes in the pH, spreadability, viscosity and drug content of the gels throughout the stability assay period. Dermal toxicity studies revealed that the *P. muellerianus* gels were not toxic to the skin (acute and repeated dose dermal toxicity tests). Wounds treated with formulations A4 and C5 showed significantly decreased wound area from the fifth day to day 15 post-injury compared to the positive and negative control groups, with an increased rate of re-epithelialization, fibroblast proliferation, collagen deposition and neovascularization. **Conclusion:** Ultimately, *P. muellerianus* gels (A4 and C5) showed tremendous wound healing activity, stability and safety.

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Introduction

Damages to the normal anatomical state and form, which may or may not affect bodily functions, are classified as wounds. There could be a breach in the skin's epithelial structure, or it may be more significant, expanding into subcutaneous tissues and injuring relevant cells like muscles, arteries, parenchymal organs, tendons and nerves (Gushiken *et al.*, 2021). Underlying connective tissues, such as muscles, bones, or nerves, may be lost (Ding *et al.*, 2021). Wounds may be characterized in several approaches, such as location, level of contamination, wound depth, duration of the wound, type of injury, and presenting symptoms and tissue loss. The body has its ways and cascades of dealing with wounds; however, when it becomes infected, the wound healing process is aided by pharmaceuticals. Synthetic agents having antimicrobial, anti-inflammatory and analgesic properties are incorporated in topical dosage forms for wound cleansing, dressing and treatment. The high expense of current pharmaceuticals used to treat wounds, as well as some of their adverse effects, has spurred the search for new innovative drugs, particularly those derived from natural sources that have minimum side effects and are relatively less expensive (Bhuyan *et al.*, 2021).

Many plants with known wound healing activities exist, and such a plant is *P. muellerianus,* which has shown tremendous activity when formulated into various topical dosage forms such as ointments and creams. Wounds treated with 0.25, 0.5, and 1% w/w *P. muellerianus* extract significantly ($p < 0.001$) reduced wound area from day 5 to 11 post-injury compared to the untreated wounds. Furthermore, the area under the curve (AUC) revealed that 0.25, 0.5, and 1% w/w *P. muellerianus* extract significantly ($p < 0.001$) reduced wound area compared to the untreated wounds (Boakye *et al.*, 2018). Transdermal systems are convenient, inexpensive, self-administered and can provide a steady drug concentration profile for an extended period. They include patches, creams, ointments, lotions, etc., of which gels are the most preferred (Apriani *et al.*, 2023). Gels are made by capturing large volumes of water or hydroalcoholic liquid in a mesh of colloidal solid particles (Nayak and Bera, 2019). Gels are known to be soluble, do not retain sweat and dry faster than ointments and creams and are ideal for people with hairy skin. Cosmetically, they are acceptable and have a bigger aqueous component than an ointment or cream base, which allows for better drug solubility and easier drug migration in a vehicle that is almost a liquid. In terms of ease of use and patient acceptability, these are superior (Bhuyan *et al.*, 2021; Kabiri *et al.*, 2018). The current study aims to develop an effective gel formulation with the aqueous extract of the leaves of *P. muellerianus* and evaluate its wound healing activity. Its wound healing properties if found comparable to standard wound healing agents, would serve as a good option for wound treatment and ultimately, reduce the cost of producing topical wound healing medications for manufacturers in Ghana.

Materials and methods

Materials

The leaves of *Phyllanthus muellerianus* were acquired in Ghana in July 2021 from Kwahu in the Eastern Region. They were authenticated at the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, with specimen voucher number KNUST/HMI/2021/L022. Carbopol 940, carboxymethyl cellulose (CMC), hydroxypropyl methyl cellulose (HPMC) and triethanolamine were obtained from UK chemicals, Kumasi. Calcium chloride, glycerol, propyl paraben, propylene glycol, methyl paraben and xanthan gum were also obtained from the stores of the Department of Pharmaceutics.

Method

Extraction of P. muellerianus leaves

Extraction was done as described by Boakye et al. (2018). Powdered *P. muellerianus* leaves (0.5 kg) were heated in 5 L of distilled water at 90 ^{o}C for fifteen (15) minutes. A vacuum rotary evaporator was used to concentrate the filtrate under decreased pressure at 45 ^{o}C after a Buchner funnel and Whatman no. 10 filter paper were used to filter the

extract. The extract was freeze dried, yielding a powdered substance which was stored in the desiccator until use.

Phytochemical analysis of P. muellerianus extract

The powdered extract of *P. muellerianus* was screened qualitatively for secondary metabolites following standard procedures according to Evans, (2009).

Test for saponins

A mass of 1 g of powdered *P. muellerianus* extract was mixed with 10 mL of water in a test tube. The mixture was filtered, and the filtrate was vigorously agitated and placed aside for five minutes and observed for froth formation.

Test for tannins

A mass of 0.5 g of powdered *P. muellerianus* extract was combined with 25 mL of boiling water and was allowed to stand for 5 minutes. The mixture was filtered and chilled. Ten drops of 1 % Lead acetate solution were added to 1 mL of filtrate. The presence of tannins was revealed by the development of a precipitate.

Test for flavonoids

Twenty milliliters (20 mL) of water were added to 1 g of powdered *P. muellerianus* extract, and the mixture was then filtered. After being dipped into the filtrate, a white filter paper strip was dried and subjected to hydrochloric acid vapors. The appearance of a bright yellow colour confirms the presence of flavonoids.

Test for alkaloids

A mass of 1 g of powdered *P. muellerianus* extract was weighed and dissolved in 1 % H_2SO_4. The solution was filtered. One drop of Dragendorff's reagent (potassium bismuth iodide solution) was added to 1 mL of the solution, and the appearance of an orange-red precipitate indicates the presence of alkaloids

Test for sterols

A mass of 1 g of powdered *P. muellerianus* extract was extracted with chloroform to obtain a $CHCl_3$ extract. Concentrated H_2SO_4 was carefully drizzled down the side of the test tube after two drops of acetic anhydride were added to 5 mL of the extract. A layer formation was observed in the sample.

Test for triterpenoids

A chloroform ($CHCl_3$) extract was obtained by extracting 5 g of powdered *P. muellerianus* extract with $CHCl_3$. A volume of 5 mL of $CHCl_3$ extract was gently poured down the edge into a test tube containing 1 mL concentrated H_2SO_4. The sample was observed for layer formation.

Test for reducing sugars

A mass of 0.5 g of *P. muellerianus* extract was reheated in 5 mL of diluted H_2SO_4 on a water bath for two minutes before being filtered. To the filtrate, four (4) drops of a 20% sodium hydroxide solution were added. The filtrate was mixed with 1 mL of Fehlings solutions A and B, which were then heated over a water bath for approximately two minutes. The appearance of a red precipitate confirms the presence of reducing sugars.

Formulation of P. muellerianus gels

Different masses (Table 1) of the gelling agents (Carbopol 940, CMC, HPMC and xanthan gum) respectively were carefully weighed and dispersed in 50 mL of a suitable solvent (deionised water for Carbopol and xanthan gum, distilled water heated to 70–90 ^oC for HPMC and 3 %w/v calcium chloride solution for CMC) in separate beakers. The beakers were set aside to allow the gelling agent to swell for half an hour and then stirred using a mechanical stirrer for 30 minutes. In 5 mL of propylene glycol, 1 g of the extract was dissolved, and in a separate beaker, 5 mL of glycerol was mixed with methyl and propylparaben. After all of the gelling agents had dispersed, 1 g of extract and preservative solutions were added to each of the gelling agent dispersions while constantly being stirred. Finally, the volumes were increased to 100 mL by adding more of the solvent. In the case of the Carbopol-based gels, triethanolamine was added in drops to the formulations to adjust the pH and consistency (Jamadar and Shaikh, 2017).

Table 1: Composition of *Phyllanthus muellerianus* gel formulations

Composition	A1	A2	A3	A4	A5	B1	B2	B3	B4	B5	C1	C2	C3	C4	C5
Extract/g	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Carbopol 940/g	0.25	0.40	0.50	0.75	1.0	-	-	-	-	-	-	-	-	-	-
HPMC/g	-	-	-	-	-	1.00	1.50	2.00	2.50	3.00	-	-	-	-	-
CMC/g	-	-	-	-	-	-	-	-	-	-	2.00	2.50	3.00	3.50	4.00
Propylene glycol/mL	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Glycerol/mL	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Methyl paraben/g	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Propyl paraben/g	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Triethanolamine/mL	qs	qs	qs	qs	qs	-	-	-	-	-	-	-	-	-	-
Deionised Water / mL	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Physicochemical evaluation of P. muellerianus gels

Physical appearance

The colour, homogeneity, and phase separation of the produced gel base and gel formulations containing the *P. muellerianus* leaf extract were visually evaluated. The texture of the gel was determined by rubbing it between the thumb and the middle finger.

Measurement of pH

Using a digital pH meter, the pH of the gel base and gel formulations was measured. One gram of gel was dissolved in one hundred millilitres of distilled water and allowed to stand for two hours. Each formulation's pH was measured in triplicate, and the mean was determined (Jamadar and Shaikh, 2017).

Spreadability

On two sets of standard-sized glass slides, a sphere with a diameter of 2.4 cm was produced. One gram of gel was sandwiched between the two slides and uniformly pressed to generate a thin layer in the centre of the sphere on the slide. The upper slide was loaded with a 100 g weight. Over one minute, the distance (new circumference to old circumference) generated by the gel spreading out under the impact of the weight was measured. The experiment was done in triplicate, with the average distance being used to determine the gel's spreadability (Helal et al., 2012).

Extrudability

The gel formulations were packed in a conventional collapsible aluminium tube with a capped end and crimped shut. The tubes' weights were recorded. The tubes were secured between two panes of glass. The slides underwent compression using a 500 g weight before the removal of the cover. After extrusion, the gel was gathered and weighed. The percentage of the extruded gel was then determined by using equation 1 (Jamadar and Shaikh, 2017):

$$\frac{wt\ of\ gel\ extruded}{wt\ of\ gel\ and\ tube - wt\ of\ empty\ tube} \times 100\% \qquad (\mathbf{1})$$

Drug content determination

One gram of *P. muellerianus* extract was dissolved in 50 mL of phosphate buffer, and after a series of suitable dilutions, the absorbance spectrum was scanned with a UV-visible spectrophotometer. The highest absorbance (peak) was determined at 279 nm and was used as a mark for the active constituent in the extract. One milliliter of each formulation was dissolved in 50 mL of phosphate buffer solution at pH 7.2. The resultant solution was adjusted to 100 mL in a volumetric flask with phosphate buffer at pH 7.2 after being filtered through Whatman number 1 filter paper. The resulting solution was appropriately diluted and using phosphate buffer with a pH of 7.2 as a blank, the absorbance was determined at 279 nm using a UV-visible spectrophotometer (Bhuyan *et al.*, 2021).

FT-IR analysis

At a scan resolution of 4 cm^{-1} and over a wave number range of 400 – 4000 cm^{-1}, IR spectra were developed for the dried powdered aqueous extract of *P. muellerianus* leaves and each of the *P. muellerianus* gel formulations with an ALPHA II FTIR Spectrometer (Amponsah et al, 2016).

Microbial content by pour plate method

Aseptically, 1 mL of the *P. muellerianus* gel was transferred into 9 mL sterile distilled water to achieve a 10-fold dilution. One millilitre (1 mL) of the 10-fold dilution was pipetted into sterile petri dishes labelled for viable aerobic bacterial and fungal counts, including pathogenic microorganisms such as *Staphylococcus aureus*, *Salmonella* species, *Pseudomonas aeruginosa* and *Escherichia coli*. Fifteen millilitre (15 mL) of stabilized Cetrimide agar, Sabouraud Dextrose agar, Mannitol Salt agar, MacConkey agar, Nutrient agar and Bismuth Sulphite agar were placed separately into their appropriate designated plates and swirled to mix. Aerobic viable bacterial and pathogenic organisms were cultured at 37 ^{o}C for 48 hours, while fungi were incubated at 25 ^{o}C for 5 days. The determination was performed in duplicates. The colonies were counted to determine the mean, then calculated for colony-forming units (cfu/ml or cfu/g).

Toxicity studies on dermal application

Test animals

Male Sprague Dawley rats (130-300 g) were purchased from the "Animal House" at the University of Ghana in Accra, Ghana. They were housed in steel cages where they were exposed to room temperature (25 °C), light, and 50 – 60 % relative humidity. Throughout the toxicity testing, they were fed rat meal and water from clean bottles on an ad libitum basis. The animals were given a week to adapt before each trial.

Skin irritation test

As specified in Organisation for Economic Co-operation and Development (OECD) standard 404, Sprague Dawley rats were used for the skin irritation test (OECD, 2015). Before each rat was caged separately, hair covering approximately 10 % of the entire back of each rat was cut with a razor blade. Skin abrasion was prevented by using only animals with intact skin. Twenty-eight (28) rats were used in this experiment. The treatment group consisted of twenty-four of the twenty-eight subjects, with each group of four getting one of the six optimal formulations, while the control group consisted of the remaining four. After that, the rats were given 72 hours to adjust to their new surroundings without being disturbed. In the treatment group, A uniform 1 mL of the gel was applied to the area that had been shaved. (about 6 cm^2). Gauze and non-irritating adhesive tape were used to keep it in place. In the control group, sterile water was applied to the shaved area, which was then secured with gauze and non-irritant tape. After a 4-hour exposure period, the covers were removed, distilled water was used to clean the test area, and the OECD scoring system was used to detect oedema and erythema symptoms at 1, 12, 24, 48, and 72-hour intervals (Nayeem *et al.*, 2021; Pedrosa *et al.*, 2017).

Repeated dose dermal toxicity test

This test was performed following the OECD guidelines 410 (OECD, 2015). About ten percent (10%) of the total surface area of the back of the rat, where the incisions will be made, was shaved. The animals were put into 7 groups of 4 rats each. These groups consisted of six (6) *P. muellerianus* gel-treatment groups and one (1) control group to which only sterile distilled water was applied. Each rat weighed between 100 and 200 g. The rats were then left alone for 24 hours before the application of the *P muellerianus* gel formulation. The test ingredient (1 mL from each formulation) was applied to the shaved region and secured with a porous gauze bandage and non-irritant adhesive tape twice a day for 21 days. The skin and fur, eyes and mucous membranes, and respiratory and behavioural patterns of the animals were observed daily. The test animals were reweighed every week. The histology of skin tissues was analysed after 21 days of the test.

Histological examination

Skin tissues were removed from two rats in each group and promptly fixed in a 10 % buffered neutral formalin solution. The tissues were placed into tissue cassettes immediately after fixation. Water was removed from the fixed tissues by concentrated ethanol solution treatment. The tissues were subsequently cleared by immersing them in various xylene concentrations to displace the ethanol. The tissues were then infiltrated with paraffin wax, which displaced the xylene in the tissues. After they had solidified, they were sliced into 5 µm thick sections with a Leica rotary microtome. The tissues were placed on cleaned glass slides and stained with haematoxylin and eosin after the paraffin was removed. After that, a light microscope was used to assess the glass slides to determine the extent of cell regeneration, re-epithelialization, and granular tissue formation. At a magnification of x40, photomicrographs were obtained (Talekar et al., 2012; Boakye et al., 2018).

Wound healing assay using P. muellerianus gel

The excision wound healing model in Sprague Dawley rats, as stated by Boakye et al. (2018), was employed to investigate the wound healing properties of the formulated *P. muellerianus* gel. Eight sets of five rats each received topical treatments of *P. muellerianus* gel and silver sulphadiazine cream (1 % w/w), while the untreated group's wounds were just cleansed with 0.9% w/v normal saline solution. The wounds were cleansed with 0.9 % w/v saline solution daily before the

topical application of the formulated gels (0.5 mL of gel per daily application). Any rat that exhibited signs of wound infection was excluded from the experiment. Wound scar tissues were harvested on day 15 post-injury and used for histological studies. Wound treatment began at twenty-four hours (24 hr) post-wounding. The wound diameter was measured with a millimeter rule on days 1, 3, 5, 7, 9, 11, 13, and 15 post-injuries. The percentage of wound contractions was determined and noted accordingly using Equation 2:

$$\% \text{ WC} = (\text{IWS} - \text{SDWS}) / \text{IWS} \times 100 \% \quad (2)$$

% WC = Percentage wound contraction

IWS = Initial wound size

SDWS = Specific day wound size

Histological examination

The histological examination was performed as described by Talekar et al. (2012) and Boakye et al. (2018). Two rats from each group on day 15 were anaesthetized with pentobarbitone, and tissues from the wound sites were taken. These tissues were fixed in 10 % buffered neutral formalin. Tissue preparation followed by immersion in ethanol, xylene and paraffin. Five micrometer (5 μm) thick sections were cut from the prepared tissue, washed in sterile distilled water and stained with haematoxylin and eosin stain. These were viewed with a light microscope. Photomicrographs were taken at x40 magnification.

Stability studies on P. muellerianus gels

The optimized formulations (A3, A4, A5, C3, C4, C5) were subjected to stability tests by the International Conference on Harmonisation (ICH) guidelines. The formulations were tested for short-term stability for three months. The samples were kept at various temperatures, including refrigeration (4 - 8 °C), room temperature (25 °C), and a temperature of 40 °C in the oven. Every month, a sample was taken and analysed for visual appearance, pH, spreadability, viscosity, and drug concentration.

Statistical Analysis

Irritation data was simply reported as visual ratings using the Draize erythema and oedema grading system, and PII was computed. GraphPad Prism was used for statistical analysis. In every analysis, a p-value of less than 0.05 was deemed statistically significant. To compare the mean of each formulation group to the control, data on wound healing were evaluated and represented as mean ± SEM, whilst data on body weight measures were expressed as mean ± SD and analyzed using one-way ANOVA followed by Tukey's multiple comparisons test.

Results and discussion

Extraction and determination of physicochemical properties of extract

The percentage yield for the extracted sample was 13.1% w/w, which is close to that of the aqueous extraction yield of 14.1 %w/w reported by Boakye et al, 2018. A phytochemical examination of the milled leaves of *P. muellerianus* revealed the presence of compounds with medicinal and physiological properties. The presence of tannins, saponins, alkaloids, glycosides, and triterpenoids were detected in the milled leaves (Table 2). Saponins are known to have anti-inflammatory properties. They can precipitate and coagulate red blood cells as well (Kumari *et al.*, 2017). Triterpenoids are anti-inflammatory, antiviral, antibacterial, and antitumoral compounds that are implicated in the mechanisms of action of many therapeutic plants (Rios, 2010). Tannins are recognized to aid the healing of wounds by binding to proteins and other organic substances and precipitating them because of their astringent polyphenolic biomolecules such as geraniin which has been reported to be the major isolate of the aerial part of the plant known for its wound healing activity (Boakye et al., 2018; Li, 2011).

Table 2: Chemical constituents of the leaf extract of *Phyllanthus muellerianus*.

Phytoconstituents	Powdered Sample
Tannins	+
Glycosides	+
Saponins	+
Alkaloids	—
Flavonoids	—
Coumarins	—
Triterpenoids	+
Phytosterols	+

Keys :(+) Detected **(—)** Not Detected

Physicochemical evaluation of P. muellerianus gel

FT-IR analysis revealed the compatibility between the extract alone and a mixture of the extract with excipients. There were no interactions between the extract and the excipients, as indicated by the intact primary peaks and stretches of all the different functional groups in the gels (Fig 2).

The Carbopol-based gel bases were colourless and transparent, whereas the gel bases of HPMC and CMC were translucent and odourless. Saroha et al. (2013) reported that the gel base in which the active ingredients(s) are incorporated should possess the physicochemical parameters expected of the final gel (including ease of spread, no lumps, non-gritty and smooth to feel) (Fig 1). The Xanthan gum gel bases were hazy, grainy, and odorous and were thus omitted from the optimized gels. All of the gel formulations developed were homogeneous and free of lumps.

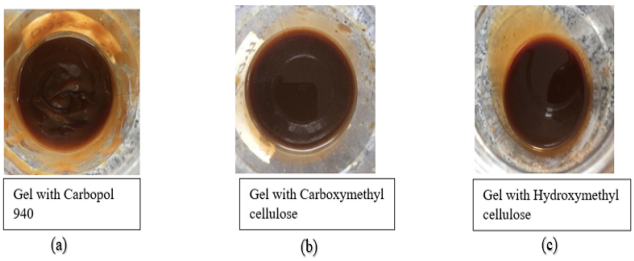

Fig 1: P. muellerianus gel with (a) Carbopol, (b) CMC and (c) HPMC

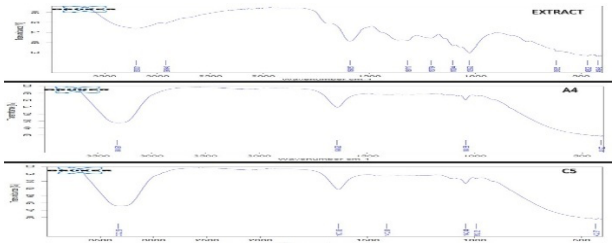

Fig 2: FT-IR of optimized gels (A4, C5) with *P. muellerianus* extract

The pH of human skin ranges from 5.5 to 6.8. To be evaluated for industrial usage, the pH of a formulation should be within or slightly above this range. Too acidic or alkaline pH levels can cause itching, redness, and scaly skin (Smaoui et al., 2017). The data suggests that as the gelling agent concentration increases, the pH of the resultant gel for the Carbopol bases increases (Table 3). The situation was unique with Carbopol-based gels since the gelling agent is naturally acidic and must be neutralized before the gel can be formed (chemical gelation). The concentration of the gelling agent employed affects the amount of neutralizers (TEA) required, which in turn influences the viscosity of the gel formed. Except for the xanthan-based gels, all of the formulated *P. muellerianus* gels had pH values that were within or slightly beyond the range of normal human skin, making them safe for usage (Table 3).

The spreadability of gels is important because it shows how the gel acts once it is removed from the packaging unit. The results obtained indicate that all of the polymers examined resulted in gels spreading by a modest amount of shear. Increasing the concentration of any of the gelling agents caused the spreadability to decline, as assessed by the smaller diameter of the spread circle (Table 3). The *P. muellerianus* gels were easily spreadable, according to the spreadability parameters of 15 – 20 gcm/s (Helal et al., 2012).

The extrudability of the gel formulations informs us of the ease with which the gels are removed from the packaging unit, which usually consists of tubes, with the application of a minimum force. More than ninety percent (90 %) of the packaged gels were extrudable, indicating excellent extrudability (Jamadar and Shaikh, 2017). Some had greater

Table 3: pH, Viscosity, spreadability, extrudability and drug content of *P. muellerianus* gels

Formulation	pH	Viscosity (centipoise)		Spreadability (gcm/s)	Extrudability	Percentage drug content
		Speed 3	Speed 6			
A1	6.55 ± 0.40	952.00±0.37	533.00 ±0.85	18.81	Excellent	91.51 ± 1.44
A2	6.31 ± 0.40	1121.00 ±0.19	772.00 ±0.23	18.11	Excellent	91.57 ± 1.47
A3	6.31 ± 0.39	2854.00 ±0.12	1768.00 ±0.66	17.87	Excellent	96.18 ± 1.38
A4	6.33 ± 0.36	3852.00 ±0.49	2631.00 ±0.02	17.76	Good	98.77 ± 1.83
A5	6.46 ± 0.31	6808.00 ±0.52	4715.00 ±0.50	17.53	Good	97.64 ± 1.32
B1	7.15 ± 0.27	711.00 ±0.21	205.00 ±0.12	23.03	Excellent	88.77 ± 0.15
B2	7.11 ± 0.29	1121.00 ±0.92	522.00 ±0.18	21.94	Excellent	89.17 ± 0.24
B3	7.12 ± 0.30	2010.00 ±0.61	1595.00 ±0.54	19.14	Excellent	91.19 ± 1.24
B4	7.18 ± 0.32	3190.00 ±0.53	2214.00 ±0.63	18.72	Good	85.90 ± 0.79
B5	7.21 ± 0.33	6401.00 ±0.18	3489.00 ±0.24	15.96	Good	87.22 ± 0.20
C1	6.62 ± 0.28	412.00 ±0.35	145.00 ±0.10	20.72	Excellent	99.22 ± 0.53
C2	6.77 ± 0.27	842.00 ±0.49	303.00 ±0.28	19.73	Excellent	101.17 ± 0.37
C3	6.69 ± 0.29	1770.00 ±0.70	900.00 ±0.71	18.94	Excellent	101.91 ± 2.97
C4	7.18 ± 0.02	2114.00 ±0.27	1150.00 ±0.18	18.34	Excellent	100.80 ± 1.72
C5	7.21 ± 0.03	3179.00 ±0.52	1276.00 ±0.24	18.17	Good	100.86 ± 2.14

Key: A1 – A5 = Carbopol Concentrations, B1 – B5 = HPMC Concentrations and C1 – C5 = CMC Concentrations

Table 4: The average weight of rats over the 21-day study period

Group	Weights of rats over 21-day study period			
	Day 1	Day 7	Day 14	Day 21
A3	138 ± 12.52	143 ± 12.17	151 ± 14.62	155 ± 12.34
A4	143 ± 13.85	151 ± 14.48	156 ± 14.29	163 ± 13.16
A5	133 ± 14.35	138 ± 13.24	145 ± 15.47	151 ± 12.72
C3	155 ± 15.31	162 ± 13.49	166 ± 15.94	168 ± 14.19
C4	141 ± 13.12	149 ± 15.62	153 ± 15.75	157 ± 12.86
C5	152 ± 13.78	155 ± 14.11	162 ± 15.51	169 ± 13.19
Control	159 ± 14.41	164 ± 13.87	168 ± 15.22	172 ± 15.25

than eighty percent (80 %) of the packaged gel extruded, indicating good extrudability. A few had greater than seventy percent (70 %) of the packaged gel extruded, indicating fair extrudability (Aiyalu et al., 2016). Some (0.8 %, 1.0 % and 1.2 % xanthan gel base) had less than seventy percent, indicating poor extrudability (Table 3).

The release of the active ingredient is also heavily influenced by numerous physical parameters, mainly the viscosity of the gel preparation. This is mostly related to the concentration of the gelling agents utilized in this study, such as Carbopol, CMC, HPMC, and Xanthan. Gels have a non- Newtonian fluid behaviour. Most non-Newtonian fluids undergo shear-thinning, which means that when shear stress increases, viscosity drops and could exhibit thixotropy (recoverable decreases in viscosity with stress over time) (Baviskar *et al*., 2013). In the study, increasing the concentration of the gelling agents resulted in a rise in viscosity for all gelling agents used (Table 3). However, increasing the speed (rate of shear) and concentration of active componentsresulted in a drop in viscosity. This demonstrates that the gels had a distinct shear-thinning feature, which makes it easy to apply.

The drug content of the gels was tested, and some of the Carbopol-based and all of the CMC-based gels ranged between 96.18 % and 101.91 % which was within the USP regulatory limits for herbal drugs (95 % – 105 %). The HPMC-based gels had the drug content below the acceptable limits (Table 3). The FT-IR of the HPMC-based gels did not reveal any interaction between the base and extract, or any other excipient and no evidence of physical interaction was observed. The data, however, suggests that the quantity of *P. muellerianus* extract decreased when incorporated in an HPMC gel base and therefore warrants further studies. The drug content analysis indicated that some of the gels contained acceptable amounts of the active *P. muellerianus* extract (Table 3) (Shiva *et al*., 2021; USP38/NF33, 2015).

Topical medications formulated with a herbal extract have specifications of the quantity of both pathogenic and non-pathogenic organisms permitted to be in them. For pathogenic organisms, none should be present in the formulation, while the quantities of non-pathogenic organisms allowed are specified (BP, 2018). No pathogenic organism was present in all the *P. muellerianus* gel formulations, and the non-pathogenic organisms were within the acceptable range for herbal formulations. Therefore, the gels can be used as a wound healing agent without the fear of wound infection and suppuration.

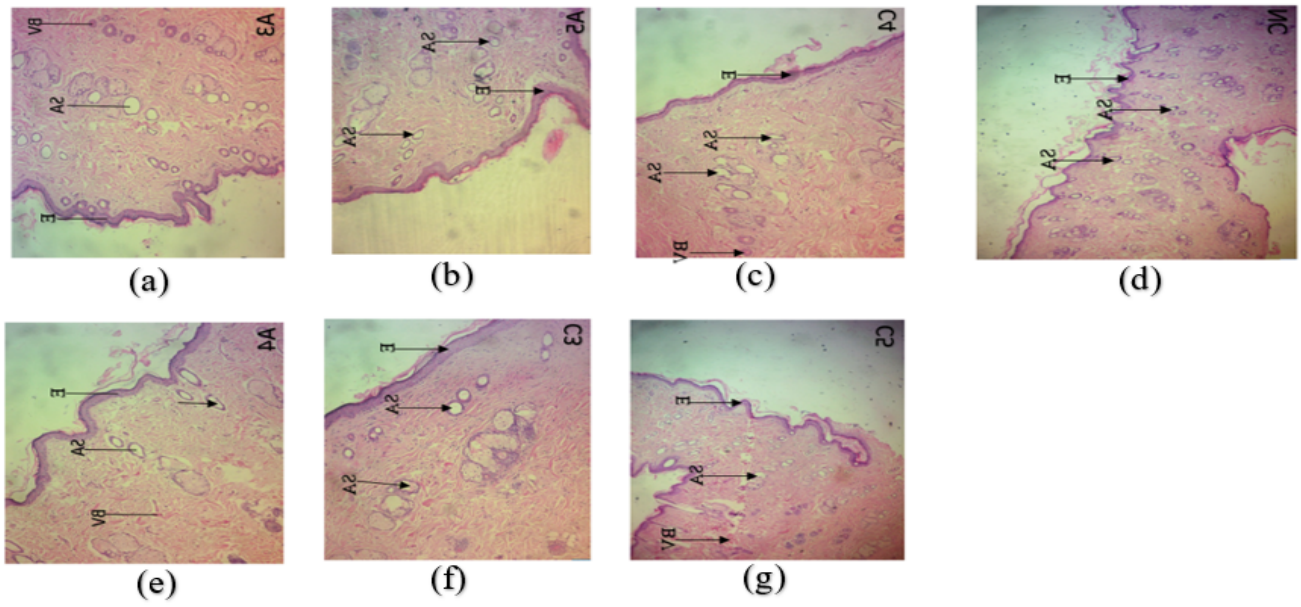

Fig 3: Histological images (x40) of excised skin tissues after 21-day treatment with *P. muellerianus* gels; (a) A3, (e) A4, (b) A5, (f) C3, (c) C4, (g) C5 and (d) negative control (NG)

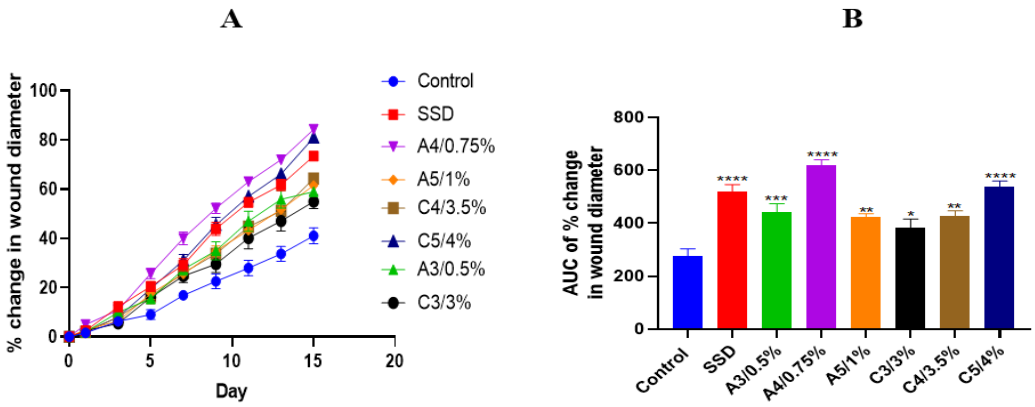

Fig 4: Influence of *Phyllanthus muellerianus* gel on the rate of wound closure

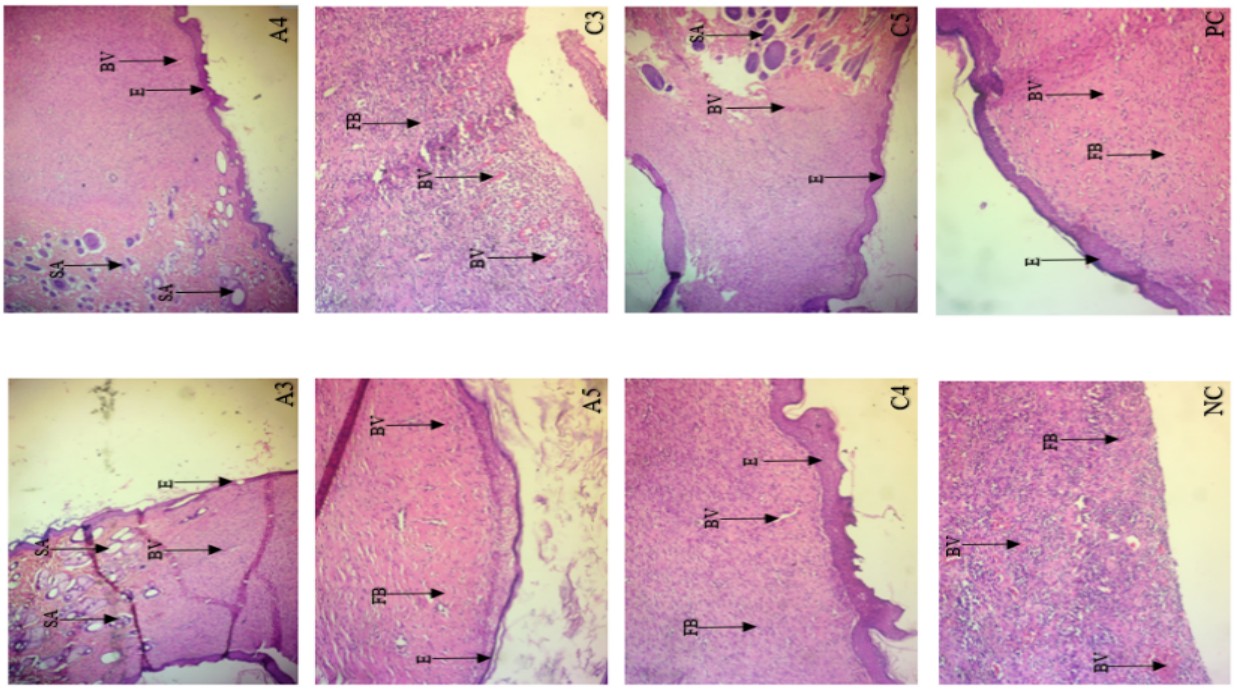

Fig 5: Histological images (x40) showing P. muellerianus gel activity in excised wound tissues from both treated and untreated rat wound

Dermal toxicity studies of P. muellerianus gel

The pharmacological ingredient present in topical formulations may have a negative impact on the skin, causing irritation and abrasion. As a result, toxicity studies on such medications are required in order to determine their safety. The gel formulations produced no oedema and redness on the rat skin 72 hours after it was applied, hence safe to use on the skin. Similarly, Ofokansi et al. (2018) found no irritation from *P. muellerianus* extract after it was applied externally to male Sprague-Dawley rats for 72 hours. Also, after the 21-day repeated dose assay, it was revealed that the relative organ weights of the test and control animals were statistically (p=0.999) similar (Table 4). Tissue histopathology after the study period revealed normal morphology of the skin (Fig 3). The epidermal layers of typical skin appendages and dermis were found to be intact in the skin tissues. In both the control and treatment groups, the hair follicles, sebaceous glands, and other skin appendages remained normal.

Wound Healing assay of P. muellerianus gel

In excised rat wounds, P. muellerianus gel demonstrated rapid cutaneous wound healing. The findings are comparable to those of an in vitro study by Boakye (2018), in which P. muellerianus showed increased fibroblast proliferation, angiogenesis, and granulation tissue formation, as well as

Table 5: pH, spreadability, viscosity and percentage drug content of optimized *P. muellerianus* gels for 3 months at room temperature

Gel Formulation	pH		Spreadability (gcm/s)		Viscosity (Centipoise)		Percentage Drug Content	
	Initial	Month 3	Initial	Month 3	Initial	Month 3	Initial	Month 3
A3	6.86 ± 0.19	6.72 ± 0.33[a]	17.87	17.69[a]	2854.00 ±0.12	2921.00±0.52[a]	96.18± 1.38	96.10±0.24 [a]
A4	6.99 ± 0.08	6.81 ± 0.61[a]	17.76	17.71[a]	3852.00 ±0.49	3885.00 ±0.73[a]	98.77± 1.83	98.49±0.13 [a]
A5	6.69 ± 0.10	6.69 ± 0.12[a]	17.53	17.60[a]	6808.00 ±0.52	6822.00 ±0.61[a]	97.64± 1.32	97.47±0.49 [a]
C3	6.73 ± 0.21	6.69 ± 0.49[a]	18.94	18.90 [a]	1770.00 ±0.70	1834.00 ±0.80[a]	101.91±2.97	101.72±0.15 [a]
C4	6.82 ± 0.30	6.87 ± 0.52[a]	18.34	18.39[a]	2114.00 ±0.27	2149.00 ±0.28[a]	100.80±1.72	100.50±0.35 [a]
C5	6.98 ± 0.43	6.98 ± 0.18[a]	18.17	18.28[a]	3179.00 ±0.52	3195.00 ±0.86[a]	100.86±2.14	100.78±0.05 [a]

Values are means ± SD (n = 3). Values were not significantly different from Initial at ([a]$p \geq 0.05$), and were significantly different from the Initial at ([c]$p < 0.01$) and ([d]$p < 0.001$).

Key: A3 - A5: Carbopol Concentrations, C3 - C5: CMC Concentrations

Table 6 pH, spreadability, viscosity and percentage drug content of optimized *P. muellerianus* gels for 3 months at refrigeration temperature

Gel Formulation	pH		Spreadability (gcm/s)		Viscosity (Centipoise)		Percentage Drug Content	
	Initial	Month 3	Initial	Month 3	Initial	Month 3	Initial	Month 3
A3	6.86 ± 0.19	6.80 ± 0.62 [a]	17.87	17.77 [d]	2854.00 ±0.12	2888.00±0.42[e]	96.18± 1.38	96.16±0.48[a]
A4	6.99 ± 0.08	6.91 ± 0.08 [a]	17.76	17.59 [d]	3852.00 ±0.49	3863.00±0.63[e]	98.77± 1.83	98.49±0.61[a]
A5	6.69 ± 0.10	6.52 ± 0.51 [a]	17.53	17.42 [d]	6808.00 ±0.52	6834.00±0.72[e]	97.64± 1.32	97.47±0.22[a]
C3	6.73 ± 0.21	6.64 ± 0.17 [a]	18.94	18.84 [d]	1770.00 ±0.70	1784.00±0.15[e]	101.91±2.97	101.72±0.05[a]
C4	6.82 ± 0.30	6.57 ± 0.62 [a]	18.34	18.3 [d]	2114.00 ±0.27	2179.00±0.75[e]	100.80±1.72	100.50±0.27[a]
C5	6.98 ± 0.43	6.71 ± 0.12 [a]	18.17	18.04 [d]	3179.00 ±0.52	3194.00±0.48[e]	100.86±2.14	100.78±0.21[a]

Values are means ± SD (n = 3). Values were not significantly different from Initial at ([a]$p \geq 0.05$) and were significantly different from the Initial at ([c]$p < 0.01$) and ([d]$p < 0.001$).

Key: A3 - A5: Carbopol Concentrations, C3 - C5: CMC Concentrations

Table 7: pH, spreadability, viscosity and percentage drug content of optimized *P. muellerianus* gels for 3 months at oven temperature

Gel Formulation	pH		Spreadability (gcm/s)		Viscosity (Centipoise)		Percentage Drug Content	
	Initial	Month 3	Initial	Month 3	Initial	Month 3	Initial	Month 3
A3	6.86 ± 0.19	6.59 ± 0.39 [a]	17.87	17.96 [e]	2854.00 ±0.12	2801.00±0.34[a]	96.18± 1.38	96.16±0.11[a]
A4	6.99 ± 0.08	6.46 ± 0.24 [a]	17.76	17.95 [e]	3852.00 ±0.49	3810.00±0.55[c]	98.77± 1.83	98.49±0.45[a]
A5	6.69 ± 0.10	6.43 ± 0.18 [a]	17.53	17.82 [e]	6808.00 ±0.52	6745.00±0.13[e]	97.64± 1.32	97.47±0.84[a]
C3	6.73 ± 0.21	6.50 ± 0.11 [a]	18.94	19.04 [e]	1770.00 ±0.70	1745.00±0.25[a]	101.91±2.97	101.72±0.61[a]
C4	6.82 ± 0.30	6.61 ± 0.15 [a]	18.34	18.47 [e]	2114.00 ±0.27	2092.00±0.11[a]	100.80±1.72	100.50±0.48[a]
C5	6.98 ± 0.43	6.57 ± 0.08 [a]	18.17	18.4 [e]	3179.00 ±0.52	3150.00±0.65[b]	100.86±2.14	100.78±0.50[a]

Values are means ± SD (n = 3). Values were not significantly different from Initial at ([a]$p \geq 0.05$), and were significantly different from the Initial at ([c]$p < 0.01$) and ([d]$p < 0.001$).

Key: A3 - A5: Carbopol Concentrations, C3 - C5: CMC Concentrations

significant re-epithelization and collagenation in the wound bed when compared to the untreated group, indicating that a P. muellerianus gel is an outstanding agent for wound care. Topical application of P. muellerianus gel (A3, A4, A5, C3, C4 and C5) resulted in a significant reduction ($p<0.001$) in wound size of excised rat wounds (Fig 4). This indicates a high rate of wound contraction. Histopathological examination confirmed the significant wound area reduction shown by P. muellerianus gel (A3, A4, A5, C4 and C5), which revealed increased neovascularization, fibroblast proliferation, and granulation tissue formation, as well as substantial collagen deposition and epithelial regeneration in comparison to the control (Fig 5). P. muellerianus gel-treated wounds produced fibroblast secretion that improved collagen deposition and crosslinking more than the untreated group. This aided in the faster healing of wounds and hence enhanced the tensile strength of the recovered skin.

Accelerated stability studies

The primary purpose of testing the stability of pharmaceuticals is to provide confidence that medicines will retain an acceptable level of quality during their duration on the market, and that they will be safe to use until the patient exhausts the product (Sengupta *et al.*, 2018). Organoleptic examinations of the formulations revealed no *muellerianus* gels in terms of colour, smell, texture, and homogeneity over the three-month stability study period. The gels remained clear, odourless, and uniform. The gels retained their clear, transparent, and translucent appearance, were smooth and homogeneous, and had no bad odour. Throughout the accelerated stability study, *P. muellerianus* gels remained stable and showed no significant alterations in pH, drug content, spreadability and viscosity when stored at room temperature (25 ^{o}C) (Table 5). However, there were statistically significant changes in pH, spreadability and viscosity from the second month when stored at higher (40 ± 2 ^{o}C) and lower (4-8 ^{o}C) temperatures (Tables 6 and 7). This suggests that storage at highly elevated and decreased temperatures, respectively, for a period of more than a month, will cause a marked decrease in product quality.

Conclusion

P. muellerianus gels with Carbopol 940 (0.75%w/v) and CMC (4 %w/v) as gelling agents had acceptable homogeneity, optimum pH values, good extrudability, viscosity and stability. The gels had no toxic effect on the skin after prolong usage, decreased wound closure time and promoted epithelisation and vascularization of the dermis. *P. muellerianus* gels could serve as an alternate wound healing agent and offer a scientific insight into future topical gel formulations.

Authors contributions

Study conception and design: OAA, MEBG, YDB, RJ
Data Collection: OAA, MLO, KAMA, WNA
Analysis and interpretation of results: OAA, MEBG, FWAO, AAE, RJ, YDB. Draft manuscript preparation: OAA, MEBG, FWAO

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Ethical approval statement

The animal study protocols were approved by the Animal Research Ethics Committee (AREC) of Kwame Nkrumah University of Science and Technology (KNUST 0041) and approved on August 02, 2023).

Conflict of interest

The authors declare that they have no conflict of interest with regards to publication of this paper.

Declaration of generative AI and AI-assisted technologies in the writing process

No AI (e.g. ChatGPT, Gemini and others) was used to improve readability and language during the preparation of this work.

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The Role of Fall Risk-Increasing Drugs in Prevalence of Fall and its Associated Factors

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Abstract

Introduction: The World Health Organization defines a fall as "an event where a person comes to rest inadvertently on the ground or another lower level". Among numerous risk factors, medication use stands out as one of the most highly modifiable risk factors for falls in adults. Medications associated with an increased fall risk have been termed Fall Risk–Increasing Drugs (FRIDs). This study aimed to determine the prevalence of falls among patients at Hospital Baling and its associated factors. **Methods:** A cross-sectional study was conducted by examining patients' history of falls and medication records for those admitted to the ward from 1st January to 30th June 2023. Systematic random sampling was used, and multiple logistic regression was performed to determine the risk factors for falls. Types of FRIDs were extracted from the Comprehensive Falls Risk Assessment Instrument (FRAI) and scored based on prescribed medications. **Results:** A total of 200 medical records were examined. The prevalence of falls among patients was 10.5%. Patients with a history of falls had higher FRAI medication scores compared to those without, although this difference was not statistically significant. Patients using diuretics were 81% less likely to experience falls while each additional comorbidity increased the odds of falling by 2.2 times **Conclusion:** While most FRIDs did not demonstrate a significant link to falls, comorbidities were a key predictor. Diuretics were unexpectedly associated with a lower fall risk. These findings emphasize the importance of personalized medication reviews by pharmacists to mitigate fall risk.

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Introduction

Fall is defined by the World Health Organization as "an event which results in a person coming to rest inadvertently on the ground or floor or other lower level (World Health Organization, 2007). Falls have become a significant public health issue due to their association with higher rates of mortality and morbidity (James et al., 2020). Those affected may experience severe debilitating effects, which in some cases can lead to fatal injuries (Appeadu & Bordoni, 2023). Falls result from a complex interaction of various contributing factors, including age, gender, number of comorbidities, history of previous falls, functional dependence, and medication burden (Tinetti et al., 1995; Zia et al., 2017). Among these risk factors, medication use is particularly important because it is both a major and modifiable contributor to fall (de Jong et al., 2013; de Vries et al., 2018). Research consistently indicates that using multiple medications significantly increases the risk of falls (Hart et al., 2020; Ramos et al., 2023).

Medications that are associated with an increased risk of falls are referred to as fall risk–increasing drugs (FRIDs). Although definitions of FRIDs may differ, they generally include drugs such as benzodiazepines and non-benzodiazepine hypnotics, antidepressants, antipsychotics and opioids (Lee et al., 2021). Although the exact mechanisms are not entirely clear, these drugs may increase the risk of falls by negatively affecting the central nervous or cardiovascular systems. This can result in symptoms such as orthostatic hypotension, dizziness, confusion, sedation, sleep disturbances, and bradycardia (de Vries et al., 2018).

According to the Falls Guideline for Hospitalized Older Adults 2019 from the Ministry of Health, Malaysia, falls are the most commonly reported incident in hospital wards, with rates varying from 1.7 to 25 falls per 1,000 patient bed days, depending on the unit. Almost half of the falls reported in hospitals result in injuries, which can vary from minor bruises to serious wounds and fractures (Ministry of Health Malaysia, 2019). Falls can lead to significant medical expenses due to their association with prolonged hospital stays and increased healthcare utilization (Dunne et al., 2014; Dykes et al., 2023).

To the best of our knowledge, there are limited studies evaluating the factors associated with falls in Malaysia, particularly those focusing on the role of fall-risk-increasing drugs. Most quantitative research in Malaysia has primarily explored intrinsic factors contributing to falls, with less emphasis on the role of medication (Alex et al., 2020; Azidah et al., 2012; Sahril et al., 2020; Yeong et al., 2016). Despite the well-documented impact of medications on fall risk, existing fall assessment tools commonly used in Malaysian healthcare settings may not adequately account for this factor. For instance, widely used tools such as the Morse Fall Scale (MFS) primarily focus on general fall risk indicators but do not include a dedicated component assessing medication-related risks. Therefore, this study aims to investigate the role of fall risk-increasing drugs (FRIDs) in the prevalence of falls, determine the median difference in Fall Risk Assessment Index (FRAI) scores between individuals with and without a history of falls, and identify factors associated with falls among patients in a Malaysian hospital.

Materials and methods

Study Design and Settings

A cross-sectional study was conducted in Hospital Baling by reviewing patients' fall history and medication records for those admitted to the ward between January 2023 and June 2023.

Study Participants

Patients aged 18 and above who were admitted to the adult medical ward between January and June 2023 were included in the study. However, patients with cognitive impairment upon admission or those with incomplete medical records were excluded.

Study Tool

MFS was developed to identify risk of falls that are easy and quick to use, and with good predictive and interrater reliability (Morse et al., 1989). The MFS assesses six crucial risk factors for patient falls. These include: (1) history of falling, (2) secondary diagnosis, (3) ambulatory aids, (4) intravenous therapy, (5) gait, and (6) mental status. Each item was coded as "yes" if the condition was present, and "no" if it was not. In clinical settings or in ward, the assessment is typically performed by the registered nurses as part of routine patient monitoring (Ministry of Health Malaysia, 2018). For history of falling, it is coded as yes if the patient has fallen

during the current admission or recently experienced physiological falls within 3 months (Ministry of Health Malaysia, 2018). More than one medical diagnosis was also coded as yes. Use of walking aids was marked yes. Patients with an intravenous line or heparin lock were similarly coded. Gait was marked yes if it appeared weak or impaired. Lastly, mental status was coded as yes if the patient overestimated their physical abilities or gave responses inconsistent with their mobility orders.

The FRAI questionnaire (Ministry of Health Malaysia, 2019) comprises four sections: history of falls, conditions (including postural hypotension and episodes of syncope/dizziness), medications (including diuretic and antidiabetic), and diagnoses (such as incontinence and cardiac disease). The medication section of the FRAI questionnaire assigns varying scores based on the types of medications. Cardiac drugs, antihypertensives, diuretics, antidiabetics, NSAIDs, weak opioids, anticonvulsants, and muscle relaxants received a score of 1. For antipsychotics, metoclopramide, dopaminergic agents, antidepressants, antihistamines, anxiolytics, and strong opioids, a score of 2 was assigned which indicates higher risk of fall. To assess medication-related fall risk, the total score for the FRAI medication component was calculated by adding the scores of all active medications prescribed during each patient's hospital stay.

Data collection

Demographic information and clinical characteristics of the patients were gathered by reviewing the online medical records through the Medical Program Information System (MPIS). In addition, the history of falls was gathered from the MFS during admission. Active medication records for each patient were retrieved from the Pharmacy Information System (PHIS) and assessed using the medication section of the FRAI.

Sample Size and Sampling Method

Sample size calculations were performed for each objective. The single proportion formula (Ariffin, 2024), was utilized to determine the prevalence of falls among patients. Using $z = 1.96$, $\delta = 0.05$, and a previous prevalence reported $p = 0.15$ (Sahril et al., 2020), a total of 196 samples were required. Two-mean formula was used to calculate the required sample size to determine the difference in FRAI scores between individuals with a history of falls and those without (Ariffin, 2024). The standard deviation of 0.95 was obtained from preliminary data within the same study sample. An expected mean difference of 2.0 was estimated based on expert input from a senior clinical pharmacist familiar with fall risk assessment in the local population. Using a significance level of 0.05 and a power of 80%, the minimum required sample size was calculated to be 10 participants. For the third objective, following Green (1991), a minimum of 200 samples was set based on the rule of thumb for any regression analysis (Green, 1991) Thus, upon comparing the samples required for all objectives, it was determined that a minimum of 200 samples was needed.

Samples were chosen using systematic random sampling. The sampling interval for each ward was calculated by dividing the total number of admissions by the required number of samples. To initiate the selection process, a random starting point was chosen using a random table. Subsequent samples were selected by repeating the same interval.

Statistical analysis

Data analysis was performed using SPSS version 29.0 (IBM Corp, 2023). Descriptive statistics were used to summarize both categorical and numerical data. Numerical data were described using means and standard deviations, while categorical data were summarized with frequencies and percentages. Mann–Whitney U test was used to compare FRAI medication component scores between individuals with and without a history of falls, as the data did not meet the assumption of normality. Additionally, to identify factors associated with falls, multiple logistic regression analysis was employed. Variable with a p-value of less than 0.25 in the univariable analysis were considered for inclusion in the multivariable analysis to avoid prematurely excluding potentially important predictors.

Table 1: Sociodemographic and clinical characteristics of the patients (n=200)

Characteristics	History of fall				
	Yes (n=21)		No (n=179)		
	n	%	n	%	p-value
Socio-demographic					
Age	62.0*	15.0#	58.0*	26.0#	0.077
Gender					0.780
Male	10	47.6	91	50.8	
Female	11	52.4	88	49.2	
Race					0.563
Malay	20	95.2	164	91.6	
Non-Malay	1	4.8	15	8.4	
Occupation					0.611
Working	3	14.3	19	10.6	
Not working	18	85.7	160	89.4	
Clinical					
Number of comorbidities	3.0*	1.0#	2.0*	2.0#	0.005
Number of Medication	6.0*	3.0#	6.0*	4.0#	0.181
FRAI Medication Component Score	3.0*	1.0#	2.0*	2.0#	0.051
Secondary diagnosis during admission					0.689
Yes	17	81	41	22.9	
No	4	19	138	77.1	
Ambulatory aids					0.009
Yes	3	14.3	9	5.0	
No	18	85.7	170	95.0	
IV devices during admission					0.489
Yes	21	100	175	97.8	
No	0	0	4	2.2	
Gait					0.818
Normal	10	47.6	90	50.3	
Weak	11	52.4	89	49.7	
Mental status					0.617
Normal	20	95.2	174	97.2	
Overestimate/ Forgetful of limitations	1	4.8	5	2.8	

*median

#Interquartile range

Results

Tables 1 and 2 summarize the sociodemographic, clinical, and medication characteristics of the patients. Overall, there were no significant differences in the variables between the two groups.

Sociodemographic characteristics

This study involved 200 patients, of whom 21 (10.5%) had a history of falls, while 179 (89.5%) did not. Gender distribution was similar in both groups, with approximately half of the patients being male. Patients with a history of falls had a higher median age of 62.0 years (IQR: 15.0) compared to 58.0 years (IQR: 26.0) in those without a history of falls. However, it was not statistically significant. Ethnic distribution showed that the majority of patients were Malay, comprising 95.2% of those with a history of falls and 91.6% of those without. Regarding employment, more than half of the patients in both groups were not working with 85.7% of those with a history of falls and 89.5% of those without a history of falls (Table 1).

Clinical characteristics

Patients with a history of falls had a higher number of comorbidities compared to those without a fall history. Likewise, patients with a history of falls use ambulatory aids more than those without a history of falls, (Table 1). Additionally, most patients were on IV devices during admission (100% of those with a fall history and 97.8% of those with no fall history), used ambulatory aids (85.7% of those with a fall history and 95.0% of those with no fall history), and had normal mental status (95.2% of those with a fall history and 97.2% of those with no fall history). Furthermore, weak gait was observed in 52.4% (n=11) of patients with a fall history and 49.7% (n=89) of those without, indicating that gait impairment was similar regardless of fall history.

Medication characteristics

Patients with a history of falls showed a higher percentage of usage for cardiac medications, antihypertensives, antidiabetics, metoclopramide, anxiolytics, opioids, and anticonvulsants, as reported in Table 2. However, they exhibited lower percentages in the usage of diuretics, antipsychotics, dopaminergic agents, and antidepressants/antihistamines.

Table 2 Type of medications for patients based on FRAI (n=200**)**

Characteristics	History of fall				p-value
	Yes (n=21)		No (n=179)		
	n	%	n	%	
Medication					
Cardiac					0.365
Yes	9	42.9	59	33.0	
No	12	57.1	120	67.0	
Antihypertensive					0.337
Yes	13	61.9	91	50.8	
No	8	38.1	88	49.2	
Diuretic					0.263
Yes	2	9.5	35	19.6	
No	19	90.5	144	80.4	
Antidiabetic					0.098
Yes	11	52.4	61	34.1	
No	10	47.6	118	65.9	
Antipsychotic					0.731
Yes	0	0	1	0.6	
No	21	100	178	99.4	
Metoclopramide					0.234
Yes	7	33.3	39	21.8	
No	14	66.7	140	78.2	
Dopaminergic					0.731
Yes	0	0	1	0.6	
No	21	100	178	99.4	
Antidepressant/ antihistamine					0.434
Yes	1	4.8	18	10.1	
No	20	95.2	161	89.9	
NSAID					
Yes	0	0	0	0	
No	21	100	179	100.0	
Anxiolytics					0.194
Yes	1	4.8	2	1.1	
No	20	95.2	177	98.9	
Opioid					0.097
Yes	6	28.6	26	14.5	
No	15	71.4	153	85.5	
Anticonvulsant					0.112
Yes	2	9.5	5	2.8	
No	19	90.5	174	97.2	
Muscle relaxant					
Yes	0	0	0	0	
No	21	100	179	100	

Comparison of FRAI Medication Component Scores by Fall History

The FRAI medication component scores were higher among individuals with a history of falls (median = 3.0, IQR = 1.0) compared to those without (median = 2.0, IQR = 2.0). However, this difference was not statistically significant (p = 0.051).

Risk factor of fall

In univariable analysis, age, number of comorbidities, FRAI score, the use of diuretics, antidiabetic, metoclopramide, anxiolytics, opioid and anticonvulsant were found to be significant (Table 3 and Table 4). Subsequently, a multivariable analysis was carried out for all significant variables to determine risk factor associated with falls. Only number of comorbidity and patient with diuretics remained significant factors. Patients using diuretics were found to be 81% less likely to experience falls (adjusted OR = 0.19, 95% CI [0.04, 0.94], p = 0.038) compared to those not using diuretics. Conversely, for each additional comorbidity, the odds of experiencing a fall increased by 2.2 (adjusted OR = 2.20, 95% CI [1.38, 3.51], p = 0.001).

Discussion

This study found that the prevalence of falls among patients with a mean age of 63 years was 10.5%, which is lower than the 18.9% prevalence reported by (Alex et al., 2020) in an urban Malaysian population aged 55 and older, specifically in Kuala Lumpur. Similarly, two studies conducted in Malaysia on the prevalence of falls among the elderly reported higher rates, as demonstrated by Ghazi et al. (2017) and Rizawati and Ayu (2008), which showed prevalences of 30% and 27.3%, respectively. Additionally, Pitchai et al. (2019) reported that the prevalence of falls in people over 65 is 53% in India, 30% in the USA, 26.4% in China and 13.7% in Japan. One possible explanation for the lower prevalence observed in this study may be the inpatient hospital setting, where patients are closely monitored and hospitals usually implement active fall prevention measures.

Table 3 Factors associated with falls based on socio-demographic and clinical characteristics (n=200)

Variable	Simple Logistic Regression				Multiple Logistic Regression			
	b[#]	Crude OR	95%CI	p-value	b[#]	Adjusted OR	95%CI	p-value
Socio-demographic								
Age	0.03	1.032	(1.002,1.062)	0.037[*]	0.04	1.04	(0.98,1.08)	0.062
Gender								
Male								
Female	0.13	1.14	(0.46,2.81)	0.780				
Race								
Malay								
Non-Malay	-0.60	0.58	(0.07,4.36)	0.569				
Occupation								
Working								
Not working	-0.34	0.71	(0.19,2.65)	0.713				
Clinical								
Number of comorbidities	0.60	1.83	(1.21,2.77)	0.004[*]	0.79	2.20	(1.38,3.51)	0.001[**]
Number of Medication	0.01	1.10	(0.91,1.33)	0.303				
FRAI Medication Component Score	0.25	1.28	(0.98,1.67)	0.066[*]	-0.283	0.75	(0.42,1.39)	0.350
Secondary diagnosis during admission								
Yes	0.23	1.26	(0.40,3.96)	0.689				
No								
Ambulatory aids								
Yes	0.69	1.99	(0.40,9.87)	0.4				
No								
IV devices during admission								
Yes	19.08	<0.01		>0.95				
No								
Gait								
Normal								
Weak	0.11	1.11	(0.45,2.75)	0.818				
Mental status								
Normal								
Overestimate/ Forgetful of limitations	0.55	1.74	(0.19,15.65)	0.621				

[#]regression coefficient
[*]statistically significant at p<0.25 at the univariable level and included in multivariable analysis
[**]statistically significant at p<0.05

Table 4 Factors associated with falls based on medications (n=200)

Variable	Simple Logistic Regression				Multiple Logistic Regression			
	b[#]	Crude OR	95%CI	p-value	b[#]	Adjusted OR	95%CI	p-value
Medication								
Cardiac								
Yes	0.422	1.525	(0.61,3.82)	0.368				
No								
Antihypertensive								
Yes	0.452	1.571	(0.62,3.98)	0.34				
No								
Diuretic								
Yes	-0.837	0.433	(0.10,1.95)	0.275[*]	-1.66	0.19	(0.04,0.94)	0.042[**]
No								
Antidiabetic								
Yes	0.755	2.128	(0.86,5.29)	0.104[*]	0.723	2.06	(0.59,7.20)	0.258
No								
Antipsychotic								
Yes	-19.066	0	0	>0.95				
No								
Metoclopramide								
Yes	0.585	0.239	(0.68,4.76)	0.239[*]	0.598	1.82	(0.32,10.41)	0.502
No								
Dopaminergic								
Yes	-19.066	0	0	>0.95				
No								
Antidepressant/ antihistamine								
Yes	-0.805	0.447	(0.057,3.53)	0.445				
No								
NSAID								
Yes	0							
No								
Anxiolytics								
Yes	1.487	4.425	(0.384,51.001)	0.233[*]	2.750	15.64	(0.63,386.06)	0.093
No								
Opioid								
Yes	0.856	2.354	(0.84,6.62)	0.105[*]	1.280	3.60	(0.73,17.76)	0.116
No								
Anticonvulsant								
Yes	1.298	3.663	(0.66,20.19)	0.136[*]	1.581	4.86	(0.61,38.89)	0.136
No								
Muscle relaxant								
Yes	0							
No								

[#]regression coefficient
[*]statistically significant at p<0.25 at the univariable level and included in multivariable analysis
[**]statistically significant at p<0.05

In this study, patients with a history of falls had higher FRAI scores, indicating greater exposure to fall risk–increasing medications. Despite the lack of statistical significance, the observed trend is consistent with previous literature, although different tools were used instead of the FRAI questionnaire. For instance, a study by Milos et al. (2014) reported that individuals with a history of falls were prescribed, on average, 1.2 more FRIDs compared to those without falls. Similarly, Berdot et al. (2009) found that 69% of patients taking more than five medications experienced falls, and Ziere et al. (2006) noted that the risk of falling increased when polypharmacy included FRIDs. While these studies used different approaches to measure medication-related fall risk, their findings support the observed association between higher FRAI scores and fall history in our study.

Each additional comorbidity was associated with more than twice the odds of experiencing a fall by 2.2 (adjusted OR = 2.20, 95% CI [1.38, 3.51], p = 0.001), highlighting the important role of comorbidities as an intrinsic risk factor. Conditions such as cognitive impairment, sensory loss, joint problems, and syncope, as outlined by the Ministry of Health Malaysia (2019) may compromise balance, coordination, or awareness, therefore increasing vulnerability to falls. This finding aligns with previous literature; for example, Sibley et al. (2014) found that having multiple chronic conditions increased the likelihood of falling by 62%. Damián et al. (2013) also reported a similar trend, a 32% increase in fall rates for each additional diagnosis, based on adjusted rate ratio, although their study used a rate-based rather than odds-based approach. In contrast, Ghazi et al. (2017) did not observe a significant association between the number of comorbidities—particularly chronic conditions—and fall risk, which may reflect differences in study populations or the way comorbidity was defined and measured.

An interesting and unexpected finding from this study was that patients prescribed diuretics were 81% less likely to experience falls. This contrasts with existing literature, which generally categorizes diuretics as FRIDs due to potential side effects such as orthostatic hypotension and cognitive changes, especially among older adults (de Vries et al., 2018). This discrepancy might be due to several reasons. It may be that patients receiving diuretics in this study were more clinically stable, or prescribers were more selective and cautious, avoiding prescribing diuretics in patients who are more likely to be at higher risk of falling. Another consideration is that these patients may have been under closer monitoring in the inpatient settings, allowing any side effects to be identified and managed more promptly.

In this study, no significant associations were found for other fall risk–increasing drugs (FRIDs) apart from diuretics. Several factors may explain this finding. The relatively small sample size for certain drug classes may have limited the statistical power to detect significant associations. Small sample sizes in some cells of logistic regression models are known to yield inaccurate risk estimates, wide confidence intervals, and potentially misleading p-values (Kumar, 2015). Additionally, the inpatient hospital setting, where patients are under closer supervision and medications are often reviewed and adjusted, may reduce the likelihood of FRID-related falls. This environment differs significantly from community or home settings, where such supports may be limited or absent. A study by Adams et al. (2020) showed that older adults have an increased risk of falls at home following discharge from acute care hospitalization, particularly within the first 90 days post-discharge.

Identifying FRIDs is essential as it lays the foundation for their potential deprescribing, which is a critical element of a multifaceted falls prevention strategy (Seppala et al., 2021). Recognizing FRIDs helps healthcare providers mitigate the risk of falls among elderly patients by addressing one of the modifiable risk factors. By systematically using a fall risk assessment tool to review and evaluate patients' medication histories, clinicians and pharmacists can more effectively identify and manage these high-risk medications. This approach not only aids in pinpointing drugs that contribute to fall risk but also facilitates targeted interventions, such as adjusting or discontinuing these medications, to enhance patient safety and ultimately reduce fall.

This study has several limitations. First, the cross-sectional study conducted over a 6-month period may not accurately represent longer-term trends in fall risk and may partly explain discrepancies between the findings of this study and those reported in other research. Second, the relatively small sample size, particularly the limited

number of patients with a history of falls, may have reduced the statistical power to detect significant associations especially those related to medication characteristics. Third, as the study was conducted in a single hospital setting, the findings may not be generalizable to other healthcare settings or broader populations.

Conclusion

This study found that diuretics are associated with a lower risk of falls, while other fall-risk-increasing drugs (FRIDs) did not show a significant association with increased fall risk. Higher FRAI scores and the presence of multiple comorbidities were linked to an increased likelihood of falling, underscoring the importance of comprehensive risk assessments. Despite the unexpected findings, particularly regarding diuretics, cautious interpretation is warranted due to the single-centre nature of the study and its modest sample size. Despite these results, thorough medication reviews by pharmacists remain crucial to ensure that all medications are optimized for individual patient needs. The findings highlight the importance of a personalized approach to fall prevention, focusing on overall medication management and patient-specific factors.

Authors contributions

Conceptualization, K.N.G. and N.S.S.; methodology, F.E.F.; software, F.E.F.; validation, F.E.F, K.N.G. and N.S.S; formal analysis, F.E.F.; investigation, K.N.G, N.S.S.; writing—original draft preparation, F.E.F and N.S.S; writing—review and editing, F.E.F and N.S.S; supervision, F.E.F. All authors have read and agreed to the published version of the manuscript."

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Ethical approval statement

The study was registered with National Medical Research Register NMRR ID-23-01987-G0W and obtained ethical approval from Medical Research and Ethics Committee (MREC).

Conflict of interest

All authors declare no conflicts of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

I hereby declare that generative AI technologies were used during the preparation of this work to enhance clarity and refine grammar. These tools were employed solely to support the quality and efficiency of the writing process, without compromising the originality or integrity of the content. All AI-generated suggestions were carefully reviewed, revised, and incorporated in alignment with my academic and creative intentions.

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Journal of Pharmacy

Stingless Bee Honey Stick Deodorant: Formulation, Antioxidant and Antimicrobial Activities

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Abstract

Introduction: Deodorant often contain ingredients like aluminium salts, triclosan, fragrances, propylene glycol, and parabens that are usually associated with skin irritation and other health conditions. This study addresses the problem by formulating and characterising a natural deodorant that was free from these ingredients by using stingless bee honey (SBH) as an antibacterial and antioxidant ingredient. SBH, recognised for its efficacy in inhibiting the proliferation of odour-producing bacteria like *Staphylococcus sp*, was integrated into a stick deodorant formulation owing to its significant benefits. **Methods:** The evaluation of SBH began with testing its antioxidant activity, including total phenolic content (TPC), total flavonoid content (TFC) and DPPH assay. Thereafter, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. Based on the MIC value, an SBH-stick deodorant was formulated and then characterised based on its pH, softening point, antimicrobial study by using well diffusion method and stability study (30 °C ± 2 °C /75% RH ± 5%) for two weeks. **Results:** The TPC and TFC in this study were 57.99 ± 0.38 mg GAE/100g and 0.132 ± 0.04 mg QE/mL, respectively. Meanwhile, the DPPH scavenging activity was 66.78 ± 0.45%. The result showed that 20% w/w and 50% w/w of SBH were needed as MIC and MBC, respectively. The formulated stick deodorant was reported to have suitable pH, softening point and exhibit its antibacterial activity towards *Staphyloccoccus aureus* after being formulated as deodorant. It was also stable during the two weeks of storage. **Conclusion:** The SBH stick deodorant was successfully formulated and demonstrated potential antibacterial activity against *Staphylococcus aureus*, a known body-odour causing bacteria. These findings highlight the potential application of SBH as a natural antibacterial agent in personal care products, offering a promising alternative to synthetic deodorants.

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Introduction

Body odour, which mainly caused by activity of apocrine sweat glands, is influenced by various factors, including sex, diet, age, and genetics (Baker, 2019). Sweat is originally odourless, but body odour arises when bacteria on the skin come in contact with sweat, breaking down specific proteins in sweat into acids, leading to the generation of volatile organic compounds (VOCs), such as volatile fatty acids and thioalcohols. The primary bacteria involved in body odour formation are *Staphylococcus sp. Staphylococcus* bacteria were involved in body odour by metabolising compounds present in sweat, especially proteins and amino acids, into thioalcohols such as 3-methyl-2-hexenoic acid, which led to a pungent, unpleasant smell commonly associated with body odour. This explains the widespread use of deodorants and antiperspirant to manage body odour.

However, the use of antiperspirants and deodorants are linked to harmful effects due to certain ingredients. These include substances like aluminium salts (aluminium chloralhydrate or aluminium chloride) and zinc salts, which form gel plugs in sweat pores. Thus, it obstructs sweat from reaching the skin's surface and hinders the body's ability to eliminate toxins. Fragrances, propylene glycol, and parabens in these products are known allergens that may cause allergies and skin irritation (Sidek et al., 2021). Additionally, triclosan, an antibacterial compound utilised in personal care items, also raises concerns due to its oestrogen-disrupting properties (Farasani & Darbre, 2020). These concerns have led to a growing interest in natural alternatives.

SBH has been reported to exhibit significant antioxidant and antibacterial capabilities, covering for both gram-positive and gram-negative bacteria due to its phenolic and flavonoid compounds (Tuksitha et al., 2018), including *Staphylococcus sp.* in many studies (Ávila et al., 2019; Rosli et al., 2020; Tuksitha et al., 2018). Due to these properties, SBH is considered an excellent option to be used as an antibacterial ingredient in deodorants to eliminate body odour-causing bacteria, such as *Staphylococcus aureus*. Thus, this research aims to formulate and characterise a natural deodorant that is free from aluminium salts, triclosan, fragrances, propylene glycol, and parabens by using SBH as an antibacterial and antioxidant ingredient, which has not yet been explored in deodorant formulations.

Materials and methods

Materials

SBH, Heterotrigona itama (Kuin Honey, Kuantan, Malaysia), Folin–Ciocalteu reagent (Merck KGaA, Darmstadt, Germany), gallic acid (R&M Chemical Company, Selangor, Malaysia), quercetin hydrate 95% (Arcos Organics, Selangor, Malaysia), potassium acetate (HmbG Chemical, Selangor, Malaysia) , aluminium chloride hydroxy hydrate (Bendosen, Selangor, Malaysia) 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) reagent (R&M Chemical Company, Selangor, Malaysia), ascorbic acid (Sigma-Aldrich, St. Louis, United States), ethanol, Cetostearyl alcohol (R&M Chemical Company, Selangor, Malaysia), Olivem 1000 (Hallstar), isoamyl laurate (Future Food, Selangor, Malaysia), candelilla wax (Kahl, Trittau, Germany), zinc ricinolate (Take it Global, Penang, Malaysia), arrowroot powder (Take it Global, Penang, Malaysia), essential oil, vitamin E, Amoxicillin/clavulanic acid (Oxoid, Hampshire, United Kingdom), *Staphylococcus aureus* (ATCC 6538), Tryptic Soy Agar, Tryptic Soy Broth. All materials used in the formulation were cosmetic grade, except cetostearyl alcohol.

Method

Antioxidant Properties of SBH

Total phenolic content

The total phenolic content (TPC) was determined via spectrophotometry method. First, 1 mL of 0.2 g/mL honey was mixed with 1 mL of Folin–Ciocalteu reagent. After 3 minutes, 1 mL of sodium carbonate 10% and 7 mL of distilled water were added and mixed using a vortex mixer, then left to stand in the dark for approximately 90 minutes. The absorbance of the solution was measured at 725 nm, which corresponds to the

maximum absorbance of the blue complex formed by phenolic compounds with the Folin-Ciocalteu reagent. Gallic acid at concentrations of 0, 20, 40, 80, and 100 μg/mL were used as positive control. Phenolic content level was measured in triplicate and expressed as mg of gallic acid equivalents (GAEs) per 100 g honey (Khalil et al., 2012).

Total flavonoid content

The total flavonoid content (TFC) was determined using aluminium chloride colorimetric method. TFC test was conducted to determine the honey's ability to neutralise free radicals. First, a 50% v/v honey sample was prepared by diluting 2 mL of SBH with 2 mL of distilled water. Next, 1.5 mL of 95% ethanol was mixed with 500 μL SBH followed by 100 μL of 10% aluminium chloride hydroxy hydrate, 2.8 mL of deionised water and 100 μL of 1M potassium acetate. The solution was mixed and allowed to sit at room temperature for 30 minutes in the dark. Its absorbance was measured at a wavelength of 415 nm. Quercetin at concentrations 0, 20, 40, 60, 80, and 100 μg/mL were prepared as a standard (Tuksitha et al., 2018).

DPPH assay

The DPPH assay was conducted to measure the ability of honey to scavenge the free radicals. First, 50% v/v honey sample was prepared by diluting 2 mL of SBH with 2 mL of distilled water. Next, 300 μL of honey sample, 300 μL of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) solution and 2.4 mL of 99% ethanol were added and mixed before being left in the dark for 30 minutes. Next, the solution was centrifuged at 4500 rpm for 5 minutes, and the absorbance was measured at 517nm. Ascorbic acid as used as positive control. The percentage of free radical scavenging activity that targeted DPPH was calculated following Tuksitha et al., (2018) using Equation 1:

$$(\%) = [1 - (A_S/A_C)] \times 100 \quad (1)$$

Here, A_S is the absorbance of the honey sample while A_C is the absorbance of the control at 517 nm.

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) procedure was conducted to determine the lowest concentration of honey sample that was able to inhibit the growth of the *Staphylococcus aureus* while minimum bactericidal concentrations (MBC) were used to identify the lowest concentration of honey that is required to kill the microorganism. An overview of these processes was illustrated in ***Fig 1.*** First, the optical density of the *Staph. aureus* was adjusted to 0.5 McFarland standard (1 to 2 × 10^8 cfu/ml) using a densitometer. A stock solution of SBH was prepared at a concentration of 70% w/w by dissolving 7 g of honey in 10 ml of tryptic soy broth (TSB). Next, the solution was filtered using 0.45μm filter to eliminate contaminating microorganisms before preparing serial dilutions of honey to achieve concentrations of 60%, 50%, 40%, 30%, 25%, 20%, 15%, 10% and 5% v/v.

In each 96-well plate, 190 μl of honey dilution was mixed with 10 μl of bacterial inoculum in triplicate for each dilution. A few control wells were prepared in triplicates which are 1) broth sterility control wells containing 200 μl of TSB alone, 2) viability control wells containing 190 μl of TSB and 10 μl of inoculum (without honey) and 3) dilution sterility controls containing 200 μl of the honey dilution in TSB (without inoculum). The plate was incubated overnight at 30 °C, and the absorbance of the wells was measured the following day at 590 nm. The percentage inhibition of bacterial growth was calculated using the formula in Equation 2:

$$Inhibition\ (\%) = 1 - \left(\frac{A_s - A_{dc}}{A_{vc} - A_{bc}}\right) \times 100 \quad (2)$$

Here, A_s is the absorbance of the test wells, A_{dc} is the absorbance of dilution sterility control, A_{vc} is the absorbance of viability control and A_{bc} is the absorbance of broth sterility control. The minimum value for the percent inhibition is 0% while the maximum value is 100%.

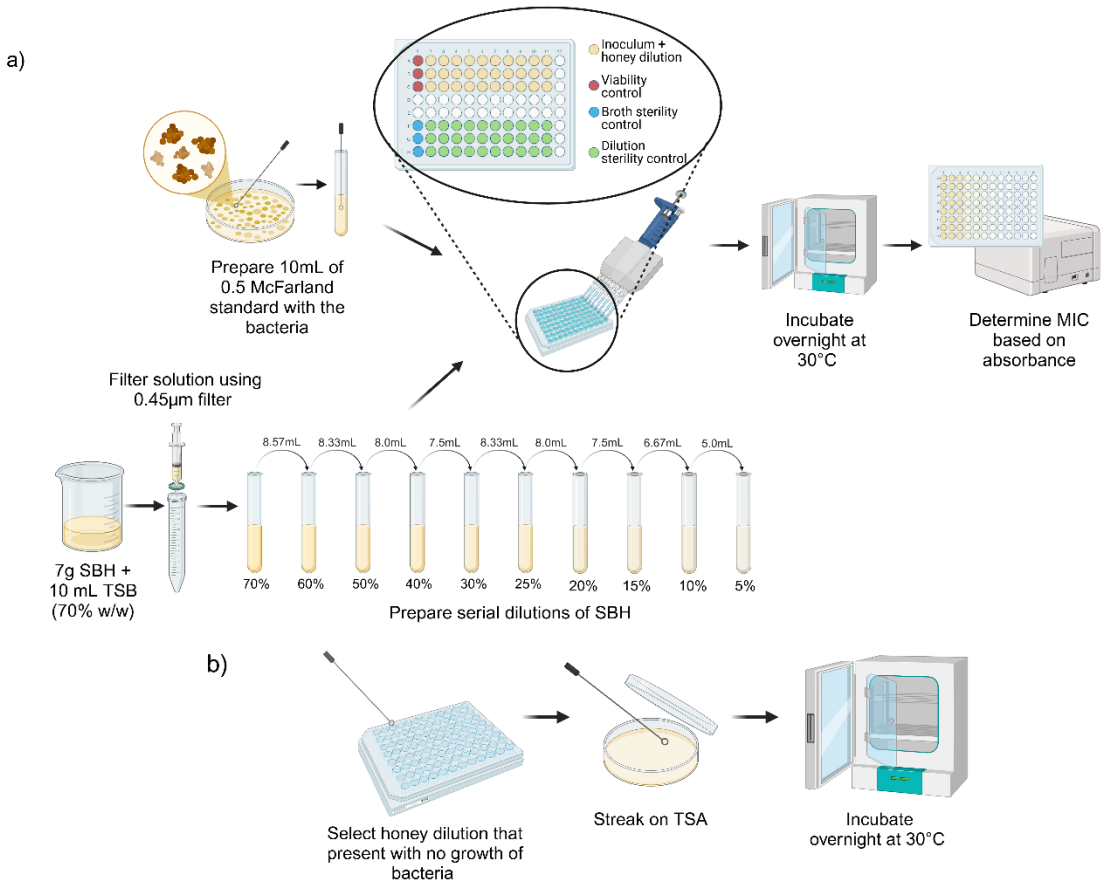

Fig. 1: Overview of (a) MIC and (b) MBC procedures

Minimum Bactericidal Concentration (MBC)

By using the streak plate method, honey dilution from each test well that showed no growth of bacteria in the MIC test was examined. A sterile wire loop was used to streak it onto the tryptic soy agar (TSA) plates. These plates were then incubated overnight at 30 °C. The minimal concentration of the diluted honey that showed no growth of the test inoculum (≥1%) was classified as the MBC (Zainol et al., 2013 as cited in Tuksitha et al., (2018).

Formulation of SBH Stick Deodorant

The ingredients were accurately weighed according to their respective percentages. Components in Phase A were combined and heated to 65 °C using a hot plate. The mixture was stirred with a magnetic stirrer until all components were fully melted and homogenised. Subsequently, the temperature was reduced to 45 °C before incorporating phase B ingredients. SBH added was

Table 1: Formulation of SBH stick deodorant

Phase	Ingredients	Function	w/w%
A	Cetostearyl alcohol	Emulsifier	15
	Olivem 1000	Emulsifier	10
	Isoamyl laurate	Emollient	17
	Beeswax	Thickening agent, binder	20
	Zinc ricinolate	Odour absorber	3
	Arrowroot powder	Thickening agent	14
B	Essential oils	Fragrance	0.8
	Vitamin E	Antioxidant	0.2
	SBH	Antibacterial	20*

* The honey added is based on the MIC value.

based on its MIC value (Komala et al., 2019), comprising 20% of the formulation, which is the lowest concentration of SBH required to effectively inhibit the growth of *Staphylococcus aureus*. This approach ensures optimal antimicrobial activity while minimising the use of SBH, thereby enhancing the cost-effectiveness. The mixture was then stirred for one minute. Finally, the prepared mixture was poured into containers and allowed to solidify at room temperature overnight. The formulation in Table 1 was labelled as F1. A blank of stick deodorant (F2), which had the same composition as F1, but did not contain SBH was also prepared to compare the antibacterial activity between the two deodorants.

Characterisation of Deodorant

Both F1 and F2 were characterised based on its pH, softening point, antimicrobial activity against *Staphylococcus aureus* and its stability at room temperature (30 °C ± 2 °C /75% RH ± 5%) for 14 days.

pH

The pH of the deodorant was measured to ensure it was suitable for application on underarm skin, which typically ranges from 4.0 to 6.8. First, 1% w/v sample was prepared by dissolving 1 g of stick deodorant into 100 mL of distilled water. The solution was heated to 40 °C and mixed using a vortex before being allowed to cool at room temperature. The pH was measured in triplicate using a calibrated pH meter (Insan & Vera, 2021).

Softening point

The softening point procedure was conducted following Debnath et al., (2011) method to determine the temperature at which the deodorant melted. The deodorant stick was cut in half lengthwise, mounted vertically in petri dishes, and placed in an oven. The temperature was then gradually increased until the sharp edges of the tip began to melt.

Antimicrobial Test

Antimicrobial test was conducted by following Debnath et al., (2011) with a few modifications. Antimicrobial test was conducted to make sure that the 20% of honey that was incorporated in the deodorant exhibits its antibacterial activities. First, 10% w/w of deodorant was prepared by dissolving 1 g of the deodorant into 9 mL distilled water. The optical density of the *Staphylococcus aureus* was adjusted to a 0.5 McFarland standard (1 to 2 × 10^8 cfu/ml) using a densitometer before swabbing it uniformly onto a nutrient agar plate with a sterile cotton swab. By using a borer, 0.5 cm wells were created in each plate and 0.15 mL of sample solution F1 and F2 was added into the well before incubating it at 37°C for 24 hours. The procedure was prepared in triplicate with amoxicillin/clavulanate as positive control. The inhibition zone was measured in triplicate and reported in centimetres (cm).

Stability test

Stability testing was conducted. A sample for each of the deodorant sticks, F1 and F2, was stored at room temperature at 30 °C ± 2 °C at 0, 7 and 14 days to measure the stability for both formulations. The room temperature conditions were controlled using an air-conditioned laboratory. The product was monitored for its organoleptic properties, including any signs of sweating, odour, changes in shape, colour, or separation of ingredients. If the stick lost its shape or exhibited the formation of oil droplets on the surface, it was deemed unstable.

Results and discussion

The antioxidant properties of SBH were assessed through the evaluation of its total phenolic content (TPC), flavonoid content, and DPPH scavenging activity and was demonstrated in **Table 2**.

Total phenolic content

The TPC of SBH was measured as 57.99 ± 0.3812 mg GAE/100g, indicating the presence of significant amounts of phenolic compounds, which were known for their antioxidant properties. The TPC value observed in this study was consistent with findings by Ya'akob et al. (2019), who reported that the phenolic content of SBH from eleven samples collected across different regions in Johor, Malaysia, ranged from 414.53 ± 3.166 mg GAE/kg to 778.23 ± 2.011 mg GAE/kg. Similarly, Ismail et al., (2021) reported TPC values for SBH derived from *Trigona sp* from Sabah and Kelantan ranged between 33.2 ± 1.2 to 60.2 ± 2.2 mg GAE/100 g. Thus, the TPC value in this study was expected to give positive effects in terms of antibacterial activity.

Table 2: Phenolic content and DPPH scavenging activity of SBH

Sample	Phenolic content		DPPH scavenging activity (%)
	TPC (mg GAE/100g)	TFC (mg QE/mL)	
SBH	57.99 ± 0.38	0.132 ± 0.04	66.78 ± 0.45

Note: The data are expressed as mean ± S.D. (n = 3)

Total flavonoid content

Meanwhile, the value of TFC for SBH in this study is 0.132 ± 0.04 mg QE/mL, which is considerably lower compared to the TFC values reported by Tuksitha et al. (2018), where three samples of SBH ranged from 12.41 ± 0.62 to 17.67 ± 0.75 mg QE/mL. However, the amount of TFC in the SBH is considered sufficient to provide antibacterial activity in this study.

DPPH assay

The DPPH scavenging activity of SBH in this study was measured at 66.78 ± 0.45%, which highlights the SBH's ability to scavenge free radicals. DPPH is a stable free radical. As the antioxidants in SBH donate their hydrogen atoms or electrons to DPPH, the free radical will reduce, resulting in the colour changes of DPPH from purple to yellow. This result was aligned with a study conducted by Mat Ramlan et al., (2021), which reported the percentage inhibition of eighteen samples of SBH from Malaysia and Australia approximately between 32.00% to 87.15%. However, this result was higher compared to Maringgal et al., (2019) and Tuksitha et al., (2018) which reported the percentage ranged between 2.77 ± 1.02% to 44.05 ± 11.04% and 17.0 ± 7.5% to 47.4 ± 3.2%, respectively. The variability of phenolic, flavonoid and antioxidant activities of SBH are due to factors such as bee species, geographical location, floral sources, and processing methods (Fatima et al., 2018; Shamsudin et al., 2019; Pimentel et al., 2021).

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The antimicrobial activity of SBH was then evaluated against *Staphylococcus aureus*, with the results presented in Fig. 2.

The MIC of SBH was found to be 20% w/w, indicating the lowest concentration required to completely inhibit visible bacterial growth of 1–2 × 10^8 cfu/mL. Meanwhile, the MBC was observed at 50% w/w, which represents the concentration necessary to eliminate bacterial growth at the same inoculum concentration.

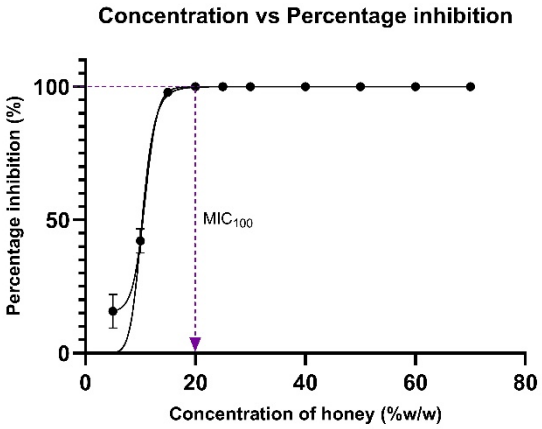

Fig. 2: Minimum Inhibitory Concentration (MIC) of SBH against *Staphylococcus aureus*.

In a study conducted by Mat Ramlan et al. (2021), it was reported that the MIC of three *Heterotrigona itama* honey samples from Malaysia and three *Tetragonula hockingsi* honey samples against *Staphylococcus aureus* ranged between 6–8% (w/w). These findings align with Sulaiman and Sarbon (2020), who reported a MIC of 6.25% (w/w), and Tuksitha et al. (2018), who reported that the MIC of SBH against *Staphylococcus aureus* ranged from 3% to 10% (w/w), while the MBC was slightly higher, ranged between 10% to 20% (w/w) across three different species of honey samples. As compared to the previous studies, the MIC and MBC levels for our honey sample are higher. This discrepancy can be attributed to the significantly higher bacterial density used in our study, which was 1–2 × 10^8 cfu/mL, compared to the bacterial density of 1 × 10^5 cfu/mL used in the previous studies. Thus, a higher concentration of honey is needed to effectively inhibit a higher concentration of *Staphylococcus aureus* growth, which results to higher MIC and MBC values.

According to studies, SBHs have stronger antibacterial properties than *Apis* honeybee honey (Rao et al., 2016; Zulkhairi Amin et al., 2018). SBH demonstrates considerable inhibitory effects against a broad spectrum of bacterial species, including gram-positive, gram-negative and multidrug-resistant strains (Ng et al., 2020; Tuksitha et al., 2018). The presence of its antibacterial activity may be influenced by a few factors. A review conducted by Nordin et al., (2018) revealed that SBH has high acidity due to its higher hydrogen ion in the honey composition, with pH ranges from 3.15 to 6.64. The

level of its acidity may be the contributor to bacterial fatality.

Other than that, studies correlate the antimicrobial properties with flavonoid content present in stingless bees, where flavonoids play a crucial role in antibacterial activity. These compounds disrupt membrane function and inhibit DNA synthesis, affecting the viability of pathogenic microorganisms. The antibacterial activity of flavonoids is closely linked to their chemical structure, particularly the hydroxy (OH) and methoxy (MeO) groups in phenolic rings. These structural components allow flavonoids to interact with bacterial proteins and membranes, leading to disruptions in the cell's structural integrity then, causing cell lysis and death (Komala et al., 2019). However, Biluca et al. (2016) reported no correlation between antimicrobial activity and antioxidant properties or phenolic compounds in honey samples. Similarly, Bueno-Costa et al. (2016) found no significant link between the TPC in honey from Rio Grande do Sul, Brazil, and its antibacterial activity against *Shigella dysenteriae, S. typhimurium, S. aureus*, and *Bacillus cereus* ($p > 0.05$).

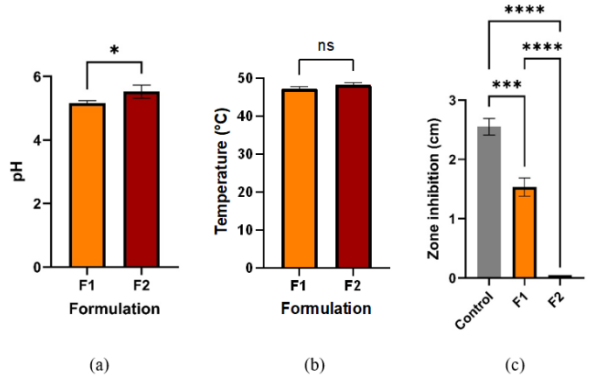

Fig. 3: (a) pH (b) Softening point (c) Zone of inhibition of stick deodorant against *Staphylococcus aureus.*

Formulation of SBH stick deodorant

The stick deodorant was subsequently formulated and characterized by evaluating key parameters, including its pH, softening point, and antimicrobial activity against *Staphylococcus aureus*, shown in **Fig. 3**. Additionally, a stability study was also conducted to assess its stability over time, as illustrated in **Table 2**.

During formulation process, SBH was added once the mixture had reached 45 °C. SBH are very sensitive to heat. In a study conducted by Mat Ramlan et al., (2021), SBH shows decreased antibacterial activities after being heated at 45 °C, 55 °C and 65 °C for an hour. However, another study conducted by Shahabuddin et al., (2022) reported that the antibacterial activity of SBH against *Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Salmonella typhimurium* remained comparable to controlled honey when it was exposed to heat, 50°C for 10 minutes. Thus, incorporating the SBH in stick deodorant at a temperature of 45 °C for 1 minute in this study may help preserve its antibacterial activity while minimising the negative impact of prolonged exposure to heat. This temperature was also used to make sure that the mixture was still in the liquid phase to facilitate the pouring process.

Characterisation of Deodorant

pH

The pH of underarm skin typically ranges from 4.0 to 6.8, which is slightly broader than the general skin pH of about 4.5 to 6.5 (Komala et al., 2019). From the results, it shows that both pH of deodorant formulation that contains honey and without honey are 5.17 ± 0.08 and 5.53 ± 0.21 respectively. The addition of honey in the formulation causes a statistically significant difference ($p < 0.05$) between pH of the two formulations. However, both remain compatible with the natural underarm pH range. Based on Costa & Horswill (2022), an acidic skin pH offers stronger defence against harmful bacteria, including *Staphylococcus aureus,* which contribute to the formation of body odour. The bacteria grow optimally at pH 7.5 but exhibit reduced growth between pH 5.0 and 6.0. Thus, it can be concluded that the acidic pH of both deodorant formulations, particularly F1, not only aligns with the natural underarm pH but may also contribute to inhibiting the growth of *Staphylococcus aureus*.

Softening point

The softening point of the deodorant in **Fig 3(b)** with honey is 47.3 ± 0.6 °C, while the deodorant without honey has a slightly higher softening point of 48.3 ± 0.6 °C. The addition of honey in the formulation does not significantly ($p > 0.05$) affect the softening point of the stick deodorant. However, compared to commercial stick deodorant, as reported by Debnath et al., (2011) with a softening point of 66 °C, the values are considerably lower. This difference suggests potential challenges during handling and storage, especially in hot climates places.

Antimicrobial test

The antimicrobial activity of the stick deodorant was evaluated again against *Staphylococcus aureus* to assess its effectiveness. The results showed F1, which contains 20% w/w of honey, exhibited a statistically significant zone of inhibition (1.53 ± 0.15 cm, $p < 0.0001$), while the blank deodorant, F2 showed no inhibition activity. This indicates that SBH ingredients can still show antibacterial activity after being incorporated into the deodorant. Meanwhile, amoxicillin and clavulanate, which act as positive control show the biggest zone of inhibition measuring 2.55 ± 0.14 cm. In comparison to F1, this difference was statistically significant ($p < 0.001$). This result was aligned with a study by Rosli et al., (2020), where the zone inhibition for SBH for eight species was reported between 10 ± 0.00 cm to 28 ± 0.58 cm.

Stability study

Table 2. Stability study of stick deodorant

Day	Organoleptic properties (Colour, odour, sweating, melting)	
	F1	F2
Day 0	 **Fig. 4:** Organoleptic properties of formulations F1 The colour is yellowish, has a good rose smell, no visible signs of sweat on the surface, and in a solid form	 **Fig. 5**: Organoleptic properties of formulations F2 The colour is yellowish, has a good rose smell, no visible signs of sweat on the surface, and in a solid form
Day 7	No changes	No changes
Day 14	No changes	No changes

Based on the results shown in Table 2, the stick deodorant for both formulations remained stable during the two-week observation period at room temperature. The colour remained yellowish with rose aroma attributed to the beeswax and fragrance composition in the formulation. There were no visible signs of sweating or melting as they remained in solid form. The absence of sweating and phase separation indicates that the formulations were effectively emulsified and structurally stable.

Conclusion

The SBH stick deodorant was successfully formulated and demonstrated potential antibacterial activity against *Staphylococcus aureus*, a known body-odour-causing bacteria. For future studies, testing the deodorant on human participants is recommended to evaluate its practicality and effectiveness. Additionally, a roll-on deodorant could be considered instead of stick deodorant as it would avoid the heating process during production, which helps retain the natural properties of SBH. Lastly, the formulated deodorant can also be compared with commercially available deodorants to evaluate its suitability in the market further.

Authors contributions

Study design, S.A.N.Z. and M.M.D.M. Direction and Coordination, M.S.H. Investigation, S.A.N.Z. Resources M.S.H and S.M.R. Writing-Original Draft, S.A.N.Z. Writing-Review and Editing M.S.H., S.M.R., A.F.H.I and S.A.N.Z. Project Administration M.S.H. and S.A.N.Z.

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Conflict of interest

The authors declare that there is no conflict of interest in writing this manuscript.

Declaration of generative AI and AI-assisted technologies in the writing process

ChatGPT was used to improve readability and language. The author then review and edit the content as needed. Turnitin was used to check plagiarism for this study.

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Green Synthesis of Silver Nanoparticles Using *Aidia densiflora* Leaf Extract: Characterisation and Bioactivities

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Abstract

Introduction: Plant-mediated green synthesis of nanoparticles has become a promising option in green nanotechnology because it is simple, cost-effective, eco-friendly, and biologically effective. This study focused on the synthesis and characterisation of silver nanoparticles (AgNPs) using *Aidia densiflora* leaf extract, as well as the evaluation of their antimicrobial and cytotoxic activities. **Methods:** AgNPs were synthesised with *A. densiflora* leaf extract and their formation was confirmed using an ultraviolet–visible (UV–Vis) spectrophotometer. Liquid chromatography-mass spectrometry quadrupole time-of-flight was utilised for phytochemical profiling. The synthesised AgNPs were characterised using a zetasizer and zeta potential analyser, scanning electron microscopy-energy dispersive X-ray, Fourier Transform Infrared, X-ray diffraction (XRD), and thermogravimetric analysis. Antimicrobial activity of AD-AgNPs was tested against six microorganisms using the disc diffusion method, while cytotoxicity against MCF-7 human breast cancer cells was evaluated via MTT assay. **Results:** AgNP formation was confirmed by XRD and UV–Vis analysis, with absorbance peaks at 399–424 nm. Optimal synthesis was achieved using 10 mM $AgNO_3$ at 60°C and pH 7. SEM showed spherical-like nanoparticles averaging 96.06 nm with significant aggregation. The zeta potential was –35.6 mV, and XRD indicated a face-centred cubic structure with a crystalline size of 6.94 nm. AD-AgNPs showed no antimicrobial activity and low cytotoxicity. **Conclusion:** *A. densiflora* leaf extract can be used to synthesise AgNPs, however, further optimisation is required for better nanoparticle stabilisation and improvement of bioactivities.

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Introduction

Nanotechnology has gained significant attention as a new research area due to its diverse applications in biomedicine, pharmaceuticals, electrochemistry, catalysis, food technology, sensors, cosmetics, and more. Nanoparticles are atomic- or molecular-scale solid particles with a size of <100 nm (Vanlalveni et al., 2021). The conversion of bulk materials to nanoparticles has demonstrated the enhancement of the properties of the parent materials due to high atomic interactions, owing to their large surface area-to-volume ratio (Singh et al., 2018). several metallic nanoparticles, such as silver nanoparticles (AgNPs), have gained significant momentum for their potential as antimicrobial agents. AgNPs have proven to be very useful, especially in the biomedical field for their antimicrobial, antibiofilm, antifungal, antiparasitic, antioxidative, and anticancer activity. Their applications have been found in topical ointments, wound dressings, ultrasound gels, bone cements, surgical implants, and medical devices because of their antimicrobial nature (Velidandi et al., 2020).

Nanoparticles have been more commonly synthesised using physical and chemical methods, which require expensive equipment, use toxic chemicals, and release hazardous by-products. The production of nanoparticles through physical and chemical methods can be toxic to both humans and the environment, which also limits their applications (Akhter et al., 2024). Therefore, researchers have developed new approaches to green synthesis of nanoparticles by utilisation of biologically friendly elements such as bacteria, viruses, yeasts, plant extracts, fungi, and algae (Singh et al., 2018). In comparison to the various green synthesis methods for metal and metal oxide nanoparticles, plant extract-based synthesis is a simpler, more efficient, and scalable approach compared to microbial methods (Singh et al., 2018). Biomolecules in plant extracts, such as polyphenols, amino acids, vitamins, enzymes, and proteins, play an important role as both reducing and stabilising agents in the stages of nanoparticle synthesis (Asif et al., 2022). Numerous studies have been conducted on the synthesis of AgNPs using various plant extracts; however, no research has yet been conducted on *Aidia densiflora* in this area.

To date, no studies have explored the phytochemical constituents, antioxidant properties, or bioactive compounds of *A. densiflora*. However, research on related species within the same genus had demonstrated the presence of various phytochemical compounds, such as alkaloids, triterpenoids, flavonoids, and coumarins, which can potentially act as reducing and stabilising agents in AgNP synthesis (Anokwah et al., 2021; Awang-Jamil et al., 2019). Additionally, *A. densiflora* grows abundantly across Malaysia, making it readily available and cost-effective for this research. This study aimed to synthesise AgNPs using *A. densiflora* leaf extract, characterise the nanoparticles, and evaluate their antimicrobial and cytotoxic activities. The antimicrobial efficacies were assessed against two Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*), two Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*), and two fungi (*Candida albicans* and *Aspergillus niger*) using the disc diffusion method, while the cytotoxicity study was conducted on MCF-7 (human breast cancer cells) using the MTT assay.

Materials and Methods

Materials

Fresh leaves of *Aidia densiflora* (Wall.) Masam (Fig. 1) were collected from Kuantan, Pahang, Malaysia in October 2024 and identified by Dr. Shamsul Khamis. The plant specimen was then deposited in the Herbarium of the Kulliyyah of Pharmacy, IIUM Kuantan, Malaysia. The test microorganisms included *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Candida albicans,* and *Aspergillus niger* obtained from ATCC, US. MCF-7, human breast cancer cells also obtained from ATCC, US. Silver nitrate ($AgNO_3$, EMSURE®, Merck, analytical grade), dimethyl sulfoxide (DMSO, EMSURE®, Merck, analytical grade), ethanol (EMSURE®, Merck, analytical grade), methanol (EMSURE®, Merck, analytical grade), sodium hydroxide (NaOH, R&M Chemicals), 3-(4,5-Dimethylthiazol-2-

yl)-2,5-diphenyltetrazolium bromide (MTT reagent, Molecular Probes), tamoxifen citrate (Calbiochem, Merck), econazole nitrate (Dr. Ehrenstorfer), kanamycin sulphate (Sigma-Aldrich), amoxicillin/clavulanic acid disc (Oxoid, Thermo Fisher) were used as chemical reagents.

Dulbecco's Modified Eagle's Medium (DMEM, Gibco), TrypLE Express (Gibco), Fetal Bovine Serum (FBS, Gibco), Penicillin-Streptomycin (Nacalai Tesque, Kyoto, Japan), phosphate-buffered saline (PBS, Sigma-Aldrich), Tryptic Soy Agar and Tryptic Soy Broth (TSA, TSB, Merck), Nutrient Agar and Nutrient Broth (NA, NB, Merck), Sabouraud Dextrose Agar and Sabouraud Dextrose Broth (SBA, SDB, Merck) were used for microbial and cytotoxic studies. The instruments used included a ultraviolet–visible spectrophotometer (Shimadzu UV-1800), LC-MS/QTOF (Agilent 1200 LC system coupled with a 6520 QTOF mass spectrometer, Agilent Technologies), FTIR spectrometer (PerkinElmer Dual), scanning electron microscope with energy dispersive X-ray (SEM, JEOL / JSM-IT200), zetasizer nano (Malvern ZN1600 Nano ZS), X-ray diffractometer (XRD, ULVAC-PHI / PHI 5000 VersaProbe II), thermogravimetric analysis (TGA, Hitachi / STA7000), high-speed centrifuge (Supra 22K, Hanil Science Industrial), microplate reader (Azure Biosystems), rotary evaporator (BÜCHI Rotavapor R-300) and sonicator (Qsonica). Other apparatus included were Petri dish (90 mm × 15 mm), CO_2 incubator (BB15, Thermo Fisher, 5% CO_2, 37°C) and 96-well flat-bottom tissue culture plates (Falcon, Becton Dickinson).

Method

Collection of plant material and extraction

Fresh *A. densiflora* leaves were dried at 40°C and ground into powder. 50 g of the dried powder was mixed with 500 mL of 80% ethanol (Eze et al., 2019) and extracted using ultrasonic-assisted extraction (UAE) with a probe sonicator at 50 kHz for 30 minutes. The extract was filtered through filter paper, followed by 0.45 µm and 0.22 µm syringe filters, and stored at 4°C.

Fig. 1: *A. densiflora* plant collected from Kuantan, Pahang, Malaysia.

Liquid chromatography-mass spectrometry time of flight (LC-MS/QTOF) analysis

The *A. densiflora* leaf extract was dried using a rotary evaporator at 50°C, diluted to 1 mg/mL in methanol, and filtered through a 0.22 µm syringe filter before injection. Chromatographic separation was conducted on an Agilent ZORBAX Eclipse Plus C18 column (2.1 × 100 mm, 1.8 µm) at 40°C. The mobile phase consisted of 0.1% formic acid in deionized water (A) and 0.1% formic acid in acetonitrile (B), with a gradient elution over 30 minutes at a 0.25 mL/min flow rate. The mass spectrometer operated in positive ESI mode with a gas temperature of 325°C, gas flow of 11 L/min, and nebulizer pressure of 35 psi. Data analysis was performed using Agilent Mass Hunter Qualitative Analysis B.05.00 software based on accurate mass measurements.

Synthesis of Aidia densiflora-silver nanoparticle (AD-AgNP)

AgNPs were synthesised at a 1:9 ratio (extract solution: $AgNO_3$), as a previous optimisation study by Jalab et al. (2021) demonstrated effective AgNPs formation at this ratio. 10 mL of plant extract was added dropwise to 90 mL of 1-, 5-, and 10-mM silver nitrate ($AgNO_3$) solution under constant stirring at 60°C for 2 hours. The formation of AgNPs was monitored through periodic sampling using a UV spectrophotometer. As reported by Alharbi et al. (2022), numerous studies have shown that AgNPs production increases with higher pH levels, with the highest yield achieved at a pH range of 7–9. Therefore, the sample with the highest absorbance peak among the three $AgNO_3$ concentration

variable was used to further investigate the effect of different pH levels on AgNPs formation. The same method was applied, keeping other variables constant. After the 2 hours synthesis, the mixture was adjusted to pH levels of 7, 8, and 9 by adding 1 M NaOH dropwise and stirred for another 30 minutes at room temperature. The final mixture was centrifuged at 8000 rpm for 10 minutes at 21°C. The pellet was rinsed by removing the supernatant, adding distilled water, and centrifuging again. This rinsing step was repeated three times.

Characterisation of AD-AgNPs

Ultraviolet–visible (UV-Vis) spectrophotometric analysis

During the reaction, 1 mL of the sample solution was analysed using a UV-Vis spectrophotometer (Shimadzu UV-1800) to confirm AgNPs formation. Wavelength and absorbance were recorded within a 300–800 nm scanning range, with distilled water as the blank. Samples were appropriately diluted to ensure absorbance readings fell within the optimal range.

Particle size and zeta potential analysis

The size, polydispersity index (PDI), and zeta potential value of the synthesised AgNPs was determined using a zetasizer instrument (Malvern ZN1600 Nano ZS).

Scanning electron microscopy-energy dispersive X-ray (SEM-EDX) analysis

The semi-solid AD-AgNPs were deposited on a glass slide, dried, and gold-coated using a vacuum sputter coater. The morphology, size, and elemental composition of the synthesised AD-AgNPs were examined using an SEM-EDX analyser (JEOL /JSM-IT200) at 20 kV. This method was retrieved from Sundar et al. (2024).

X-ray diffractometer (XRD) analysis

XRD analysis was conducted to assess the crystallinity, phase, and average crystallite size of AD-AgNPs using XRD (ULVAC-PHI / PHI 5000 VersaProbe II). The analysis was performed using CuKα radiation (λ = 1.5405 Å) over a 2θ range of 20°–100°, with an accelerating voltage of 45 kV and an applied current of 40 mA. The average crystallite size (D) was calculated using the Debye-Scherrer equation: $D = K\lambda/\beta \cos \theta$, where K is the Scherrer constant (0.9), λ is the X-ray wavelength (1.5405 Å), β is the full width at half maximum (FWHM) in radians, and θ is the diffraction angle.

Thermogravimetric analysis (TGA)

The dried powder of the AD-AgNPs was subjected to TGA using TGA (Hitachi / STA7000) under a nitrogen atmosphere with a flow rate of 10 mL/min. The sample was heated from room temperature to 700 °C at a heating rate of 10 °C/min.

Fourier Transform Infrared (FTIR) analysis

FTIR analysis was conducted to identify functional groups and biomolecules involved in AgNPs synthesis. Spectra were recorded using FTIR spectrometer (PerkinElmer Dual) in the range of 4000–400 cm^{-1} in Attenuated Total Reflectance (ATR) mode.

Antimicrobial assay

The antimicrobial activity of AD-AgNPs was assessed using the disc diffusion assay against two Gram-positive bacteria (*B. subtilis*, *S. aureus*), two Gram-negative bacteria (*E. coli*, *P. aeruginosa*), and two fungi (*C. albicans*, *A. niger*). Bacteria were inoculated on nutrient agar and incubated at 37 °C for 24 hours, while *C. albicans* and *A. niger* were cultured on TSA and Sabouraud dextrose agar at 35 °C for 5 days. The bacteria were then subcultured in nutrient broth, *C. albicans* in TSB, and *A. niger* in Sabouraud dextrose broth under the same conditions. The turbidity of all cultures was standardised to ~1.5×10^8 CFU/mL by adjusting UV absorbance at 625 nm to 0.08–0.1 (Arsène et al., 2023; Karu et al., 2020; Safarpoor et al., 2018)

Test samples included 10 mg/mL and 5 mg/mL AD-AgNPs, 5 mg/mL *A. densiflora* leaf extract, 5 mg/mL $AgNO_3$, and distilled water as a negative control. All samples were sterilised using a 0.22 μm syringe filter. Sterile discs were immersed in the samples and placed on the plates (Safarpoor et al., 2018). Amoxicillin-clavulanate and econazole discs were used as positive controls for antibacterial and antifungal assays, respectively (Pallavi et al., 2022; Safarpoor et al., 2018). Plates were incubated at 37 °C for 24 hours (bacteria) and at 35 °C for 5 days (fungi), after which the zone of inhibition (ZOI) was

measured in mm. The assay was conducted in duplicate.

Cytotoxic activity

The MTT assay was performed to evaluate the cytotoxic activity of AD-AgNPs and $AgNO_3$ against MCF-7 cells. The cells were first cultured in tissue culture flasks with complete medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin, then incubated at 37°C in a 5% CO_2 atmosphere. Once the cells reached 80% confluence, trypsinisation was performed to detach them from the flasks. Subsequently, the cells were seeded into a 96-well plate at a density of 2×10^4 cells/well and incubated for 24 hours. Following incubation, the cells were treated with serially diluted AD-AgNPs, $AgNO_3$, and tamoxifen (positive control) at concentrations of 5, 2.5, 1.25, 0.625, and 0.312 mg/mL, then further incubated at 37°C in 5% CO_2 for 24 hours. Afterwards, 10 µL of MTT solution (5 mg/mL) and 90 µL of complete medium were added to each well, and the plate was re-incubated for 4 hours. In this step, viable cells with active mitochondria converted MTT into purple formazan crystals. To dissolve the crystals, 100 µL of dimethyl sulfoxide (DMSO) was added, followed by an additional 30-minute incubation in the CO_2 incubator.

Absorbance was measured at 570 nm using a microplate reader. Higher absorbance indicated greater cell viability (lower cytotoxicity), whereas lower absorbance suggested reduced viability (higher cytotoxicity). The percentage of cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{(\text{OD of treated cells})}{(\text{OD of control cells})} \times 100 \qquad (1)$$

OD = optical density

This method was adapted from Fadhillah et al. (2024).

Statistical analysis

Statistical analysis was conducted using Jamovi. Data from the tests were reported as mean ± standard deviation (SD). One-way ANOVA was performed to determine significant differences, with $p < 0.05$ considered statistically significant.

Results and Discussion

LC-MS/QTOF based profiling of A. densiflora extract

Fig. 2 presents the LC-MS profile of *A. densiflora* leaf extract, identifying 99 compounds, with 12 considered major (≥1% volume).

Fig. 2 presents the LC-MS profile of *A. densiflora* leaf extract, identifying 99 compounds, with 12 considered major (≥1% volume). Among them, only 4 compounds are known, while the remaining 8 remain unidentified as presented in **Table 1**. According to Nguyen et al. (2023), active phytochemicals such as phenolics, terpenoids, flavones, alkaloids, amino acids, polysaccharides, and alcoholic compounds may contribute to the reduction of AgNPs by donating electrons to convert Ag^+ to Ag^0. Similarly, Zuhrotun et al. (2023) highlighted the role of polyphenols as the reducing and stabilising agents in AgNPs formation. Among the major compounds detected, the polyphenolic compounds epifisetinidol-4α-ol, 1,3,4,5-tetracaffeoylquinic acid, and proanthocyanidin A5′ are likely key contributors to AgNP formation. Additionally, minor constituents in the extract (≤1% volume) may further support the synthesis process, either by enhancing the reduction efficiency or improving nanoparticle stability.

Characterisation of AD-AgNPs

Visual observation

The addition of *A. densiflora* extract to the $AgNO_3$ solution resulted in colour changes, indicating the formation of AgNPs. As shown in **Fig. 3**, the extract initially exhibited a deep green colour (a), which turned light yellow (b) upon mixing with $AgNO_3$. Over time, the solution gradually changed to brown (c), becoming darker as the reaction progressed. This change suggests the reduction of Ag^+ ions and the excitation of surface plasmon resonance (SPR). Further adjustment of the pH using sodium hydroxide resulted in a deep brown colour (g), indicating potential effects on nanoparticle stability and growth. These observations align with previous studies by Liaqat et al. (2022) and Asif et al. (2022), that correlate colour changes with AgNPs synthesis.

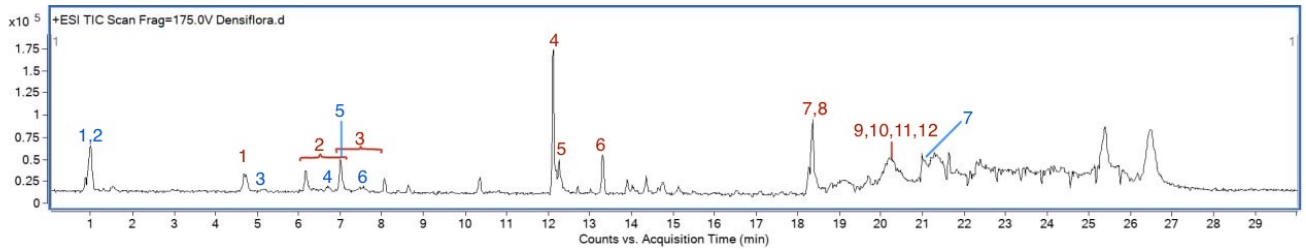

Fig. 2. LC-MS profile of *A. densiflora* leaf extract. Chromatographic separation was conducted on an Agilent ZORBAX Eclipse Plus C18 column (2.1 × 100 mm, 1.8 µm) at 40°C. The mobile phase consisted of 0.1% formic acid in deionized water (A) and 0.1% formic acid in acetonitrile (B), with a gradient elution over 30 minutes at a 0.25 mL/min flow rate. The mass spectrometer operated in positive ESI mode with a gas temperature of 325°C, gas flow of 11 L/min, and nebulizer pressure of 35 psi. Compounds are labeled based on their relative abundance: Red labels: major compounds with a percentage

Table 1: Compounds identified in LC-MS analysis.

No.	RT	Name of Compound	Formula	Mass	Volume (%)	Classification
1.	0.957	D-Sorbitol	$C_6H_{14}O_6$	182.0794	0.35	Sugar alcohol
2.	0.991	Quinic acid	$C_7H_{12}O_6$	192.0633	0.25	Polyphenol
3.	4.702	Epifisetinidol-4α-ol	$C_{15}H_{14}O_6$	290.0778	2.04	Flavonoid (Polyphenol)
4.	5.137, 6.152	Epicatechin-(4β→6)-epicatechin-(2β→7,4β→8)-epicatechin	$C_{45}H_{36}O_{18}$	864.1879, 864.1914	0.60	Proanthocyanidin
5.	6.166, 6.761, 6.995, 7.559	Proanthocyanidin A5'	$C_{30}H_{24}O_{12}$	576.1258, 576.1245, 576.1259, 576.1247	4.49	Proanthocyanidin (polyphenol)
6.	6.404, 6.533	Butein 4'-arabinosyl-(1→4)-galactoside	$C_{26}H_{30}O_{14}$	566.1616, 566.1630	0.17	Flavonoid glycoside
7.	6.993	6-(3,4-Dihydroxyphenyl)-6a,12b-dihydro-3,10,11,12-tetrahydroxy-[2]benzopyrano[3,4-c]benzopyran-8(6H)-one	$C_{22}H_{16}O_9$	424.0783	0.14	Flavonoid
8.	6.711, 6.918, 7.470, 7.638, 7.993	1,3,4,5-Tetracaffeoylquinic acid	$C_{43}H_{36}O_{18}$	840.1905, 840.1909, 840.1925, 840.1893, 840.1889	1.00	Chlorogenic acid derivative, polyphenol
9.	7.363	Lippioside I	$C_{25}H_{30}O_{13}$	538.1684	0.12	Terpenoid glycoside
10.	12.107	C16 Sphinganine	$C_{16}H_{35}NO_2$	273.2665	7.50	Sphingolipid / Glycoside
11.	12.256	Unknown compound 1	-	317.2913	1.58	-
12.	13.302	Unknown compound 2	-	386.1691	2.24	-
13.	18.346	Unknown compound 3	-	278.1503	3.02	-
14.	18.349	Unknown compound 4	-	148.015	2.48	-
15.	20.066	Unknown compound 5	-	657.5013	1.08	-
16.	20.136	Unknown compound 6	-	596.4487	1.68	-
17.	20.207	Unknown compound 7	-	569.4489	1.59	-
18.	20.266	Unknown compound 8	-	508.3963	1.22	-
19.	21.139	Momordicoside K	$C_{37}H_{60}O_9$	648.4217	0.07	Triterpenoid saponin

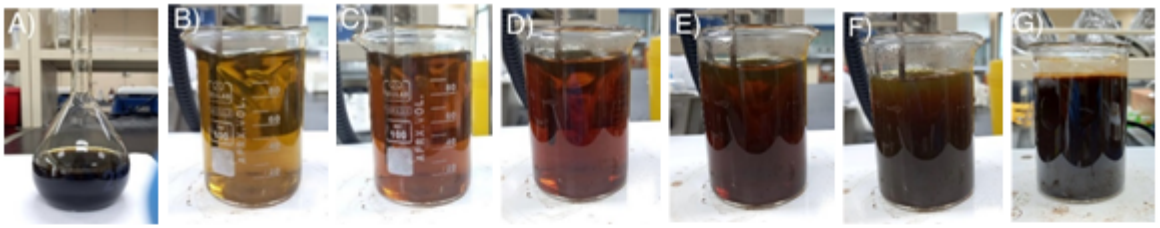

Fig. 3: (a) *A. densiflora* leaf extract. AD-AgNPs at (b) 10 min, (c) 20 min, (d) 30 min, (e) 1 h, (f) 2h, and (g) after pH adjustment.

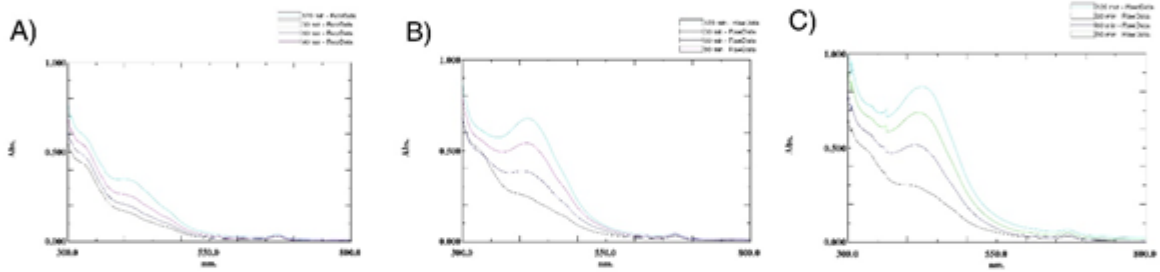

Fig. 4: Absorbance peaks of AD-AgNPs synthesised with (a) 1, (b) 5, and (c) 10 mM AgNO over time.

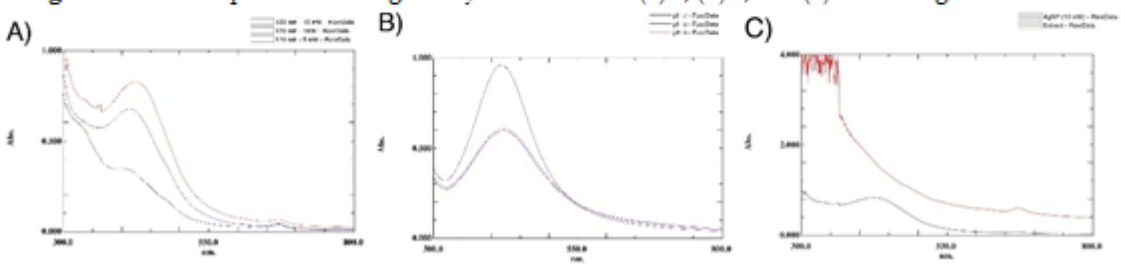

Fig. 5: (a) Absorbance peaks of AD-AgNPs synthesised with 1, 5, and 10 mM $AgNO_3$. (b) Absorbance peaks of AD-AgNPs synthesised at pH 7, 8, and 9. (c) Absorbance peaks of AD-AgNPs and plant extract.

UV–vis analysis

The formation of synthesised AD-AgNPs was further confirmed using UV–visible spectroscopy. Fig. 4 and 5 shows that peak absorbance increases as the reaction time increases. The characteristic peaks of AD-AgNPs synthesised with 1, 5, and 10 mM $AgNO_3$ after 2 hours synthesis (Fig. 4) were observed at wavelengths between 399–424 nm. This confirms the formation of AgNPs, as the SPR peak of AgNPs is typically falls within the 400–450 nm range (Sukweenadhi et al., 2021). The absorbance readings between the three samples of varying $AgNO_3$ concentration indicate that the sample synthesised with 10 mM $AgNO_3$ exhibits the highest absorbance peak, suggesting the highest yield compared to those synthesised with 1 and 5 mM $AgNO_3$. Consequently, 10 mM $AgNO_3$ was selected to investigate the effects of different pH levels on AgNP synthesis.

Fig. 5 (b) presents the absorbance peaks of AD-AgNPs synthesised at pH 7, 8, and 9. The sample synthesised at pH 7 showed the highest absorbance of 0.961 compared to pH 8 and 9 with absorbance of 0.593 and 0.604 respectively. Additionally, the absorbance peaks for pH 8 and 9 slightly decreased 30 minutes after pH adjustment, suggesting potential aggregation at higher pH levels. These findings were also observed in a previous study by Liaqat et al. (2022), where pH 7 was found to be the optimum pH for AgNP formation, and agglomeration was observed at very basic pH levels.

Furthermore, **Fig. 4** shows all samples exhibited two peaks: one within the 399–424 nm wavelength range, which is the characteristic peak of AgNP, and another minor peak between 611–671 nm, which may correspond to compounds in the *A. densiflora* extract. **Fig. 5 (c)** compares the UV peaks of the *A. densiflora* extract and AD-AgNPs, further confirming that the peak observed at ~600 nm originates from compounds in the extract.

Particle size, and zeta potential analysis

Fig. 6 shows the size distribution of AD-AgNPs synthesised at varying $AgNO_3$ concentrations and pH levels, while **Fig. 7** presents their corresponding zeta potential measurements. As the $AgNO_3$ concentration increased, the particle size and polydispersity index (PDI) value decreased. According to H. B. Kim et al. (2024), a PDI value closer to 1 indicates polydisperse and aggregated particles, while a PDI between 0.1 and 0.4 suggests uniform size and low aggregation.

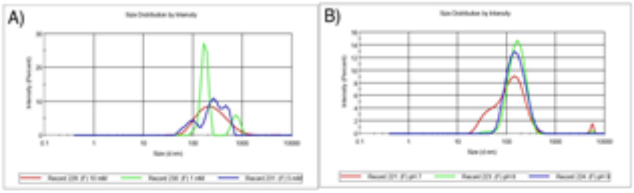

Fig. 6: Size distribution analysis of AD-AgNPs: (a) at different $AgNO_3$ concentrations and (b) at different pH levels.

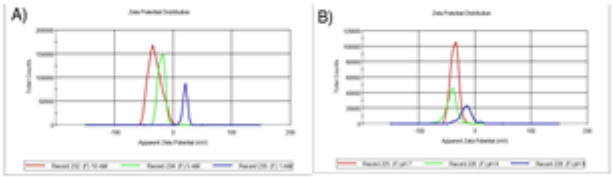

Fig. 7: Zeta potential analysis of AD-AgNPs: (a) at different $AgNO_3$ concentrations, and (b) at different pH levels.

Among all samples, AD-AgNPs synthesised at pH 9 exhibited the smallest particle size (139.8 nm) with a uniform distribution (PDI = 0.164), and the highest stability (-41.5 mV), as shown in **Table 2**. According to Liaqat et al. (2022), a minimum zeta potential of ±30 mV is required for a stable nanosuspension. The negative zeta potential suggests effective capping by phytocompounds present in the plant extract, leading to electrostatic repulsion between particles, which prevents agglomeration.

By comparing particles size and zeta potential measurements, it can be concluded that AD-AgNPs synthesised with 10 mM $AgNO_3$ at pH 9 exhibit the highest stability. However, at pH 7, the highest absorbance peak was observed in UV-Vis analysis, suggesting a high nanoparticle yield, despite the slightly larger particle size (153.1 nm), higher PDI (0.311), and lower zeta potential (-35.6 mV) compared to pH 9.

Table 2: Particle size and zeta potential of AgNPs.

Variables	Z-Average (nm)	PDI	Zeta potential (mV)
1 mM	1652	1.000	-17.1
5 mM	629.5	0.591	-19.9
10 mM	198.1	0.271	-31.0
pH 7	153.1	0.311	-35.6
pH 8	177.2	0.308	-18.3
pH 9	139.8	0.164	-41.5

SEM and EDX analysis

Among the tested samples, AD-AgNPs synthesised at pH 7 were selected for further analysis, as they exhibited the highest absorbance in UV spectroscopy analysis, while maintaining a fair

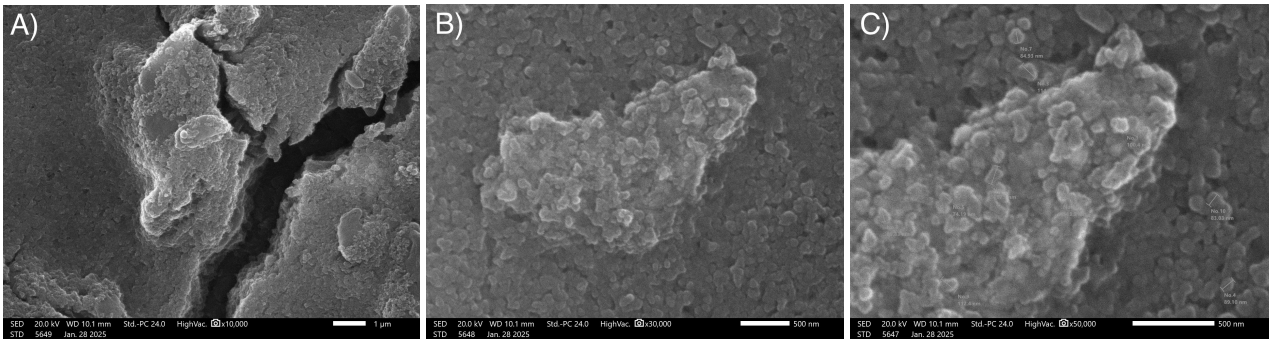

Fig. 8: SEM profile of AD-AgNPs at (a) 10 000, (b) 30 000, and (c) 50 000 magnifications.

particle size and good stability.

SEM profiles of AD-AgNPs under different magnifications are depicted in **Fig. 8.** The images show that AgNPs have a spherical-like shape with an average size of 96.06 nm but appear highly aggregated. Although the synthesised AgNPs are considerably small and within the nano range, the significant aggregation has led to the formation of larger structures. These findings suggest that while *A. densiflora* leaf extract demonstrates strong reducing capabilities for AgNP synthesis, its stabilising properties appear to be insufficient, leading to nanoparticle aggregation.

EDX analysis identified the purity, and the complete chemical composition of elements present in AD-AgNPs, as shown in **Fig. 9**. The analysis revealed the significant proportions of silver (68.25%) alongside oxygen (31.75%). The presence of oxygen is likely due to the oxidation of AgNPs or the strong binding of organic compounds from *A. densiflora* extract, capping the nanoparticle surface (Femi-Adepoju et al., 2019).

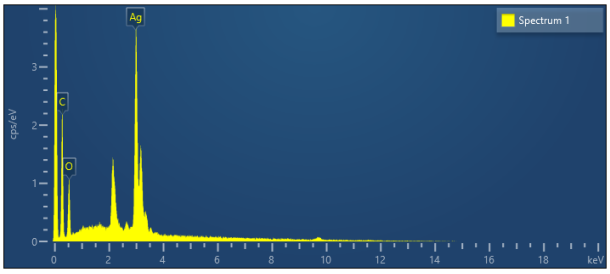

Fig. 9: EDX profile of AD-AgNPs with percentage weight of each element.

XRD analysis

The XRD spectrum (**Fig. 10**) revealed distinct peaks at 2θ angles of 38.14°, 44.07°, 64.49°, 77.31°, and 81.56°, corresponding to the (111), (200), (220), (311), and (222) lattice planes of the face-centred cubic (FCC) structure of AgNPs. These findings align with previously reported values for crystalline AgNPs (Lanje et al., 2010; Revathi et al., 2024) confirming the crystalline nature of AD-AgNPs. The crystallite sizes were calculated, and the results are summarised in **Table 3**, with an average size of 6.94 nm.

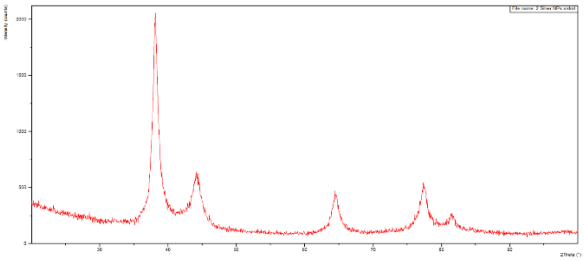

Fig. 10: The XRD spectrum revealed distinct peaks at 2θ angles of 38.14°, 44.07°, 64.49°, 77.31°, and 81.56°, corresponding to the (111), (200), (220), (311), and (222) lattice planes of the face-centred cubic (FCC) structure of AgNPs.

Table 3: The crystallite size of AD-AgNPs.

2θ [°]	hkl	FWHM Left [°2θ]	d-spacing [Å]	D (nm)
38.1356°	111	0.9079	2.35791	9.67
44.0680°	200	1.7884	2.05327	5.00
64.4886°	220	1.2590	1.44378	7.79
77.3120°	311	1.6769	1.23318	6.34
81.5649°	222	1.8629	1.17929	5.88
		$D_{average}$ (nm)		6.94

TGA

The TGA profile **(Fig. 11)** shows that AD-AgNPs exhibited a slight weight loss from room temperature to 100°C, which may be attributed to the evaporation of surface moisture. The sample then gradually degraded, and by 290°C, 50% of the it had already decomposed. A significant weight loss was observed starting at 300°C, with complete decomposition occurring at 371°C. Previous studies have associated weight loss between 100–350°C with the degradation of organic biomolecules, such as phenolics and flavonoids, which serve as capping agents on AgNPs (David & Moldovan, 2020). The melting point of pure silver is reported to be 960.54°C (Sampaio & Viana, 2018). However, the

complete degradation of AD-AgNPs observed at 371°C suggests a few possible explanations.

Firstly, AD-AgNPs may contain only a minimal amount of pure silver, with an excessive presence of organic capping molecules. Moreover, this analysis was performed using a limited sample size, which may have influenced the results. Given the already small initial weight and the minimal amount of pure silver, any residual mass may have been below the detection limit of the instrument. Therefore, for more accurate quantification, future TGA experiments should be conducted with a larger sample size.

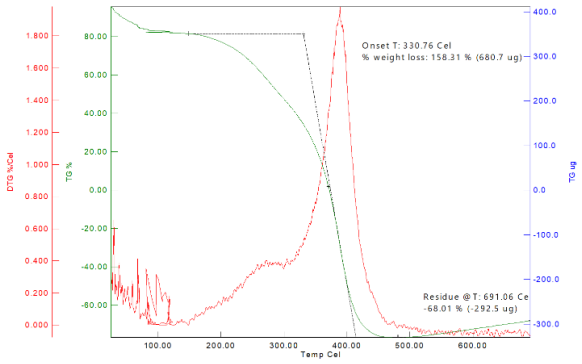

Fig. 11: TGA graph of AD-AgNPs. The initial sample weight was 0.43 mg, heated from room temperature to 700 °C at a heating rate of 10 °C/min.

FTIR analysis

FTIR analysis was carried out to identify the functional groups responsible for AgNP formation by examining shifts in spectral bands between the plant extracts and the synthesised AgNPs (Dhaka et al., 2023). **Fig. 12** presents a comparison of the IR spectra of *A. densiflora* leaf extract and synthesised AgNPs.

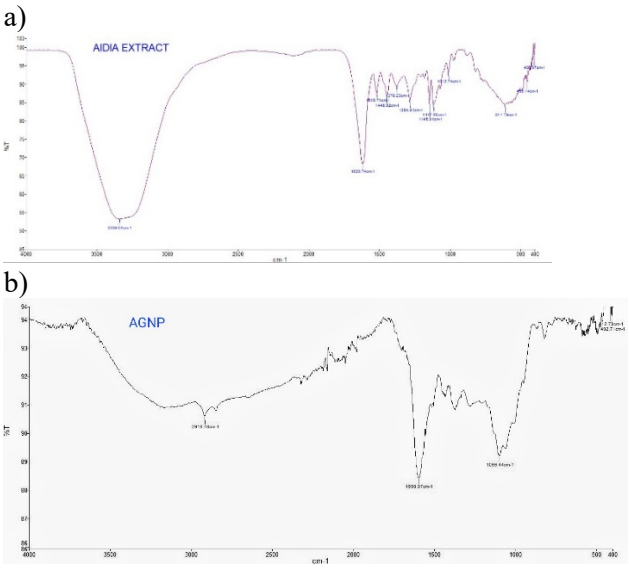

Fig. 12: FTIR profile of (a) *A. densiflora* leaf extract, and (b) AD-AgNPs. Spectra were recorded using an FTIR spectrometer in the range of 4000–400 cm^{-1} in Attenuated Total Reflectance (ATR) mode.

The FTIR spectrum of the extract revealed an O–H stretching vibration at 3339 cm^{-1}, which disappeared after AgNP synthesis. Instead, a new peak emerged at 2918 cm^{-1}, suggesting strong interactions between hydroxyl groups and AgNPs. Additionally, a peak shift from 1620 cm^{-1} to 1598 cm^{-1} indicates C=O stretching from the carbonyl group, while another shift from 1117 cm^{-1} to 1099

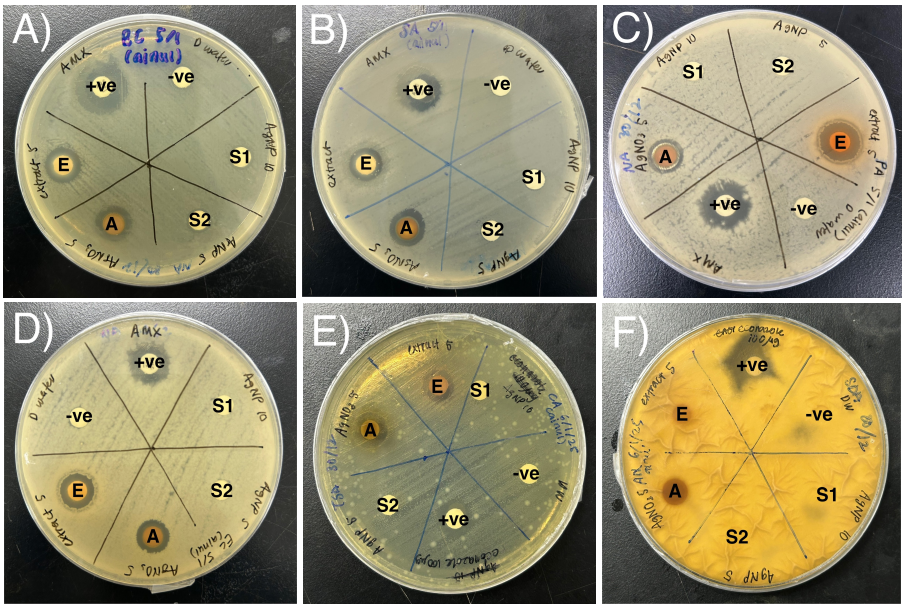

Fig. 13: Antibacterial activity of AD-AgNPs, *A. densiflora* leaf extract, and $AgNO_3$ against (a) *B. subtilis*, (b) *S. aureus*, (c) *E. coli*, (d) *P. aeruginosa*, (e) *C. albicans*, and (f) *A. niger*. (S1: 10 mg/mL AD-AgNPs, S2: 10 mg/mL AD-AgNPs, E: *A. densiflora* extract (5 mg/mL), A: $AgNO_3$ (5 mg/mL), -ve: Negative control (distilled water), and +ve: Positive control).

cm^{-1} suggests C–O stretching from ether or alcohol groups. These spectral changes indicate interactions between phytochemicals and AgNPs. The observed shifts suggest that phytocompounds from *A. densiflora,* particularly those containing alcohol, alkene, carbonyl, and ether functional groups, may play a role in the reduction and stabilisation of AgNPs. These findings align with previous studies that have also identified similar functional groups in plant-mediated AgNPs (Cherukuri & Kammela, 2022; Khan et al., 2023). Furthermore, the presence of characteristic peaks at 2918.43 cm^{-1}, 1598.97 cm^{-1}, and 1099.44 cm^{-1} in the IR spectrum of AD-AgNPs supports the assumption that biomolecules from the plant extract are bonded to the surface of AgNPs, acting as capping agents and influencing nanoparticle stability (Asong et al., 2023).

Antimicrobial assay

Fig. 13 shows ZOI for all microbes; however, excessive fungal overgrowth in *A. niger* (**Fig. 13(f)**) prevented accurate measurements despite multiple experimental repetitions. This suggests the disc diffusion method may be unsuitable for this strain due to its rapid mycelial growth.

Fig. 14 shows that AD-AgNPs did not exhibit significant inhibition against the tested microbes. Interestingly, $AgNO_3$ solution and the plant extract exhibited good antimicrobial activity, suggesting that although both precursors possessed antimicrobial properties, the synthesised AgNPs did not retain or enhance these effects. This contradicts the findings of Roy et al. (2019), who reported that when capping agents exhibit antimicrobial properties, a synergistic effect between the nanoparticles and the phytocompounds may occur.

Several factors may contribute to these findings, primarily the aggregation and stability of the synthesised AgNPs. According to Bruna et al. (2021), AgNPs size and surface characteristics significantly influence the release rate of silver ions, thereby affecting antimicrobial properties. A study by Korshed et al. (2019) demonstrated that smaller AgNPs exhibit stronger antimicrobial effects due to their larger surface area, which facilitates direct interaction with bacterial cells. Additionally, surface charge plays a crucial role in nanoparticle stability. AgNPs with low stability tend to aggregate, forming larger particles, which in turn reduces their antimicrobial efficacy (Bruna et al., 2021). In this study, SEM images revealed significant aggregation of AD-AgNPs. Furthermore, after the centrifugation step during synthesis, the resulting pellet appeared highly aggregated, requiring an additional 24-hour stirring to improve dispersion. This further proves the tendency of AD-AgNPs to aggregate, potentially leading to larger particle sizes and reduced antimicrobial effectiveness.

The antimicrobial effect of silver nanoparticles primarily results from the continuous release of silver ions (Ag^+). Due to electrostatic attractions, Ag^+ interacts with negatively charged molecules on bacterial cell walls, leading to membrane disruption, and eventually cell death (Yin et al., 2020). A previous study by Kim et al. (2022) observed low antimicrobial activity in pine needle extract-mediated AgNPs, presuming this to be excessive surface capping of polyphenols which may have reduced the electrostatic interaction between the nanoparticles and bacterial cells. Similarly, in this study, EDX analysis detected a high oxygen content in the AD-AgNPs, suggesting extensive phytochemical capping. This excessive surface capping may have prevented the release of Ag^+, thereby limiting the antimicrobial activity of AD-AgNPs.

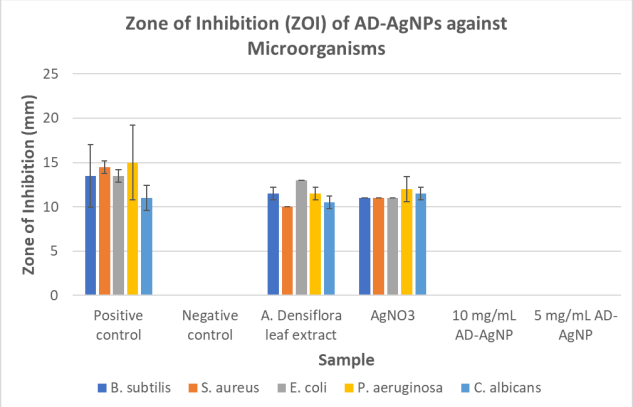

Fig. 14: Zone of Inhibitions of AD-AgNPs against different microorganisms.

Cytotoxicity assay

The MCF-7 cells were exposed to various concentrations of AD-AgNPs for 24 hours, and cytotoxicity was assessed using the MTT assay. **Fig. 15** illustrates the cytotoxic effects of AD-AgNPs, $AgNO_3$, and tamoxifen (positive control) at various concentrations. AD-AgNPs exhibited a low cytotoxic activity as even at the highest concentration (5 mg/mL), the cell viability remained relatively high (81%). Statistical analysis ($p > 0.05$) indicates no significant difference among treatments, suggesting that all treatments including

the positive control, exhibited low cytotoxic effects against MCF-7 cells.

While a dose-dependent trend was expected, fluctuations were observed across concentrations. The unexpected decrease in cell viability at lower concentrations of AD-AgNPs (79% at 0.625 mg/mL, 75% at 0.312 mg/mL) suggests inconsistencies, possibly due to uneven nanoparticle dispersion, slight variations in cell counts, or potential contamination during handling.

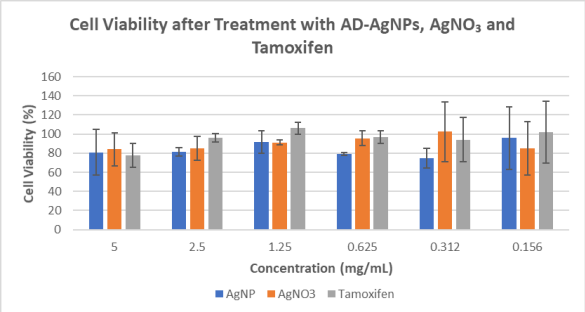

Fig. 15: Percentage cell viability of MCF-7 cell after treated with AD-AgNPs, $AgNO_3$, and *A. densiflora* leaf extract using MTT assay.

Previous studies have demonstrated that plant extract-mediated AgNPs exhibit strong anticancer properties against various human cancer cell lines, including MCF-7 (human breast adenocarcinoma) (Yeşilot & Dönmez, 2021), HeLa (human cervical cancer, ATCC CCL-2), A375, and SK-MEL-3 (human melanoma) (Radzikowska-Büchner et al., 2023). Therefore, low cytotoxicity of AD-AgNPs against MCF-7 cells may be attributed to aggregation, surface capping effects, or low bioavailability, which require further investigation.

Conclusion

This study demonstrates that AgNPs can be successfully synthesised using *A. densiflora* leaf extract, as confirmed by UV-vis spectroscopy, EDX, and XRD. LC-MS/QTOF identified several possible compounds that may play a role in the synthesis, including flavonoids, proanthocyanidins, terpenoids, steroids, and sugar alcohols. FTIR revealed the presence of alcohol, alkene, carbonyl, and ether functional groups on AD-AgNPs, which are most likely compounds from the extract acting as reducing and capping agents.

The average particle size was found to be relatively small (96.06 nm) with a good zeta potential value (-35.6 mV). XRD confirmed their crystalline structure, while SEM images revealed significant aggregation. The antimicrobial assay showed no ZOI for AD-AgNPs, whereas $AgNO_3$ and the plant extract exhibited some activity, suggesting that the synthesised AgNPs failed to retain or enhance the antimicrobial properties of their precursors.

The findings of this study suggest that, although AgNPs were successfully synthesised, their high level of aggregation may have reduced their biological activity. Future studies should focus on optimising the synthesis process to improve AgNP stability, minimise aggregation, and enhance biological activity.

Authors contributions

A.H.: Investigations, methodology, writing—original draft. **M. T., M.T.M.J.:** Supervision, funding acquisition. **J.M.J.:** Funding acquisition. **D.D., J.K.:** reviewing the draft. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author used ChatGPT in order to improve readability and language. After using this tool/service, the author reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Optimisation of Supercritical Fluid Extraction for Fatty Acids from Benincasa hispida Seed Oil: A Response Surface Approach

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Abstract

Introduction: The goal of this study was the optimisation of the process parameters for the extraction of *Benincasa hispida* seed extract using the supercritical carbon dioxide. **Methods:** Response surface methodology was carried out using the design expert software with the implementation of process parameters including the temperature (40°C – 70°C), pressure (100 bar – 400 bar) and supercritical carbon dioxide flow rate (2 g/min – 10 g/min) in this study due to their significant impact towards the oil yield and the polyunsaturated fatty acids. **Results:** The optimised parameter for this supercritical fluid model is 70°C, 247 bar and 7 g/min while 0.36 bar and 40 °C has been chosen from previous studies as the optimised parameter for Soxhlet extraction. The oil yield (33% from Soxhlet extract and 9.67% from supercritical fluid extract) obtained was quite similar with previous studies, however, the polyunsaturated fatty acids obtained throughout this optimisation were much higher indicating that this study provided better output of the polyunsaturated fatty acids obtained from the seed oil. Moreover, the polyunsaturated fatty acids contents were also compared between the extract obtained from the conventional Soxhlet extraction versus novel supercritical fluid extraction techniques. **Conclusion:** The result shows the polyunsaturated fatty acids in the supercritical fluid extraction were significantly higher than the Soxhlet extraction due to the advantages and suitability of the polyunsaturated fatty acids extraction using the supercritical fluid extraction method.

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Introduction

Benincasa hispida or also known as komora (Assamese) or kushmanda (Sanskrit) is a fruit that is widely produced and consumed in the North-East India (Mondal *et al*., 2020). The fruit has proven to be beneficial to human health due to its potential for medicinal and pharmaceutical purposes. For instance, antioxidant, anti-ulcer, anti-inflammatory, anti-obesity, anti-compulsive and anti-diarrheal activities that can be exerted by *B. hispida* (Shakya *et al*., 2020). India and South-East Asian countries are the two most cultivation places for this fruit (Gade *et al*., 2020).

B. hispida is considered as a fruit vegetable which is rich in nutritional components as functional food and nutraceutical market products. Two cultivars of Kundur are grown in Malaysia, which are the round shaped and elongated. *B. hispida* is one of the Cucurbitaceae family. *B. hispida* can be characterized by its large leaves, an annual creeper and bristly hairs on its thickly covered fruit skin (Megashree *et al*., 2017).

Despite growing interest in the beneficial properties of natural plant-based oils for their nutritional and therapeutic properties, there is limited scientific data on the optimal extraction of *B. hispida*. Traditional extraction such as solvent extraction and cold pressing often results in a much lower yields and degradation of the targeted compounds (Hou et al., 2024).

Additionally, the application of modern and greener types of extraction [supercritical fluid extraction (SFE)] is preferable. Moreover, most studies related to this plant only focused more on the beneficial effect of *B. hispida* seed oil rather than the optimization process and the fatty acid contents (Muzahid et al., 2023; Boniamin et al., 2024). Therefore, the implementation of optimization process by using statistical approach (response surface methodology) is tailored to enhance the extraction efficiency and preserving the quality of the targeted active compounds.

There was one study of SFE of *B. hispida* that was done for the optimization purposes (Bimakr et al., 2013). The parameters involved in that study were pressure (150-300 bar), temperature (40-50˚C) and dynamic extraction time (60-120 min) towards the oil yield using response surface methodology (RSM) for optimization. Therefore, the aim of this study was to optimize different extraction parameters of the *B. hispida* seed extract (BHSE) using RSM and to compare the polyunsaturated fatty acids (PUFAs) content between the extracts from both SFE and Soxhlet extraction (SE) and their fatty acid (FA) profile.

Materials and methods

Materials

Whole winter melons (*B. hispida*) were purchased from a local market at Kuantan, Pahang Malaysia. The fruits were chosen with similar characteristics such as the color, size and absence of surface defects. N-hexane (Merck, Germany) was used as the solvent for the SE. The supercritical fluid extractor (SFE Waters Thar, USA) with the carbon dioxide (CO_2) industrial grade (GasWORLD, Malaysia) were implemented. Besides that, sodium methoxide (Thermo Fisher Scientific, USA) and fatty acid methyl ester (FAME) standards (Sigma Aldrich, Germany) were used for gas chromatography analysis and all chemicals were purchased either in chromatography or analytical grade.

Method

Seed preparation method

The fruits were sliced, and the seeds were separated manually from the fruit. Later, the seeds were washed under running tap water. Then, the seeds were dried at 40°C in a ventilated oven for 24 hours and stored at ambient temperature in the dark container (Bimakr *et al*., 2013). Grinder mill (300 RPM) was used to grind the seeds into powder form to reduce the particle size and thus enhance the extraction efficiency (Bimakr *et al*., 2013).

Supercritical fluid extraction (SFE)

SFE was carried out using the supercritical fluid apparatus including 500 mL extraction vessel, automated back pressure regulator and CO_2 pump. ICE software was used to control the flow rate of

CO_2, extraction temperature and pressure while the extraction time was measured using the stopwatch. Samples of ground *B. hispida* seeds (10 g) and glass beads (120 g with 2.0 mm in diameter) were mixed and placed into the extraction vessel.

SFE of BHSE was performed on laboratory scale at the INHART, Gombak Malaysia. Based on Fig. 1, firstly, the liquid CO_2 released towards the cooling bath circulator to ensure that the liquid CO_2 was maintained at liquid state. Then, it was transferred using CO_2 pump to the heat exchanger to convert the liquid CO_2 into supercritical fluid. The vessel was heated up to the desired temperature while pumping the supercritical solvent phase into the vessel to reach values set by system (Bimakr *et al*., 2011).

Then, the solvent was pumped into the extractor vessel where the supercritical stream dissolved targeted components and moved towards the fraction collector. After the pressure nearly reached the targeted set value, the extraction process starts by the automatic opening of the automated back pressure regulator (ABPR) which allows the continuous flow of solvent through the extractor while carrying the solubilized components to the separator. Lastly, the CO_2 will be released through the last valve (Rovetto and Aieta, 2017). 100-400 bars of pressure, 40-70°C of temperature and 2-10g/min flow rate were used for the extraction process. The extraction time was maintained at 90 minutes.

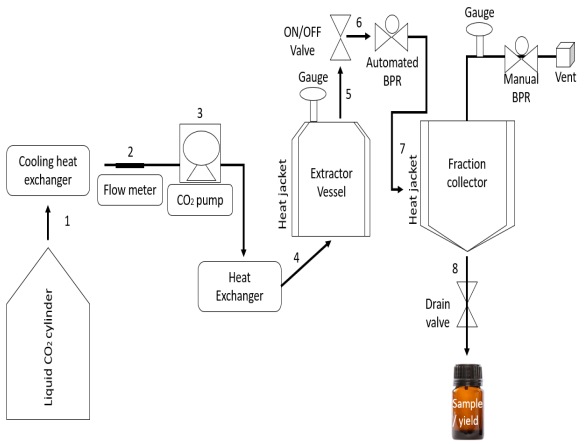

Fig. 1: General diagram for SFE

After the desired temperature and pressure have been obtained, the *B. hispida* seeds were soaked in the solvent for 30 min to equilibrate the mixture at desired pressure and temperature to enhance solvent penetration into the cellular walls and increase the oil yield (Bitwell et al., 2023). The static extraction time was applied at different desired pressure and temperature for each conducted run. During the dynamic extraction time, CO_2 carrying the released solutes will flow out of the unit and the extract was collected in the pre-weighted collection flask. The procedure was carried out in triplicate for a total of 17 runs for one complete cycle (Table 2). The extract was weighed using analytical balance and the total extractable components (TEC) were calculated using following Equation 1 (Nawaz et al., 2020):

$$\text{TEC (\%)}: \frac{\text{Weight of Extract}}{\text{Weight of Sample}} \times 100\% \quad (1)$$

Soxhlet extraction (SE)

300 mL of n-hexane was measured and poured into the round bottom flask containing a small amount of boiling chips. The extraction process was done for a duration of 6 hours (Al-Juhaimi and Özcan, 2017). After 6 hours, the solvent containing the extracts was filtered using Whatman filter paper. Then, it was evaporated using the rotary evaporator with 360 mbar pressure and 40°C temperature (Buchi, 2018) for approximately 1 hour. Finally, the extract was dried again in the drier at 40°C for 1 hour (Bimakr et al., 2013) to remove the remaining solvent in the extract.

Preparation of Fatty Acid Methyl Esters (FAME)

Samples were pre-treated to a temperature between 50°C - 60°C and homogenized thoroughly before the testing to obtain the FAME. An aliquot of the sample (100 µL) was mixed with 1mL hexane in a 2mL vial. An aliquot of sodium methoxide (1 µL, 1% w/v) was added to the vial and the mixture was mixed vigorously using a vortex mixer. The mixture was clear at first and then became turbid at the bottom of the solution due to the precipitation of sodium glyceroxide which contains the non-soluble compounds. After a few minutes, the clear upper layer of methyl esters which contains the solubilized fatty acids was pipetted off and can be injected into

the gas chromatography (GC) for further analysis (Bimakr et al., 2013).

Gas chromatography Flame Ionization Detector (GC-FID) analysis

Samples were converted to methyl esters which contain the solubilized fatty acids and were injected into the GC for further analysis. The GC analysis was performed using Hewlet-Packard 6890 gas chromatograph, equipped with flame ionization detector (FID) and a BPX70 GC column. Oven temperature was programmed isothermally to 60°C during 0.5 min, and raised to 10°C/min till 180°C for 5 min and then at 5°C/min till 215°C. Then, the temperature was raised to 220°C and finally, the temperature increased to 240°C at 5°C/min and held this temperature for 16 min.

Carrier gas, Helium was used as a carrier gas as the type of column used in this GC will be the capillary column (Alinafiah *et al*., 2021) and its flow rate was 1 mL/min. The injection volume was 1 µL. Standard FAME (Sigma Aldrich, Germany) was used as the authentic sample. The FA determination was accomplished by comparing it with standards and values as percentage of each FA. The determination of FA was performed in triplicate for each sample and expressed as means ± standard deviation.

Experimental designs and statistical analysis

The optimization of the extraction parameters (pressure, temperature and flow rate) for the SFE was done using the Design Expert software. After choosing the ranges of the parameters from previous studies, those values were incorporated into the RSM statistical tool for optimization purposes. Based on Table 1, pressure, temperature and flow rate were chosen as the independent variables and extraction yield was the only response. All the other parameters such as the extraction time (90 min), weight of sample (10 g) and extract collection time (20 hours) were kept constant throughout the experiments.

A three-factor-3-level face-centered design was chosen in the extraction of *B. hispida* seed extract (BHSE), which resulted in a total of 17 runs (Table 2) consisting of 8 factorial points, 6 axial points and 3 center points. For the evaluation of whether the constructed models were adequately fitted with the experimental data, corresponding analysis of variance (ANOVA) was applied using software which functions as numerical optimization for the determination of SFE's optimum conditions.

Results and Discussion

Prediction and optimization using RSM

RSM-central composite design (RSM-CCD) was applied to optimize the SFE conditions of the BHSE. The experimental data obtained according to the model is presented in Table 2. The experimental data was analysed and fitted to linear, interactive (2FI), quadratic and cubic models based on previous studies (Sodeifian et al., 2017). The adequacy of model was tested for response and shown in Table 3.

Table 1: The coded and uncoded values of the independent variables used in the experimental design

Independent Variables	Pressure (Bar)	Temperature (°C)	Flow Rate (g/min)
Coded levels	Level of factor		
+1	100	40	2
0	250	55	6
-1	400	70	10

The result showed that the linear and interactive models had lower R^2, predicted R^2, adjusted R^2 and high F-values compared with the quadratic model which also can be seen in previous studies (Rivas *et al*., 2021; Chalipa et al., 2024; Kumaran et al., 2024). Moreover, the cubic mode was found to be aliased. Therefore, the quadratic model was chosen to describe the current study. Quadratic model has been chosen from most of the published studies related to the optimization using RSM to describe their experimental results (Gan et al., 2020).

Model fitting and statistical analysis

ANOVA was used to indicate which terms are statistically significant and the validity of the experimental models (Abassi *et al*., 2020). Besides the ANOVA result, F-value and corresponding p-values along with the estimated coefficients were presented at Table 3. A higher model which the F-

value is 40.85 and the associated lower p-values ($P < 0.0001$) demonstrated that the corresponding coefficients are more significant and illustrate the best fit for both experimental and actual values of the developed model (Peng *et al*., 2019).

In this model, a R^2 value of 0.9813 (very close to 1) shows that there was a good agreement between the experimental and predicted yields of BHSE and the model can reasonably explain the changes of the oil yield under different sets of experimental conditions.

On top of that, the adjusted coefficient of determination (Adjusted $R^2 = 0.9573$) and predicted coefficient of determination (Predicted $R^2 = 0.8567$) were also close to 1 which reflects the result of highly correlated of both experimental and predicted yields and lead to the conclusion that the model had fully fitted the data as agreed by a study (Kothari *et al*., 2021).

The lack of fit was not significant based on the F-value (1.90) and p-value (0.3795) to the pure error. This finding was in line with previous study where the lack of fit was considered as a reference besides R- values and p-values to indicate that the model values provide significance of the regression model to infer the effect of the input variables (Nadeem *et al*., 2021).

A second order polynomial equation was fitted to the experimental data. The constants and coefficients were fitted into the equation 2 below so that the regression equation as a function of independent parameters, namely pressure (A), temperature (B) and flow rate (C) while Y is the oil yield and was obtained as follows (in terms of coded levels):

$$Y = +6.13 - 0.1090A + 0.4620B + 0.9670C + 0.3037AB - 0.1038AC + 0.7987BC - 4.52A2 + 3.76B2 - 4.16C2 \quad (2)$$

In this CCD model, the significance determination for each model was assessed by both p-value and F-value. The influence of the model term on the oil yield was considered significant if the p-value is < 0.05 and larger F-value with small p-value indicates greater significance of the corresponding model item as observed in a study (Louaer *et al*., 2019). According to the result tabulated in Table 3, the linear terms of pressure and temperature showed insignificant impact towards the oil yield while the flow rate showed a significant ($P < 0.05$) impact.

In terms of the interaction terms, interaction terms between temperature and flow rate (BC) were the only terms with significant impact towards the oil yield as agreed previously (Suryawanshi and Mohanty, 2018). Meanwhile, the interaction terms between pressure and temperature (AB) and pressure and flow rate (AC) were not significant.

In a comprehensive analysis of each model item, although the linear terms for the temperature, pressure and flow rate had no significant impact towards the *B. hispida* oil yield, the interaction term of BC and the quadratic terms of those three independent variables ($P < 0.0001$) had significant effects towards the oil yield indicating that these selected variables were also part of the indispensable factors.

Previous studies have found that the quadratic terms of the pressure, temperature and flow rate were significantly interacted, thus proving its effectiveness towards the oil yield (Ishak et al., 2021 as agreed in this study where the three quadratic terms were significantly interacted towards the SFE of BHSE.

Validation of the model was carried out by carrying out SFE using three different sets of parameters which were generated from the design expert software and each of the sets were different from the 17 runs. The result of the validation showed that by using parameters of 70°C, 247 bar and 7 g/min, running in triplicate, result in 8.13% of relative error at 90% confidence interval which was the lowest compared with the other two set of parameters (26.76% & 11.94%). Therefore, the optimised parameter for this model is 70°C, 247 bar and 7 g/min.

Table 2: The experimental designs of CCD and the oil yield measured at different conditions along with the predicted oil yield

		Factor 1	Factor 2	Factor 3	Actual Yield	Predicted Yield
Std. Order	Space Type	A: Pressure (bar)	B: Temperature (°C)	C: Flow Rate (g/min)	Yield (%)	Yield (%)
1	Factorial	100	40	2	0.7	0.8934
2	Factorial	400	40	2	0.01	0.2754
3	Factorial	100	70	2	0.03	0.3876
4	Factorial	400	70	2	0.01	0.2094
5	Factorial	100	40	10	1.66	1.44
6	Factorial	400	40	10	0.01	0.4044
7	Factorial	100	70	10	3.64	3.35
8	Factorial	400	70	10	3.75	3.53
9	Axial	100	55	6	0.99	1.73
10	Axial	400	55	6	2.15	1.51
11	Axial	250	40	6	10.06	9.43
12	Axial	250	70	6	9.63	10.35
13	Axial	250	55	2	1.25	1.01
14	Axial	250	55	10	2.61	2.94
15	Centre	250	55	6	6.36	6.13
16	Centre	250	55	6	6.63	6.13
17	Centre	250	55	6	5.6	6.13

Parameters affecting the response

For the model diagnosis results, the normal distribution of residuals and the comparison between experimental and predicted values showed good linearity which also reflected that the model was capable in expressing the relationship among the selected parameters as shown in Fig. 2 (a) & Fig. 2 (c) (Dao *et al*., 2019). Fig. 2 (c) shows that the experimental and predicted values were close to the regression line, and it reflects the high R^2 value (0.9813) thus the changes of oil yield can be reasonably explained based on this graph.

Additionally, Fig. 2 (b) shows the normal plot of residuals vs run which indicates that the residuals of the experimental yield were scattered randomly thus, the model is suitable for optimization. The same result can also be found in another study, indicating the appropriateness of the model to suggest the observation of original value is unrelated to the response values (Falowo *et al*., 2019).

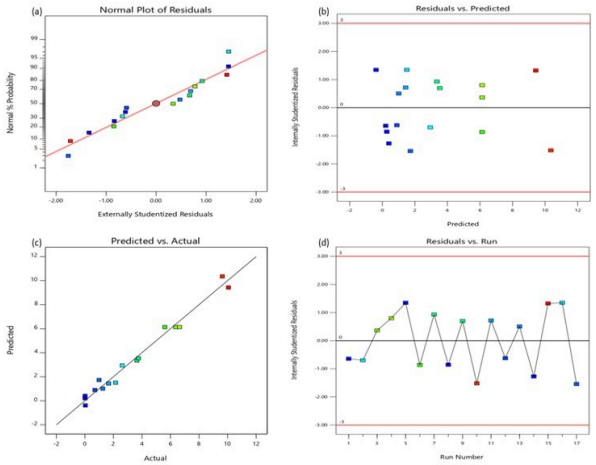

Fig. 2: Statistical analysis of the surface quadratic model

For the determination of large residuals in runs, the standard residuals in the outlier plot should fall into three standard deviations intervals as observed in Fig. 2 (d) and similar results were recorded in previous study (Zhang *et al*., 2019). Any outlier that exceeds this interval could result in a hidden error in the model or operational error in the experiment (Yingngam *et al*., 2021).

Table 3: Response surface model adequacy and ANOVA analysis

Source	Std. Dev.	R^2	Adjusted R-square	Predicted R-square	p-value	PRESS
Linear	3.55	0.0660	-0.1495	-0.5031	0.8203	264.12
2FI	3.98	0.0998	-0.4404	-2.6226	0.9432	636.56
Quadratic	0.6848	0.9813	0.9573	0.8567	<0.0001	25.18
Cubic	0.4457	0.9966	0.9819	0.7651	0.1723	41.28

Source	Sum of Squares	df	Mean-square	F-value	p-value
Model	172.44	9	19.16	40.85	< 0.0001
A-Pressure	0.1188	1	0.1188	0.2533	0.6302
B-Temperature	2.13	1	2.13	4.55	0.0703
C-Flow Rate	9.35	1	9.35	19.94	0.0029
AB	0.7381	1	0.7381	1.57	0.2499
AC	0.0861	1	0.0861	0.1836	0.6812
BC	5.10	1	5.10	10.88	0.0131
A^2	54.70	1	54.70	116.63	< 0.0001
B^2	37.81	1	37.81	80.62	< 0.0001
C^2	46.33	1	46.33	98.78	< 0.0001
Residual	3.28	7	0.4690		
Lack of Fit	2.71	5	0.5425	1.90	0.3795
Pure Error	0.5705	2	0.2852		
Cor Total	175.72	16			

Fig. 3 shows the three-dimensional (3D) plots along with their two-dimensional (2D) projections of response surfaces for the graphical representations of regression equation obtained from the RSM analysis. The constant level of the three independent variables (250 bar, 55°C and 6 g/min) of each operating parameter (pressure, temperature and flow rate) was implemented for the interactive analysis of any of the two parameters through the 3D plots.

Different shapes of the 2D contour plot including elliptical and circular shape can be observed in Fig. 3. Elliptical contour plots mean the interactions between the involved parameters were significant while circular contour plots indicate the interactions were negligible. Previous studies also proved the presence of both shapes providing the visual relationships between response and the experimental levels of each variable and their interactions (Zhai *et al.*, 2021). As from my observations, the elliptical contour can be seen at Fig. 3 (c) while Fig. 3 (a) and Fig. 3 (b) produced more circular contour plots.

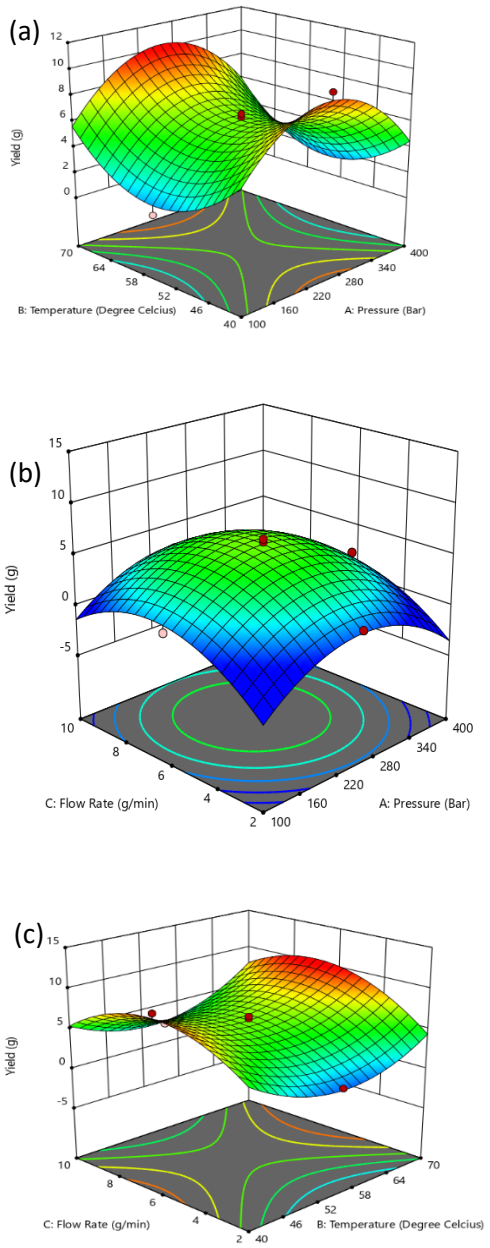

Fig 3: 3D graph and 2D plots show the effects of two-parameter interactions on BHSE

Temperature

As observed in Fig. 3, the impact of temperature was unique as the oil yield increased at both lowest and highest temperature. There are two extraction conditions which affect the impact of temperature towards the oil yield which are the above and below crossover pressure. When there is an increased in temperature, the oil yield increased

due to effect of temperature on SC-CO_2 density became less than the vapor pressure at above crossover pressure (Patil et al., 2017). However, below the crossover pressure, the effect of temperature on density supersedes the vapor pressure and at lower temperature, higher SC-CO_2 density contributed to the high solvent power of SC-CO_2 and producing higher oil yield as observed (Nde and Foncha, 2020).

From Table 2, low oil yield was obtained at the lowest temperature (40 ˚C) and pressure (100 bar) As the temperature increased from 40 ˚C to 70 ˚C, the oil yield became reduced at 100 bar and this is because of a reduction of solvation effect to disperse into the sample matrix (Ishak et al., 2021).

Additionally, when there was an increase of pressure from 100 bar to 250 bar at the same temperature (40 ˚C), the oil yield increased due to an increased in SC-CO_2 density and solvation strength (Ishak et al., 2021). However, the significant reduction of oil yield at high temperature (70 ˚C) and pressure (400 bar) decreases the SC-CO_2 density and the contacts between the seed matric and SC-CO_2 thus minimizing the interaction between the solvent and the sample thus lowering the mass transfer during extraction (Suryawanshi and Mohanty, 2018).

In this study, the oil yield was lower as the temperature was reduced as observed in Fig. 4, but as it reached 55˚C, the oil yield started to increase. Similar finding was found, where the oil yield started to be increased from 19.13% to 21.63% when the temperature was increased from 40˚C to 60˚C considering the effect of temperature is ruled by both solvent density and vapor pressure of the oil (Ferrentino *et al*., 2020).

Fig. 3 (c) shows the 3D surface graph of the effects of temperature and flow rate towards the oil yield at fixed pressure (250 bar). The effect of temperature in this study also can be seen at the lowest temperature (40 °C) which produces high oil yield. This research finding is found to be similar with the SFE of chia seed where the oil yield was higher between 40 °C – 45 °C (Ishak *et al*., 2021).

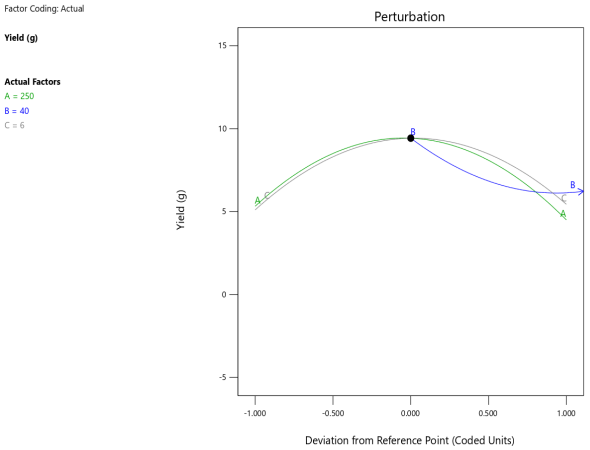

Fig. 4: Effect of individual parameters towards *B hispida* seed oil yield

This situation can also be explained by the positive correlation between yield and extraction temperature when combined with pressure. At 100 bar, the oil yield decreased when the temperature increased, dropping from 0.7% at 40 °C to 0.03% at 70 °C because at critical pressure, oil solubility decreases when the temperature increases, due to the predominant effect of density on solute vapor pressure. Conversely, the oil yield increased from 0.01 at 40 °C to 3.75% at 70 °C because above the critical pressure, the vapor pressure effect dominates, resulting in an increase in solute solubility. The compound's solubility is thus favored or disfavored by increasing the temperature, depending on whether the pressure is lower or higher than the crossover pressure (Allay et al., 2025).

Pressure

Fig. 4 (curve A) shows an increase in pressure at constant flow rate and temperature which did not result in a further increase in the oil yield. The highest yield obtained was at 250 bar, 40 °C and 6 g/min flow rate (10.06%), indicating the minimal impact of pressure towards the oil yield. Nevertheless, the effect of quadratic term of pressure increased significantly at high pressure (greater than 250 bar). The possible reason is that the highly compressed CO_2 led to the reduction in the fluid diffusion coefficient. This counteract effect often resulted in little impact towards the oil yield with high pressure being implemented (Gan *et al*.,

2020).

On top of that, the increasing extraction pressure caused a reduction in the solvent diffusivity, hence the convective mass transfer attendant to the extraction process, leading to lower oil recovery (Ahangari *et al*., 2021).

The 3D surface graphs of the effect of pressure and temperature on the yield of *B. hispida* seed at a constant flow rate (C = 6 g/min) are shown at Fig. 3 (a). At the given temperature, the increased in pressure caused the density of the supercritical solvent CO_2 to be higher, which resulted in an increase in the solubility of CO_2 and ultimately increased the oil yield. Higher extraction pressure may result in the elevation of vapor pressure, facilitating the travel of oil onto the surface of the seeds thus improving the oil yield (Peng *et al*., 2019). Thus, the yield of *B. hispida* seed increases significantly with the increase in pressure.

Previous findings have proved the positive impact of the pressure towards the oil yield and their findings were aligned with the current findings where the oil yield increased from 0.99% to 6.36% when the pressure increased from 100 bar to 250 bar. Similarly, the increase of pressure of 200 to 300 bar has resulted in the highest yield of extraction in previous studies (Teixeira *et al*., 2020; Santos et al., 2020). Additionally, the increase of extraction pressure from 100 bar to 169 bar was shown to raise the recovery of sunflower oil from 47.54% to 52.08% (Daraee et al., 2018).

Flow rate of supercritical carbon dioxide

Maximum oil yield can be obtained from the increased fluid flow rate because of the reduction of resistance to mass transfer until the exiting fluid became saturated, consequently, leading to the solute solubility equilibrium (Acevedo-Correa *et al*., 2018). This occurrence can be observed in Fig. 4 (curve C), where the maximum flow rate which resulted in maximum oil yield was at 6 g/min.

In Fig. 3 (c), under the given pressure, the increase in the CO_2 flow could increase the cycle extraction time in the pipeline, thus resulting in an increase of the oil yield. However, as the CO_2 flow bypassed 6 g/min rate, the residence time of CO_2 was too short so the contact time with the extract was reduced which was not suitable to improve the oil yield (Chouaibi *et al*., 2020).

Besides that, the extraction yield of wheat germ oil and raspberry seed oil was the highest at solvent flow rate of 0.4 kg CO_2/h or 6.67g/min (Teslić et al., 2020; Pavlić et al., 2019) which is in-line with the result of the current study because of the increase in concentration gradient and consequently improving the extraction rate.

Identification and Quantitation of Fatty Acid Composition

Table 4 shows the comparison of the FA contents from extracts of both SFE and SE. There were no significant differences in terms of the FA contents for both SE and SFE except for the linoleic acid (LA), myristic acid and oleic acid.

The total content of saturated fatty acid (SFA) (Table 4) was 29.9% from SE and 26.86% from SFE with palmitic acid (13.137 ± 1.077 for SE, 13.32 ± 0.2150 for SFE) accounted to be the highest FA content and these results are also recorded in previous findings (Anwar *et al*., 2011; Bimakr *et al*., 2013). For comparison, the SFAs extracted from the SE were much higher than those extracted using SFE even though they are not statistically significant except for Myristic acid which were statistically significant ($P < 0.05$).

The same result was obtained in previous study (Rosa *et al*., 2019) where the application of different extraction methods can be the reason and lower percentage of PUFAs could contribute to proportional increase in SFA and monounsaturated fatty acid (MUFA) contents (Rosa *et al*., 2019). The amount of SFAs were lower than the unsaturated fatty acids (UFAs) because of the unfavorable high temperature for the extraction of SFAs as observed in Table 4 (Souza *et al*., 2020).

Additionally, the UFAs' extracted using SFE were higher (72.2%) compared to those UFAs extracted using SE (70.5%). These data were agreed by a study (Anwar *et al*., 2011), in which this study used the same type of seed with only differences in the parameters chosen for the extractions. PUFAs were accounted for the highest content of FAs for

both types of extractions with PUFAs from SFE were significantly higher than in the SE ($P \leq 0.001$) which is similar with previous studies (Rajei *et al*., 2005; Gustinelli *et al*., 2018).

Table 4: Comparison of fatty acids contents between different extraction methods

Fatty Acid Category	Compound Name	Carbon Number	Fatty Acid Composition (%)	
Extraction Type			SE	SFE
TEC (%)			32.667*** ± 0.333	9.623*** ± 1.688
Saturated Fatty Acids	Octanoic Acid	8:0	0.36 ± 0.032	0.33 ± 0.103
	Decanoic acid	10:0	-	-
	Lauric acid	12:0	0.093 ± 0.009	0.073 ± 0.003
	Tridecanoic acid	13:0	0.207 ± 0.0351	0.56 ± 0.02
	Myristic acid	14:0	1.073* ± 0.078	0.207* ± 0.02
	Pentadecanoic acid	15:0	0.447 ± 0.035	0.247 ± 0.009
	Palmitic acid	16:0	13.367 ± 0.622	13.317 ± 0.124
	Heptadecanoic acid	17:0	0.407 ± 0.007	0.297 ± 0.003
	Stearic acid	18:0	11.36 ± 0.07	10.78 ± 0.103
	Arachidic acid	20:0	1.977 ± 0.02	1.05 ± 0.175
	Behenic acid	22:0	0.587 ± 0.055	-
Total			29.9%	26.86%
Unsaturated Fatty Acids	Myristoleic acid	14:1 n-5	-	-
	Palmitoleic acid	16:1 n-7	0.87 ± 0.017	0.933 ± 0.007
	Elaidic acid trans	18:1	0.497 ± 0.041	-
	Oleic acid	18:1 n-9	5.467**** ± 0.179	2.257**** ± 0.192
	Linoleic acid	18:2 n-6	58.453**** ± 0.423	68.507**** ± 0.151
	Alpha Linoleic acid	18:3 n-3	1.66 ± 0.08	1.44 ± 0.3
	Eicosenoic acid	20:1	1.123 ± 0.019	-
	Erucic acid	22:1 n-9	2.03 ± 0.04	-
Total			70.5%	72.2%

Each value represents the mean ± SEM of triplicate experiments. (*) $P \leq 0.05$, (**) $P \leq 0.01$, (***) $P \leq 0.001$ and (****) $P \leq 0.0001$.

The content of the LA and the alpha-linoleic acid (ALA) were analyzed using the optimised parameter from the RSM. Previous findings also showed that majority of the FA content extracted using the SFE was LA and ALA. The parameters affecting the LA and ALA contents are pressure and temperature. Firstly, in terms of the pressure, the change in pressure of a study from 200 bar to 250 bar resulted in an increase of the LA content in raspberry seed oil for 20% and similar trend was observed for the ALA (Campalani *et al*., 2020). This finding was aligned with the result of this current study where the optimised pressure was 247 bar to produce the high yield of the PUFAs.

Besides that, the second parameter which is the temperature, , 70˚C was found to be the best temperature for the extraction of FAs in wild strawberry pomace as well as for the *B. hispida* seed as the application of lower temperature such as 40˚C and 50˚C caused a much lower FA content because of the solubility of solute mainly affected by the increasing vapor tension rather than the CO_2 density reduction (Campalani *et al*., 2020). Also, an increase in temperature from 20˚C to 60 ˚C resulted an increase in LA content for 45% showing the positive impact of the high temperature towards LA (Hernández-Fernández *et al*., 2019).

Conclusion

As a conclusion, based on the CCD model for the optimisation, three parameters were selected and optimised according to the range of values for those parameters. Thus, the set of parameters (70°C, 247 bar and 7g/min) with 8.13% relative error was determined to be the optimised parameter for the BHSE extraction. As for the fatty acid identification, the result shows that the PUFAs were detected mostly in both types of extraction but the BHSE from SFE produced higher PUFAs' content (9.84% differences) and accompanied with advantages of the SFE over the SE especially in terms of its safety towards the operator and the environment because of the solvent recovery and avoidance of the released of harmful solvents to the environment. However, the primary limitation for the SFE is higher operating cost and excessive consumption of energy to convert and maintain the supercritical state of the solvent.

Authors contributions

Conceptualization, H.A.H. and Z.I.S.; methodology, Z.I.S., H.A.H. and R.Z.R.; resources, H.A.H.; writing—original draft preparation, R.Z.R.; writing—review and editing, H.A.H.; supervision, H.A.H. and Z.I.S.; funding acquisition, H.A.H. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors report there are no competing interests to declare.

Declaration of generative AI and AI-assisted technologies in the writing process

This manuscript is completed without any usage of AI or AI-assisted technologies during the writing process.

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Journal of Pharmacy

Evaluating the Wound Healing Activity of Fabricated Stingless Bee Honey Hydrogels in an Animal Model

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Abstract

Introduction: Honey is a well-established treatment for wound healing and had been used for ages. However, there are limitations when it is directly applied to the wound such as inconsistent dosage and pain during dressing changes. The incorporation of honey into hydrogel could overcome these problems. The study is aimed at developing a suitable honey-based hydrogel formulation for wound healing application by using systematic experimental design from response surface methods (RSM). **Methods:** The hydrogel's base was made from polyvinyl alcohol (PVA), polyethylene glycol (PEG), glycerol and agar. A two-level factorial design was selected to screen the factors followed by centred composite design for optimization. The characterizations of the optimized formulation were observed in term of hydrophilicity and rheological. The optimized formulation was further assessed in an in vivo wound healing study in New Zealand albino rabbits. **Results:** PEG and agar concentration was found to be the most important process variable based on the screening result. The optimised hydrogel has a good hydrophilicity ability and rheological property. In in vivo healing study, the healing in the honey incorporated hydrogel treated group was significantly faster than the no treatment group, as demonstrated in wound closure percentage and histological assessment. From the results, the wound in the honey hydrogel treatment group has entered the remodelling phase compared with the control group that was still in the proliferation phase. **Conclusion:** Based on all of these results, stingless bee honey incorporated hydrogel has a promising application as an efficient wound dressing.

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Introduction

Wound healing remains one of the major concerns among healthcare practitioners and scientists to this day. Poor wound healing not only causes trauma to the patients, but the process itself becomes time-consuming and can place a significant strain on healthcare resources. For example, in the United Kingdom the National Health Services (NHS) estimated that the cost for managing wound cases was approximately £8.3 billion per annum, where 81% of the total annual NHS cost was covered in the community(Guest et al., 2020).This number may rise in the future, since the prevalence of wounds is increasing due to the ageing population, in addition with other comorbid factors that could hinder the healing processes such as cardiac disease, diabetes, and obesity (Versey et al., 2021). Therefore, a careful wound healing management therapy should be considered seriously to reduce the burden and improve the outcome of wound healing cases.

Over the years, there have been a lot of methods and inventions introduced to overcome wound healing problems. Since the beginning of the 20th century advanced technologies in polymerisation research have contributed refreshing materials for wound healing products. A few studies indicated that a moist environment is the ideal condition to improve wound healing process (Farahani & Shafiee, 2021; Gao et al., 2021; Liang et al., 2021; Tottoli et al., 2020). Therefore, a dressing that can imbibe sufficient water content was developed, and it is now known as hydrogel. Hydrogel also has a promising attribute in drug delivery systems due to their high-water content that can imitate similar environment as the human body tissue and provides good biocompatibility to encapsulate hydrophilic drugs (Asadi et al., 2021).

Numerous types of honeys have been identified and one of them is stingless bee honey that are widely used in Latin America, the mainland of Australia, Eastern and Southern Asia, and Africa (Engel et al., 2017). The therapeutic effect of honey that contribute to the wound healing process is mainly due to the broad-spectrum antibacterial activity (Domingos et al., 2021; Dżugan et al., 2020; Wasihun & Kasa, 2016). This antibacterial property is contributed by the nature of honey, which contain high osmolarity,(Al-Masaudi et al., 2020) acidity (Bouhlali et al., 2019) and strong non-peroxide activity (Guttentag et al., 2021). Furthermore, with these characteristics, stingless bee honey is able to protect or confront against pathogenic microbial colonization at the injury site, which, if not handled properly, may lead to chronic wound formation (Rao et al., 2016).

Stingless bee honey also possesses numerous good phytochemical components such as phenolic acids, flavonoids, glucose oxidase and catalase enzymes (Ávila et al., 2018; Sousa et al., 2016).) These compounds are highly associated with the antioxidant activities from honey. Besides antibacterial activity, antioxidant activity could also enhance the healing process by protecting the wound site against the detrimental effects from oxidative stress (Abd Jalil et al., 2017).

Direct application of honey is inefficient, as tissue rapidly absorbs the fluid, leading to inconsistent dosing and the therapeutic concentration is not constantly maintained. Besides, when it is combined with traditional dressings materials, such as gauze or bandage, it would cause discomfort to the patient due to the frequent dressing change that might be painful during the dressing replacement (Resch et al., 2021). These predicaments can be solved by incorporating the honey within a hydrogel formulation.

The incorporation of honey with hydrogel could not only provide an ideal moist environment on the wound but also good fluid absorption that promotes permeation of the nutrient content of the honey (Ahmad et al., 2021; Gull et al., 2019). Furthermore, due to its jelly-like structure that is similar to the granulation tissue, it would reduce the pain and provide a soothing effect (Phaechamud et al., 2015). Hydrogel also can allow a sustained delivery of the bioactive substances to the wound over time.

The conventional optimization method for hydrogel development involves changing one factor at a time while keeping the other factors constant. The disadvantage of this method is it may take a long period and can be costly (Kaith et al., 2014).

Therefore, response surface method (RSM) can be used as an alternative method, whereby various factors can be designed simultaneously, and they can be related with the dependent response by using a design matrix that eventually produces an optimal formulation (Karkare et al., 2022).

In this study, stingless bee honey incorporated into a hydrogel made from synthetic polymer (PVA-PEG) and natural polymer (agar) was developed by using response surface methodology. A few optimized formulations developed were further characterized to observe the robustness as a wound healing agent. Then, the in vivo assessment was conducted on the most effective formulation to support the healing activity. Previously, there are plenty of other combination between hydrogel and different types of honey such as Medihoney® using manuka honey(Woodward, 2019), Malaysia Gelam honey (Mohd Zohdi et al., 2012), Egyptian Arabic honey (El-Kased et al., 2017), Korean Chestnut honey (Park et al., 2017), and Indonesian Euphoria longana sp. Honey (Kosimaningrum et al., 2020). However, this study is the first combination of hydrogel and stingless bee honey.

Materials and methods

Materials

The materials and chemicals used throughout this study were Muller-Hinton agar (MHA) (Merck, Germany), Muller-Hinton broth (MHB) (Merck, Germany), ethanol (R&M Chemicals, UK), polyethylene glycol 400 (PEG 400) (Merck, Germany), polyvinyl alcohol (PVA) with molecular weight of 195,000 and 99% hydrolyzed (Sigma-Aldrich, USA), protein-free agar (1st Base, Singapore), distilled water (Brandon, Malaysia), phosphate-buffered saline (PBS) tablets (Sigma-Aldrich, USA), stingless bee honey (Bris Trigona, Malaysia), Ketamin (Ilium, Australia), Xylazine (Ilium, Australia), normal saline solution (Opticare, Malaysia), 37% formaldehyde (Merck, Germany), 100% ethyl alcohol (Fisher Scientific, UK), toluene (Fisher Scientific, UK), xylene (Fisher Scientific, UK), Mallory's trichrome staining kit (DiaPath, Italy), haematoxylin (Surgipath® Leica Microsystem, USA) and eosin (Surgipath® Leica Microsystem, USA).

Stingless bee honey hydrogel preparation

The methods in preparing and characterizing the hydrogel were adapted from the study conducted by Hwang et al., (2010) for the number of freeze and thawed cycle, Kamoun et al., (2015) for the use of PVA- PEG excipient and Mohd Zohdi et al., (2012)for the use of agar as excipient and honey as main active ingredient of the hydrogel formulation. Ten percent PVA was mixed with 6% PEG (w/v) together with 1% protein-free agar solution (w/v), 1% glycerol (w/v), and 40% honey (w/v). To prepare the PVA aqueous solution, 100 g of PVA was dissolved in 600 mL distilled water for 1 h until the solution was clear. Later, 10 g of agar was dissolved in 330 mL distilled water and was heated for about 1 h until the solution was clear. The solution was stirred continuously before 60 g of PEG was added to create an agar solution. Then, 1 g of glycerol was dissolved in the mixture. After that, the mixture of the PVA aqueous solution and the agar solution was submerged in an ultrasonic water bath at 37 °C for 1.5 h for degassing purpose. When the temperature reached 40 °C, 40 g of honey was added. To prepare a control hydrogel, a similar mixture was prepared without honey. They were kept in the freezer at -20 °C for 18 h and then thawed at 25 °C for 6 h to complete a cycle (F–T cycle). They were grouped into first, second, and third cycles. Fig. 1 summarize the preparation of hydrogel.

Experimental design and model for PVA-agar-honey development

The swelling ratio of the prepared hydrogel could be influenced by many factors such as the number of cycles, temperature during the freezing cycle or percentage of PEG, agar and glycerol. It would be time consuming to test each of these factors one at a time. Therefore, the introduction of factorial design may provide a better solution to screen the effects of each factor. In addition, the usage of this design could also study both antagonistic and synergistic factor interactions (Anderson & Whitcomb, 2017).

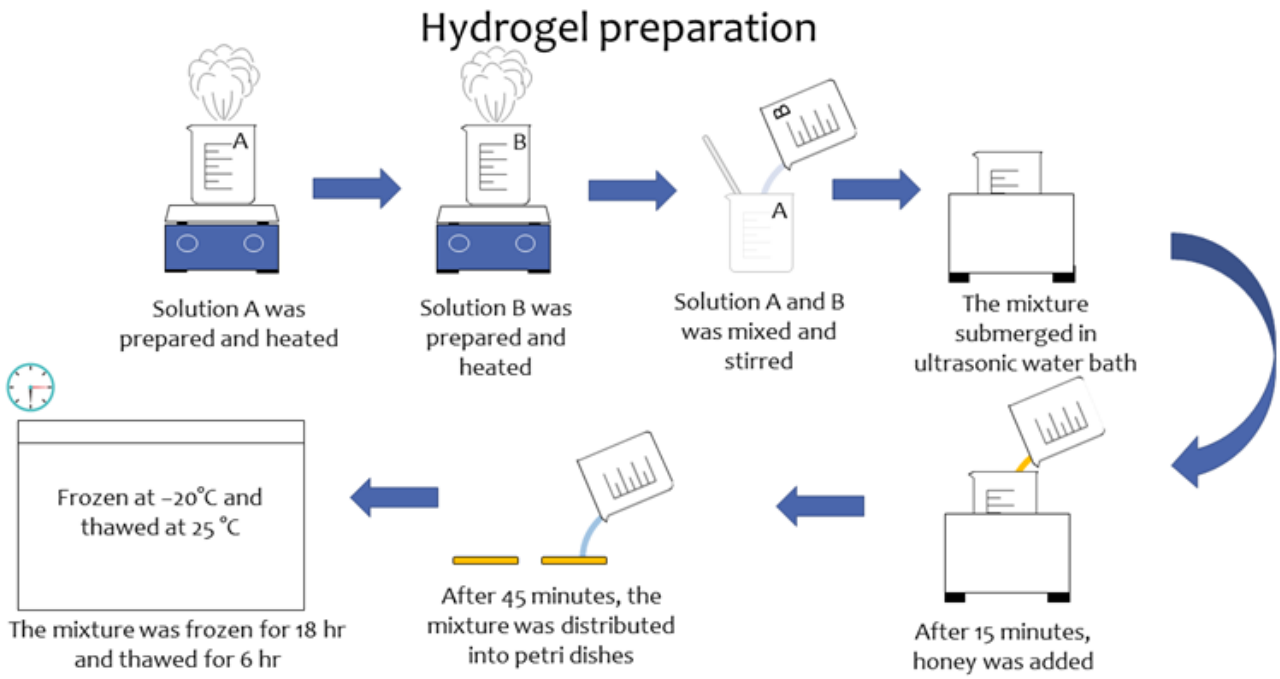

Fig. 1: Summary of hydrogel preparation. Solution A consist of PVA aqueous solution while solution B consist of agar and PEG solution.

Table 1: List of factors with their maximum (+1) and minimum (−1) points.

Factors	Factor significance	Minimum Level (−1)	Maximum Level (+1)
X1	PEG % (w/v)	3	5
X2	Agar % (w/v)	0	1
X3	Glycerol % (w/v)	0	1
X4	Temperature (°C)	−80	−20
X5	Cycles	1	3

Table 2: List of factors with their maximum (+1), central (0), and minimum (−1) points.

Factors	Factor significance	Minimum Level (−1)	Central Level (0)	Maximum Level (+1)
X1	PEG % (w/v)	4.5	5.0	5.5
X2	Agar % (w/v)	0	0.5	1

For two-level factorial design, five variables were arranged at their maximum (+1) and minimum (−1) points to improve the swelling percentage of the hydrogel formulation. Table 1 shows the list of formulations that was generated by the software. Significant variables that maximized the swelling percentage were selected by using Pareto charts. Sequential ANOVA modelling was used to fit the empirical data while the significance of the model was evaluated based on p-values. Then, based on the screening result, the two most significant process variables (PEG and agar percentage) were optimized using central composite design (CCD). Table 2 shows a list of factors with their maximum (+1), central (0), and minimum (−1) points.

Response tests

From the formulation list, a swelling ratio test was conducted as a response test to study the influence of the selected factors toward the formulation.

Determination of swelling ratio

The hydrogel samples (2 × 2 cm) were dried at 60 °C in the oven for 12 h (Wa). Then, they were soaked in pH 7.4 PBS and put inside an oven at 37 °C (Ws) for 24 h. The swelling ratio (SR) was calculated using Eq. (1) below.

$$\text{Swelling Ratio \%} = \frac{W_s}{W_a} \times 100 \quad (1)$$

where Wa is the weight of hydrogel samples dried for 12 h at 60 °C and Ws is the weight of hydrogel samples soaked in PBS at 37 °C.

Characterization of optimum formulation

Based on the results from the optimized formulations, the composition of the hydrogel was sorted as shown in Table 3. The formulations were characterized in terms of swelling and rheological properties.

Rheological Properties

This test was conducted using a Rheometer (HAAKE MARS, Thermal analysis, Germany) and analysed using the RheoWin version 3.61.0000 software. The instrument uses cone and plate geometries and the base plate temperature was maintained at 32 ± 0.05 °C using a universal temperature controller. The spindle is PP35 Ti with a diameter of 35 mm. The linear viscoelastic region (LVR) was measured through oscillation stress sweep test. The shear rates for the preformed stress sweep test were between 0.01 to 1000 Pa.

Table 3: Hydrogel composition for each formulation

Ingredient	F1	F2	F3	F4
PVA % (w/v)	10	10	10	10
PEG % (v/v)	6	6	6	6
No. of F–T cycle	3	3	3	3
Honey conc. % (w/v)	Distilled water	40	40	40
Agar conc. % (w/v)	0.25	0.25	0.25	0.5
Glycerol % (w/v)	1	1	Distilled water	1

In vivo healing efficacy test on the animals

Animal care and handling was carried out as described by Azis et al., (2017). Males New Zealand White Albino rabbits weighing from 1.7 to 2.5 kg were used for the study. The rabbits were placed individually in aluminium cages throughout the study in a holding room with the temperature at 23±2°C, relative humidity of 45% to 55% and 12-hours light/dark cycles. The acclimatization period for the animals were seven days. Animal study was approved by Institutional Animal Care and Use Committee (IACUC), International Islamic University Malaysia with approval number IIUM/IACUC- 2019 (7).

Wound induction

The hair on the dorsal thoracic region of the rabbit was shaved at the beginning of the test. Then, rabbits were anesthetised with intramuscular injection of Ketamine: Xylazine (0.4 mL: 0.1 mL). The shaved area was cleaned and disinfected with 70% ethanol. By using a biopsy punch, a 4 mm diameter excision wound was inflicted. (Azis et al., 2017) The wounding day was considered as Day 0. The wounds were categorized as no treatment, blank hydrogel treatment and honey incorporated hydrogel treatment. The wounds were treated once daily until they completely heal. The arrangement of the dressings was shifted randomly in Animal 1 to Animal 4 to avoid bias in term of location of the wounds or environmental effect that could influence the assessment of healing process.

Measurement of wound area

At the Day 0, Day 3, Day 6 and Day 9, the image of the wounds was captured using the videoscope probe of the Dermal Lab (Dermalab Series Skinlab Combo, Denmark) and their size were measured.

The relative wound size reduction was calculated according to Eq. (2).

$$Relative\ wound\ size\ reduction\ (\%) = \frac{(A_\circ - A_t)}{A_\circ} \times 100 \quad (2)$$

where Ao is wound size at the initial time and At is the wound size at the predetermined time.

Histopathological studies

After nine days of treatment, the rabbits were sacrifice by carbon dioxide overdose (Leary, Underwood & Anthony, 2013). The wound tissues were extracted and fixed immediately in 10% formalin. The tissues were dehydrated by using ethanol and toluene solution before embedded in paraffin wax. The paraffin-embedded tissues were sliced by using a microtome (RM 2135, Leica, Germany) for staining either by Haemotoxyllin & Eosin or Mallory's Trichrome. The thickness of the epidermis and dermis layer of the regenerated wound on the histology slide was measured using Leica Application Suite software (version 4.0) together with Image-J software (version 1.50i) at 10 different random locations

Statistical Analysis

Statistical evaluation was carried out using the IBM SPSS statistics version 20 and Microsoft Office Excel 2016. Significant differences between the treated groups and the control were determined by one-way ANOVA using Kruskal-Wallis test, with a significance level of $p < 0.05$.

Results and discussion

Fig. 2. Stingless bee honey-based hydrogel.

Fig. 2 displayed the hydrogel that been prepared. The PVA types were carefully selected based on several literature related to hydrogel formulations and after a few trials PVA with relatively lower molecular weights (145,000–195,000 MW and 146,000–186,000 MW) were omitted from this study since they were unable to produce a stable hydrogel with good characteristics. The concentration of PVA was capped to 10% due to the risk of irritation to the skin and eye (Rowe et al., 2009). A relatively high molecular weight, PEG 400, was selected due to its flexible chain structure, leading to a better hydrophilic portion to the hydrogel chain (Ghobadi Jola et al., 2018). The PEG plays an important role in the fabrication of the hydrogel where it acts as a plasticiser to assist the mobility of the molecular chain and reduces the hydrogel stiffness (Laboulfie et al., 2013). On the other hand, the concentration of PEG was considered based on the results from the Design of Experiment (DOE) software in which the higher concentration of PEG was predicted to produce better hydrogel characteristics.

Recently, the combination of natural and biopolymers with synthetic polymers as a product had gained increasing attention. This due to its better characteristics in terms of the increased number of polymer chains, chemical derivation convenience, and better biocompatibility (Kamoun et al., 2017). Agar was an example of a biopolymer selected to enhance the cross-linking between the PVA–PEG and eventually improve the mechanical structure of the hydrogel. The hydrogel with agar incorporated displayed better strength and flexibility. In addition, agar as a polysaccharide polymer, has a good response towards blood, mucosa, and tissues. Hence, it can be a good drug carrier and may improve drug release as well (Das & Pal, 2015; Koneru et al., 2020).

Screening of the factors for hydrogel synthesis

Table 4 indicated three factors that could influence the swelling ratio response which are PEG concentration (X1), agar concentration (X2), and number of cycles (X5). The main effects of these factors also are presented by the Pareto chart provided in Fig. 3. The bar lengths in this chart are proportionate to the 95% confidence level of the estimated effects to the absolute value, in which the orange bars denote positive influence and the blue bars denote negative influence. According to the Pareto chart, the number of cycles (X5) displayed the most significant and positive effect on the swelling ratio in which an increase in the number of cycles will increase the swelling ratio value. PEG concentration (X1) also gave a positive and significant effect on the response, while agar

concentration (X2) contributed significantly to a

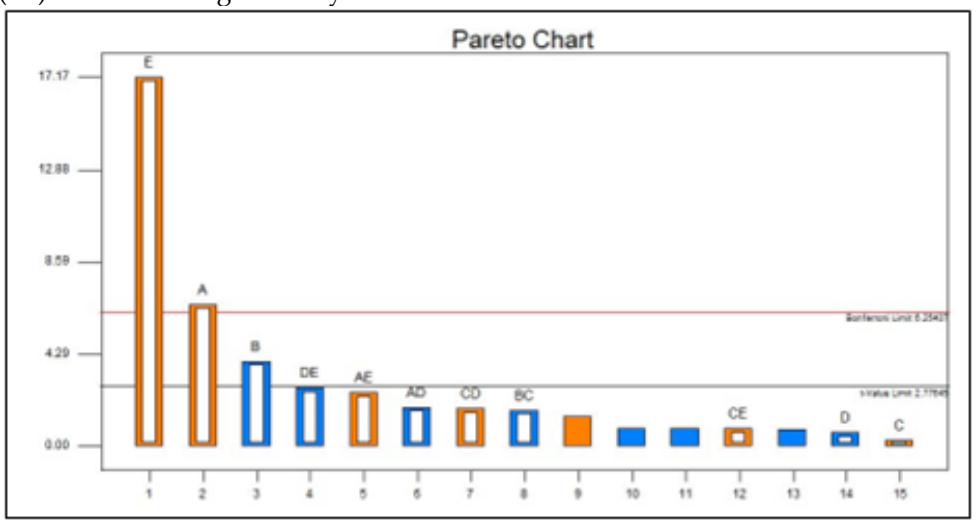

Fig. 3 Pareto chart showing significant factors that could influence the swelling ratio of the formulation where A) PEG, B) agar, C) glycerol, D) temperature, and E) number of cycles.

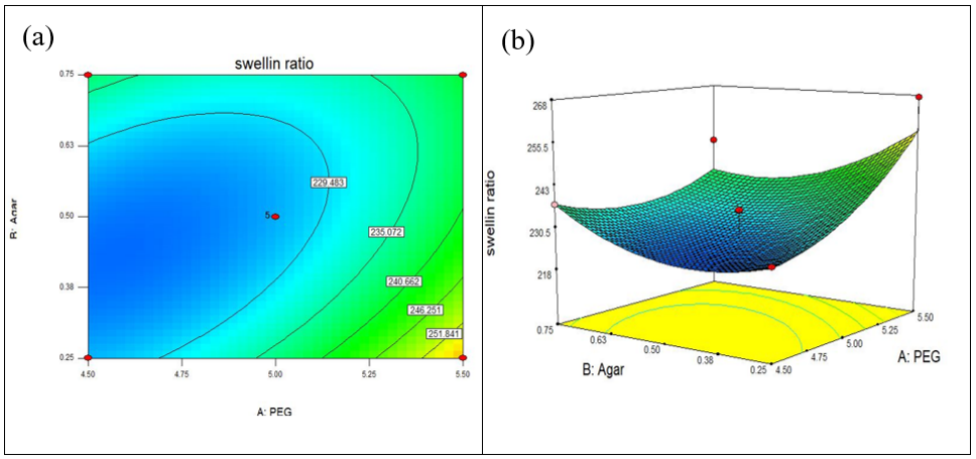

Fig. 4 Contour plot (a) and response surface (b) of swelling ratio as function of PEG and agar concentrations. The blue colour (darkened region) represents the area where the prediction is unreliable while brighter region (green and yellow), represent the area where the prediction is reliable due to enough information collected.

Table 4. Analysis of variance (ANOVA) of the factors.

Source	Mean Square	*F* value	*p*-value Prob > *F*
Model	2248.51	34.40	0.0019*
A-PEG	2860.31	43.76	0.0027*
B-Agar	1021.14	15.62	0.0168*
C-Glycerol	5.23	0.080	0.7913
D-Temperature	26.69	0.41	0.5576
E-Cycles	19273.41	294.84	< 0.0001*
AD	219.61	3.36	0.1407
AE	410.40	6.28	0.0664
BC	187.36	2.87	0.1657
CD	199.89	3.06	0.1553
CE	44.14	0.68	0.4574
DE	485.47	7.43	0.0527

*p < 0.05, significant value

negative effect on the response. The other factors were below the t-value limit line are considered as not significantly influencing the swelling ratio response.

Central composite design (CCD)

To determine the optimum value for PEG and agar concentrations for the swelling ratio response, an experiment was designed according to a face-centred CCD with the two variables following RSM. Each variable was varied at 5 levels, which are -α, -1, 0, + 1, + α, resulting in 13 experimental runs.

The contour plot and their corresponding three-dimensional (3D) response surface for swelling ratio percentage against PEG and agar are shown in Fig. 4. The swelling ratio percentage ranging from

maximum (267.51%) and minimum (218.75%).

Table 5 summarizes the ANOVA and p-value that were used to estimate the coefficients of the model in assessing the significance and interaction strength of each parameter. From the ANOVA analysis, the confidence level is greater than 95% and the p-value of the model is 0.0139, demonstrating that the model is suitable for this experiment. The R2 and adjusted-R2 are 0.8251 and 0.7002, respectively, which displayed in Table 6 indicated that the estimated model fits the experimental data satisfactorily. Since R2 for these response variables is close to 100%, which is 83%, it showed that the regression models explain the mechanism well and contains a good correlation between the predicted and observed values(Diwan et al., 2021).

Table 5. ANOVA for response surface quadratic model.

Source	Mean Square	*F* Value	*p*-value Prob > *F*
Model	537.50	6.61	0.0139*
A-PEG	800.05	9.83	0.0165*
B-Agar	40.15	0.49	0.5050
AB	188.07	2.31	0.1722
A^2	776.22	9.54	0.0176*
B^2	1325.21	16.29	0.0050
Residual	81.36		
Lack of Fit	96.25	1.37	0.3720
Pure Error	70.19		
Correction total			

*p < 0.05, significant value

Table 6. The value of fitting the model parameter.

Parameter	Value
Standard deviation	9.02
Mean	239.55
CV percent	3.77
R^2	0.8251
Adjusted-R^2	0.7002
Predicted-R^2	0.1218
Adequate precision	6.464

Characterization of hydrogel formulation

Swelling ratio

Formulation 2 with 40% (w/v) honey that contained glycerol indicates a significantly ($p<0.05$) higher swelling ability than others, while the control formulation without honey displays the lowest swelling ability as been shown in Fig. 5. The swelling ratio determines the capacity of the formulation to absorb fluids such as the wound exudate when applied as a wound dressing. A high value of swelling ratio indicates a better absorption capacity. As the number of different components in the formulation increases the swelling ratio decreases since the porosity of the hydrogel may be higher. This reduction is likely due to enhanced intermolecular interactions and a denser cross-linked polymeric network, which can limit the free volume available for water absorption. However, the addition of PEG 400 could promote the absorption capacity of the formulation and it is postulated the presence of agar can hinder its swelling ability. Pal et al., (2007) suggested that a swelling capacity of 260% for a hydrogel membrane causes it to be super absorbent.

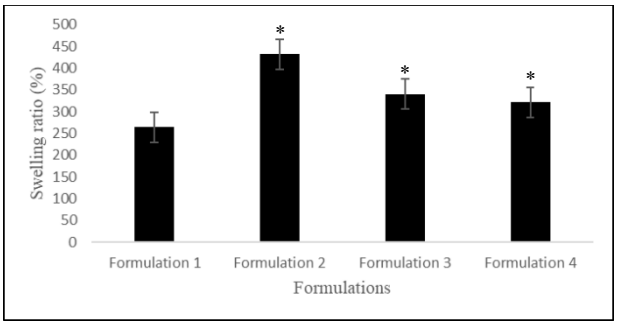

Fig 5. Effect of the formulations on the swelling ratio. The asterisks (*) indicated significant differences ($p< 0.05$) compared to the control group (Formulation 1). Results are expressed as mean ± standard error mean (SEM) (n = 3).

Rheological Properties

Fig. 6a, 6b, 6c, and 6d display the rheological properties for the Formulation 1,2,3 and 4respectively. Storage modulus, represented by G′, is the maximum stored energy available to pull back when shear stress is applied. Loss modulus, represented with G″, is the deformation energy that dissipates when shear stress is applied (Engleder et al., 2014).

The control formulation (Formulation 1) has a poor rheological property due to its low critical strain point, which was approximately 85 ± 5 Pa. The critical strain point is the point at which the network structure of the gel is disrupted. Formulations 2 and 3, with the highest concentration of honey, showed the highest value of critical strain point, which were approximately 900 ± 50 Pa. Formulation 3 that did not contain glycerol, on the other hand, showed a better and clearer LVR line. Therefore, Formulation 3 possess the best rheological properties among the formulations.

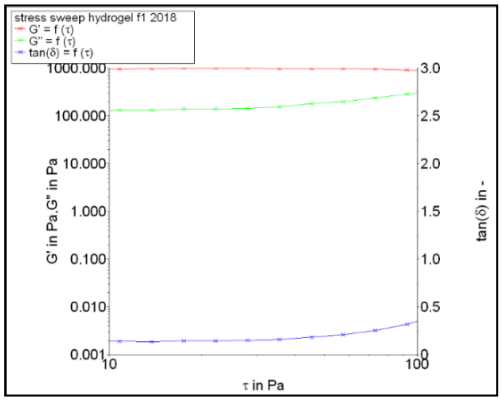

Fig. 6a. Formulation 1

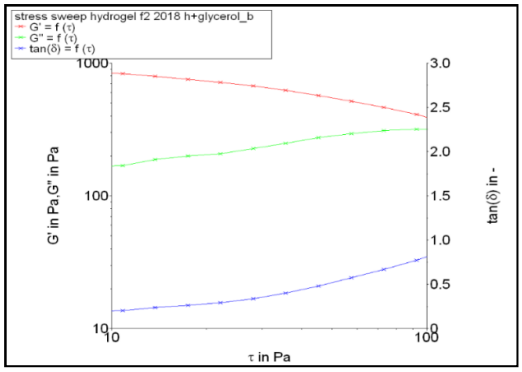

Fig. 6b: Formulation 2

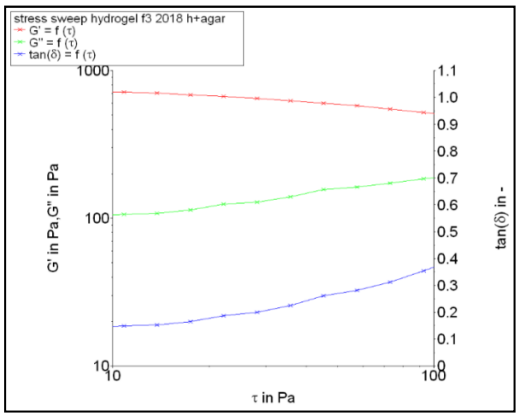

Fig. 6c: Formulation 3

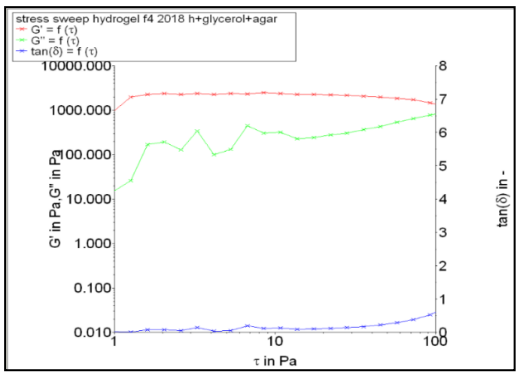

Fig. 6d: Formulation 4

The rheological properties of these hydrogel were studied in terms of liner viscoelasticity range (LVR) by using stress sweep test. The LVR indicates the range of shear stress that would cause the network between the polymer to still be interconnected. Whereas, beyond the LVR range, the cross-linking between the polymer network is broken, thus reducing its elasticity (Weiss, 2014). From the rheological testing results, the values of G′ in all formulations were relatively higher than the value of G″. This indicates that these formulations possess viscoelastic behaviour and are stable upon storage due to their higher elasticity compared to their viscosity.

In vivo wound healing test

Wound contraction is determined by the reduction of the unhealed area, where a higher contraction indicates a better rate of healing. Fig. 7 displays the percentage of contraction over time for the three different group of rabbits, whereby the rate of healing varied among the groups. Formulation 3 was selected for in vivo experiments since it possessed a lot of good physicochemical properties compared with other formulation.

On day 3 the percentage of wound contraction in the honey hydrogel group was significantly higher ($p<0.05$) than in the no treatment group (18.8 ± 3.3% vs. 11.3 ± 2.0%) and this trend continued in day 6 (66.1 ± 2.3% vs. 36.0 ± 5.1%) and day 9 (98.6 ± 2.3% vs. 88.8 ± 3.2%). It is important to point out that on day 6, the percentage of wound closure in the honey hydrogel group was also significantly higher than the blank hydrogel (66.1 ± 2.3% vs. 40.7 ± 0.7%).

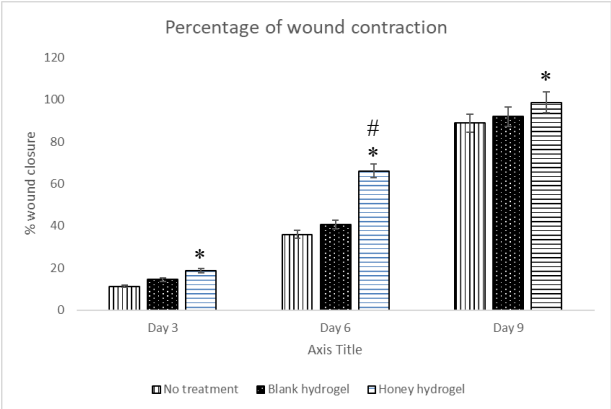

Fig 7 Percentage of wound closure for different groups form day 0 to day 9. The asterisks (*) indicated significant differences ($p< 0.05$) compared to the no treatment group. The hashtag (#) indicated a significant difference compared to the blank hydrogel group ($p<0.05$). Results are expressed as mean ± standard error mean (SEM) (n = 4).

Histological analysis

Histopathological study is used to observe the pathological changes at a microscopic level. The wound tissues were stained with H&E as well as

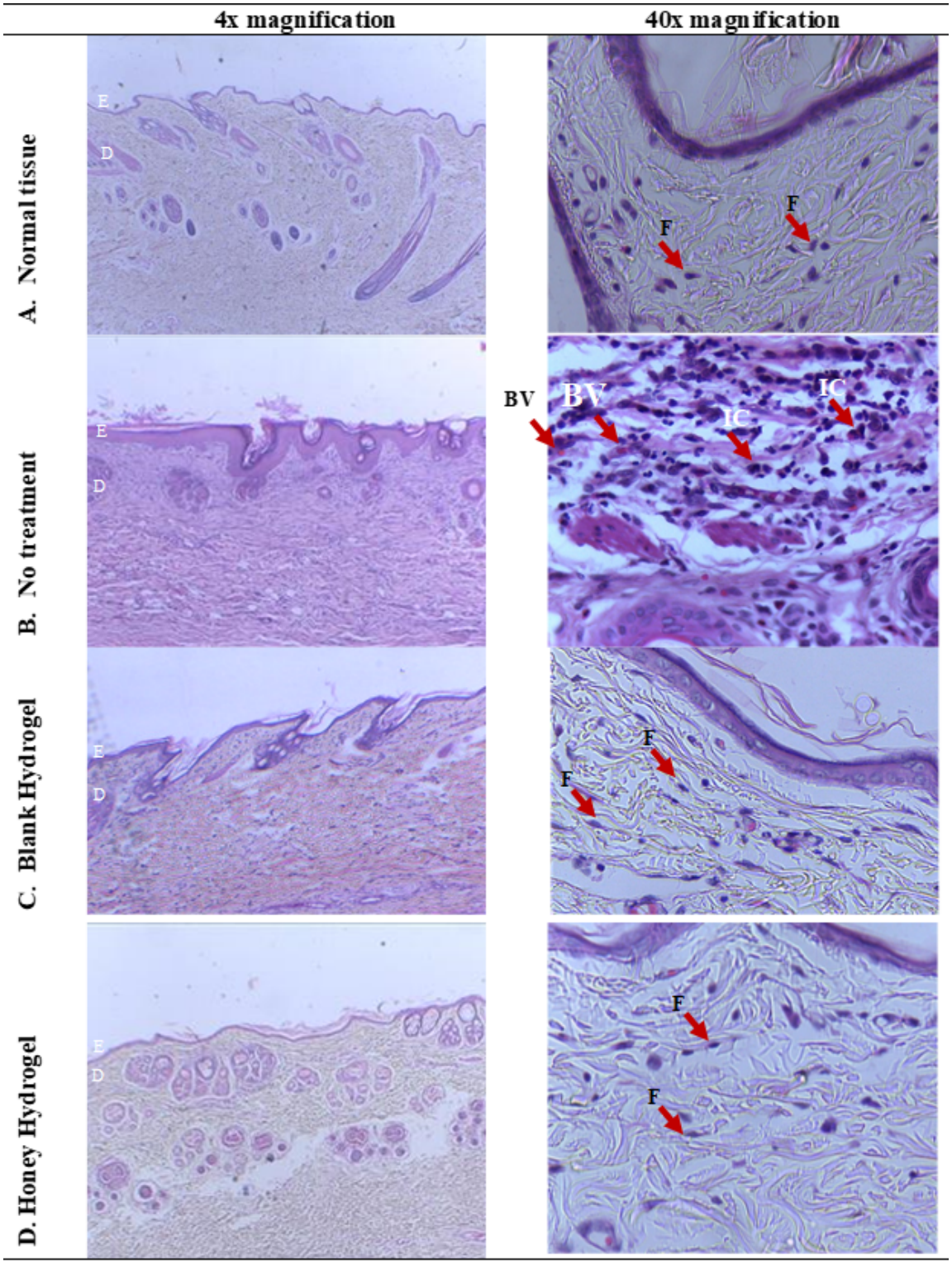

Fig 8. Histological observation of the skin at day 9 under 4x and 40x magnification for H&E. E = epidermis layer; D = dermis layer; BV = blood vessel, IC = inflammatory cells, F = fibroblast. Scale: 10325.9 pixels/cm.

Mallory trichrome. In addition, normal skin tissue was also stained with H&E and Mallory trichrome for baseline comparison with the wounded tissue. In normal tissue histology (Fig 8), it can be observed that there is a thin epidermis and the presence of many skin appendages such as hair follicles and sebaceous glands.

In the no treatment group, there were a lot of blood vessels and inflammatory cells seen when compared to the honey hydrogel group. Clumps of multinucleated cells, which are the distinguishing

feature of inflammatory cells, can be observed with H&E staining (Fig. 8B). On the other hand, in the stingless bee honey hydrogel group (Fig. 8D) the presence of inflammatory cells was much lower, and the arrangement of the fibroblasts, which are single nucleated cells, were much more organised. The higher number of fibroblasts represents an ongoing active healing process at the wound site as they play an important role in restoring damaged tissue. Therefore, it can be postulated that on day 9 the wounds in the no treatment group were still in the transition between the inflammation and proliferative phases while for the other two groups they have progressed to the following phase, which is the remodelling phase.

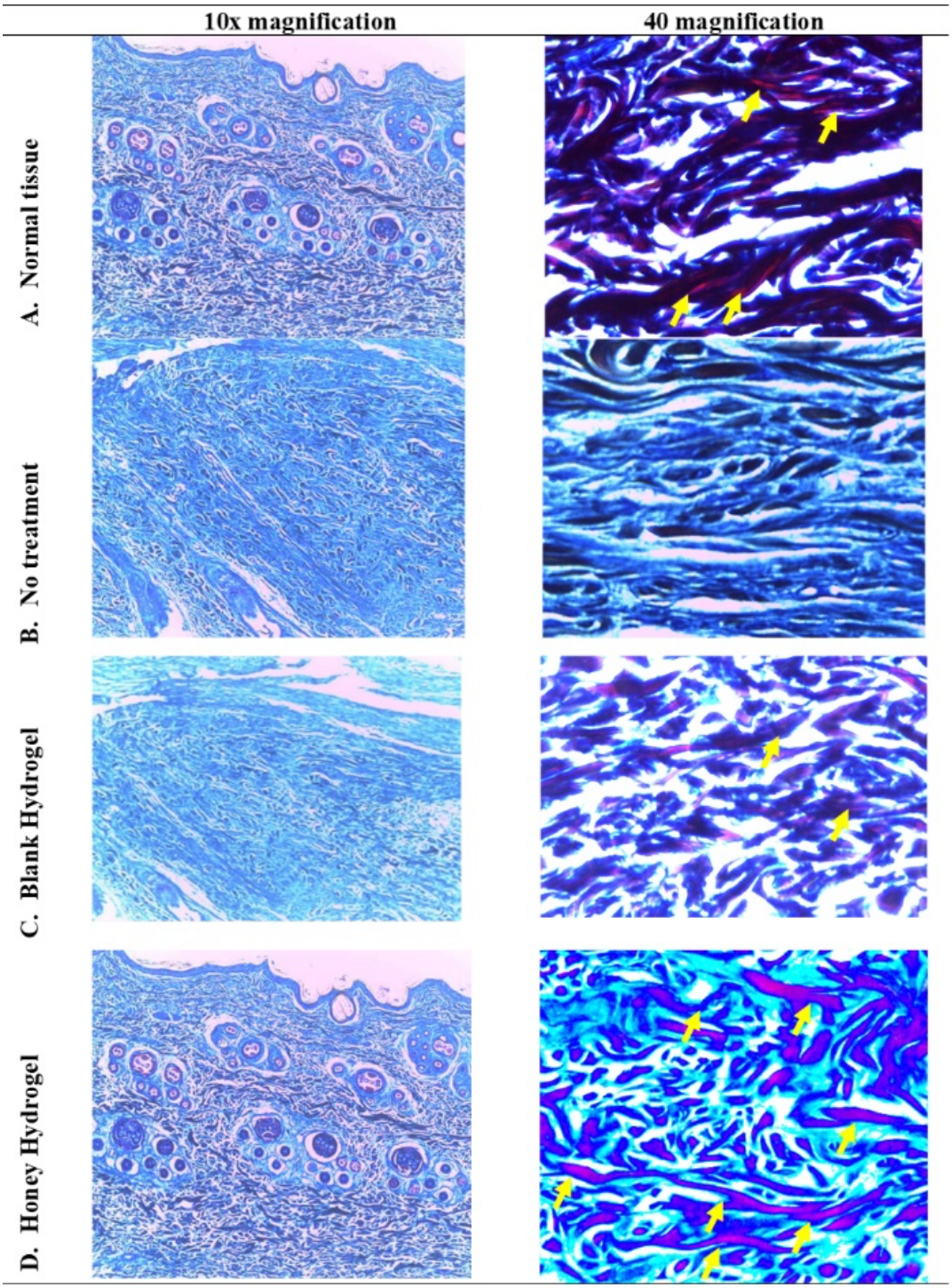

Fig 9. Histological observation of the skin at day 9 under 10x and 40x magnification with Mallory trichrome staining. Yellow arrows (➡) demonstrated the presence of collagen fibres. Scale: 10325.9 pixels/cm.

In the remodelling phase, the collagen fibres, fibroblast and keratinocytes will be elevated, leading to a well-organised structure that eventually resembles normal tissue. There is a balance between the synthesis and degradation of collagen and other proteins at the wound site to improve the wound structure. In the end, the type 1 collagen present during the healing phases will be replaced with mature type 3 collagen that resembles normal tissue (Singh et al., 2017).

In Fig. 9, using Mallory trichrome staining, the histological arrangement of collagen fibres can be observed in all tissues, except in the no treatment group (Fig. 9B). The collagen fibres are much more well-structured and in greater amount in the stingless bee honey hydrogel group, thus, it can be postulated that the structure of the tissue in this group would be stronger when compared to the other groups due to the role of collagen that supports the tissue structure (Maçin, 2021).

Fig. 10a shows the dermis thickness of the no treatment group is significantly higher than the normal group. Besides fibroblast, collagen deposition also leads to dermal thickness. In the proliferative stage, the collagen and fibroblast were disorganised in contrast with remodelling phase, where the cross-linkage between collagen fibres rearrange the structure of the dermis (Lee et al., 2017). In contrast, the thickness of honey hydrogel group closely resembles the normal tissue. That can be an indication of collagen fibres in this group is more organised than the others.

In Fig. 10b, the epidermal thickness layer of skin in the no treatment group was significantly ($p<0.05$) higher than the normal tissue. The main reason for the increased epidermal thickness is the changes in cytokine concentration that affect the ability of epithelial cells to proliferate more rather than differentiate, in order to overcome the loss of epidermal structure after a wound insult (Jacków et al., 2016). Eventually, in the remodelling phase, the thinning of epidermis would occur by the cornification of epithelial cells to reinstate the structure as before (Sullivan & Myers, 2021). Since the thickness of epidermis in honey hydrogel group is significantly ($p<0.05$) thinner than the no treatment and closely mirrors normal tissue, this can support the fact that the wound treated with honey hydrogel was at the more advance remodelling phase as compared to the no treatment group.

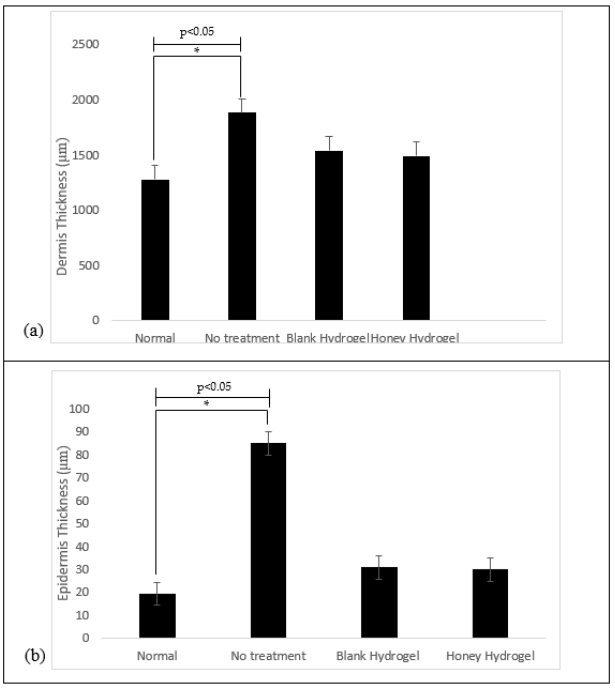

Fig 10a. Thickness of (a) dermis and (b) epidermis layers. The asterisks (*) indicated significant differences ($p< 0.05$) compared to normal tissues.

In wound contraction percentage, stingless bee honey-based hydrogel displayed a significant ($p<0.05$) increase in healing than the no treatment group. The result was consistent with the histological observations of the wound (Fig. 8 and Fig. 9) together with the dermal (Fig. 10a) and epidermal (Fig. 10b) thickness measurements, where the stingless bee honey-based hydrogel features were similar with the normal tissue. These findings were proven by gross observation via the videoscope images generated by Dermal Lab (Dermalab Series Skinlab Combo, Denmark) as seen in Fig.11.It can be observed that at day 9, the wound is completely healed for the stingless bee honey hydrogel treatment group when compared to the others. Furthermore, the closure of the wound in stingless bee honey hydrogel treatment is much better compared to the others from day 3 onwards. The stingless bee honey incorporated hydrogel displayed a significant improvement in the rate of wound healing, primarily due to the therapeutic effects of honey in the formulation. Honey stimulates immune cells, promoting wound debridement and accelerating healing. (Masad et al., 2021). In addition, the presence of honey positively influences the fibroblast proliferation, granulation tissue formation and collagen synthesis (Majtan, 2014) which could mediate the process of wound contraction. After the wound insult, growth factors (TGF- β and PDGF) stimulates the proliferation of fibroblast which subsequently results in the

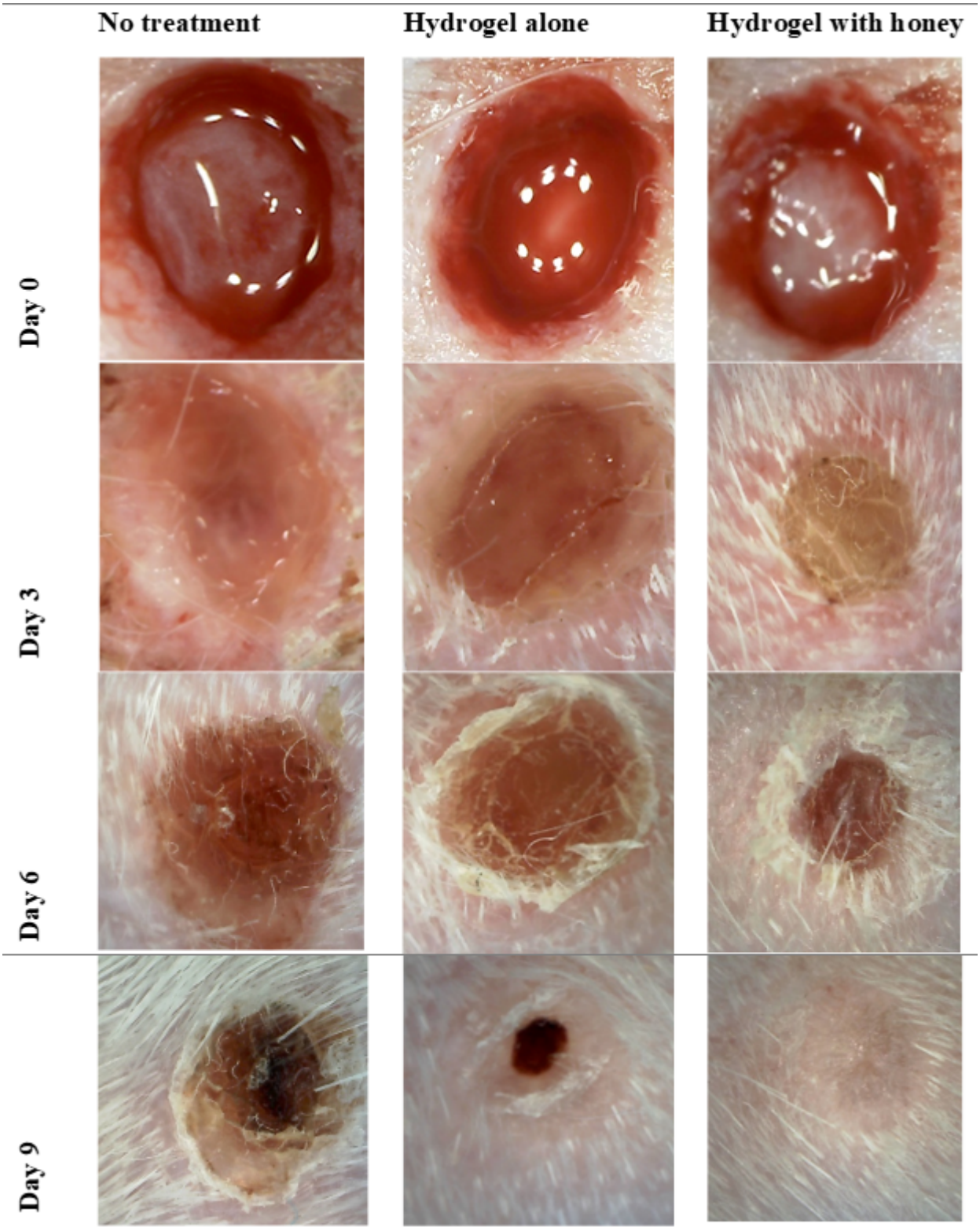

Fig 11 Gross wound observation from day 0 to day 9 Scale: 1834.72 pixels/cm.

formation of granulation tissue and an abundance of collagen. Collagen play an important role in providing strength to the matrix for contraction of the wound to occur. The action of actin and myosin in the matrix will draw the edge of the wound closer and thus decrease the wound size (Maçin, 2021). Presence of hydrogel also plays an essential part in promoting wound healing. As previously mentioned, hydrogel could provide a moist condition at the wound site, which could reduce dermal necrosis and promote wound reepithelialisation (Ahmad et al., 2021; Gull et al., 2019). Reepithelialisation of wound also occurs during the wound contraction later. Contraction of the wound must occur for the number of epithelial cells to be reduced significantly in the wound area and for epithelialisation to follow. Keratinocytes will then migrate from the edges of the wound and proliferate to reconstruct the epidermis structure. The epithelialisation period is essential to the fate of the wound, where a longer period will result in a scar formation at the later phase over many weeks (Loh et al., 2018).

In the epidermal and dermis layers, the blank hydrogel and honey did not display any significant difference, but in term of wound contraction, there were significant differences ($p<0.05$) at day 6. This indicated that blank hydrogel only promotes the thinning of the skin structure while honey hydrogel promotes thinning of the skin and the arrangement of the collagen fibres to assist in the wound contraction.

Honey could promote the wound contraction by stimulating fibroblast and re-epithelization that eventually promotes proliferation of keratinocytes to the skin surfaces(Oryan et al., 2016). The hydrogen peroxide possessed by honey can stimulate the fibroblast proliferation which contributes to the arrangement of the cells and collagen during the proliferation phase of the healing process (Al-Jadi et al., 2014). In addition, the glucose content and minerals in honey could promote epithelial migration across the wound surface and keratinocyte proliferation (Al-Masaudi et al., 2020). .

Conclusion

The experimental design by using RSM was successful in developing stingless bee honey-based hydrogel. After performing the characterization tests, Formulation 3 displayed good swelling ability, rheological property and antimicrobial activity. For wound healing assessment, stingless bee honey-based hydrogel demonstrated promising healing properties as proven by using gross appearance and wound contraction percentage where it was significantly ($p<0.05$) better than the untreated group. This result was further supported qualitatively via histological observations and quantitatively via epidermal and dermal thickness measurements. The result suggested that stingless bee honey-based hydrogel was able to promote full thickness wound healing by promoting wound contraction, re-epithelisation as well as collagen formation. In addition, since blank hydrogel consisted of PVA-PEG-agar composition that was able to imbibe water content, it has the ability to maintain a moist condition and provide an occlusive effect to the wound. Therefore, it implies that the introduction of stingless bee honey hydrogel could provide a synergistic effect with the PVA-PEG hydrogel to enhance the rate of wound healing where honey hydrogel promotes thinning of the skin structure which immediately followed by better wound contraction.

Authors contributions

Conceptualization, M.A.A.J and H.H.; Experimental studies M.A.A.J., M.L.M.I, U.A and H.H.; Data and statistical analysis, M.A.A.J and M.L.M.I; Manuscript preparation, M.A.A.J and U.A; Manuscript editing, M.A.A.J, U.A., K.A.H and H.H; Manuscript review, K.A.H, H.H and M.A.B. All authors have read and agreed to the published version of the manuscript.

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Ethical approval statement (if applicable)

The animal study protocol was approved by the

Institutional Animal Care and Use Committee (IACUC), International Islamic University Malaysia with approval number IIUM/IACUC-2019 (7).

Conflict of interest

The authors declare that there are no conflicts of interest to disclose

Declaration of generative AI and AI-assisted technologies in the writing process

A few artificial intelligent tools (e.g ChatGPT, Gemini and DeepSeek) were used to assist in improving the readability and language in certain parts of this work. The authors have reviewed and edited the content as necessary and take full responsibility for the final content of the publication.

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A Phytochemical Profiling and *in vitro* Antimicrobial Evaluation of Methanolic Extract and Fractions of *Dicranopteris linearis* Leaves

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Abstract

Introduction: *Dicranopteris linearis*, locally known as resam, has been recognized for its potential health benefits, primarily due to its rich phytochemical content. Traditionally used for medicinal purposes, the leaves are known to possess antioxidant and antimicrobial properties. This study aimed to screen the phytochemical composition and evaluate the antimicrobial activity of the methanolic extract of *D. linearis* leaves, with potential applications in the medicinal industry. **Materials and Methods:** The dried and ground leaves of *D. linearis* were macerated in 100% methanol to extract the phytochemicals. The extract was subjected to qualitative phytochemical profiling. Total Phenolic Content (TPC) was determined by the Folin-Ciocalteu method and Total Flavonoid Content (TFC) was measured using the $AlCl_3$ method. The methanol extract was fractionated by Vacuum Liquid Chromatography (VLC) with ethyl acetate (100%), ethyl acetate: methanol (5:5) and methanol (100%). Antimicrobial activity of the crude extract and fractions was assessed against *Escherichia coli* and *Staphylococcus aureus* using the disc diffusion assay and broth microdilution techniques. **Results:** Phytochemical profiling of the methanol extract revealed the presence of phenolic compounds, flavonoids, tannins, and saponins. The TPC and TFC assays showed that the methanolic extract contained 225.43 ± 4.16 mg GAE/g of phenolic compounds and 50.20 ± 4.78 mg QE/g of flavonoids. Fractionation of methanol extract was afforded three fractions, F1, F2 and F3. Antimicrobial testing demonstrated that the extract exhibited stronger activity against *S. aureus* (MIC = 1.563 mg/mL) compared to *E. coli* (MIC > 50 mg/mL). For the fraction, F1 exhibited both microbes with promising activity. **Conclusions:** The methanolic extract from *D. linearis* leaves contain bioactive phytochemicals with significant antioxidant and antimicrobial properties. These findings suggest that *D. linearis* may serve as a valuable source for the development of natural antimicrobial agents in the pharmaceutical industry

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Introduction

Dicranopteris linearis, commonly known as resam, is abundant in Southeast Asia and has been used traditionally for its medicinal benefits. The plant contains various bioactive compounds, including terpenoids, tannins, saponins, flavonoids, alkaloids, steroids, phenols, and glycosides (Rajesh et al., 2016). Previous studies have demonstrated that *D. linearis* leaves, rich in these phytochemicals, offer various potential applications, including antioxidant, anti-inflammatory, and hepatoprotective properties (Kamisan et al., 2014). One particularly promising application is its antimicrobial activity.

Wound pathogens are critically important in antimicrobial studies because they are key contributors to infection, delayed healing, and complications in clinical settings. They can be caused by bacteria such as *Staphylococcus aureus* (including MRSA), *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella* spp. Studying them helps in the development, testing, and evaluation of antimicrobial agents, especially in the context of antibiotic resistance and wound management. Studying wound pathogens also helps in testing new antimicrobials or alternative therapies e.g. herbal extracts and phage therapy. With the increasing prevalence of antibiotic-resistant pathogens, the need for natural, safe antimicrobial agents is urgent. Lai et al. (2021) investigated the antimicrobial properties of *D. linearis* methanolic and acetonic extracts, finding activity against pathogens like *M. luteus*, *E. coli*, *P. aeruginosa*, and *S. aureus*. However, these studies used multiple organic solvents and a broad range of bacterial strains.

Our study aims to provide more focused results on the phytochemical composition and antimicrobial activity of *D. linearis* leaves. (**Fig. 1**)., particularly against common wound pathogens. By narrowing our investigation to clinically relevant bacteria which are *S. aureus* and *E. coli*, we intend to evaluate the specific potential of *D. linearis* as a natural antimicrobial agent for wound management. In contrast to previous broad-spectrum studies, we will utilize a single extraction method to ensure consistency and better isolate the correlation between the extract's phytochemical content and its antibacterial efficacy. This targeted approach aims to contribute valuable insights toward the development of plant-based alternatives in combating wound infections, especially in light of increasing antibiotic resistance

Fig. 1: *D. linearis* leaves (picture taken on 18 October 2022)

Phytochemical Profiling Methods

Phytochemicals from plant material, usually extracts of plant, need identification to predict the potential and provide evidence that support medical claims against various ailments. Gas Chromatography (GC), Liquid Chromatography (LC), and High-Performance Liquid Chromatography (HPLC) are advanced techniques that can be helpful for identification and characterizing phytochemicals both qualitatively and quantitatively. However, conventional methods are still utilized to do preliminary profiling of phytochemicals when these methods are unavailable or unaffordable. The qualitative profiling of the phenolic compounds can be done with iodine test as simplest test. The procedure is to add a few drops of iodine solution to plant extract. The result will indicate positive test if it shows a transient red color. The qualitative profiling of tannins can be done using various tests, namely Gelatin test, Braymer's test, and another tests. It is done in a simple procedure, mostly using specific reagents. The test will be observed as positive when the color changes into a specific color that indicates positive test (Shaikh & Patil, 2020). The qualitative test for saponin can be done with foam high test. The procedure of this test is to use distilled water and a few drops of extract dissolved in solvent to a test tube and shake vigorously. Forming a foam means it contains saponin and no foam means absence of saponin (María et al., 2018).

Antimicrobial Activity Methods

The Kirby-Bauer method is an antimicrobial susceptibility test developed in 1966 by Alfred Bauer, William Kirby, and other associates. This method is suitable to test the antimicrobial activity of aqueous suspensions of plant extract. Several disks containing plant extracts would be placed in an agar plate that has been inoculated with the target organism. The plates are incubated to permit bacterial growth and diffusion of the antimicrobial agent into the agar. As the drug is diffused into the agar, a concentration gradient is formed. If the organism is susceptible to the agent, a clear area, also called an inhibition zone, will form around the disc, showing that the concentration is sufficient to inhibit growth. The advantages of this method are simple, low cost, and easy to interpret the results. However, this method lacks automation and is not suitable for fastidious bacteria, it also not the appropriate methods to determine the Minimum Inhibitory Concentration (MIC) because it's impossible to quantify the antimicrobial agent amount diffused in agar medium (Salem et al., 2016).

The serial dilution methods are the most appropriate methods to determine the Minimum Inhibitory Concentration (MIC) of certain antimicrobial agents. MIC is defined as the minimum concentration of antimicrobial agents to inhibit the growth of microorganisms. Either broth or agar dilution method may be used to quantitatively measure the *in vitro* antimicrobial activity against bacteria and fungi. In this test, microtiter plates containing serial dilutions of antimicrobial agents are inoculated with the target microorganisms and incubated to allow microbial growth. The clear tubes or lowest MIC result indicated that the agents successfully inhibit microbial growth. The advantages of this test are the simple determination of MIC, the convenience of having prepared panels, and the miniaturization of the test. The disadvantages of this test are some inflexibilities of drug selection available in standard panels (Balouiri, Sadiki, & Ibnsouda, 2016).

Bacteria strain for Antimicrobial Test

Staphylococcus aureus is a Gram-positive bacterium known to be an aerobic pathogen involved in various diseases affecting both humans and animals. Its growth depends on temperature, typically ranging between 18°C and 40°C. Remarkably, it can survive in freezing conditions below –20°C. The optimal pH for *S. aureus* growth falls between 4.0 and 10.0 (Rasheed & Hussein, 2021). Additionally, it has been reported that 21.8% of *S. aureus* strains are resistant to oxacillin (Bessa, Fazii, Di Giulio, & Cellini, 2015).

Escherichia coli is a Gram-negative, coccobacillus-shaped microorganism that naturally resides in the digestive tracts of humans and animals, as well as in their feces, where it acts as a decomposer. However, *E. coli* contamination in food can lead to serious health issues, including haemolytic uremic syndrome, haemorrhagic colitis, food poisoning, and diarrhea. The bacterium grows best at temperatures between 35°C and 37°C and within a pH range of 7.0 to 7.5 (Romadhon, 2016).

Materials and methods

Extract Preparation

D. linearis leaves (voucher specimen no: UKMB-PP 01248) as seen in **Fig. 1** were collected from the forest area in Kulliyyah of Pharmacy, Kuantan, Pahang. The leaves were separated from the stem and air-dried for several days. Subsequently, the leaves were oven-dried at 50-60°C for 48 hours to remove moisture completely. The dried leaves were ground into powder and macerated in 100% methanol for 48 hours (Azwanida, 2015). The mixture was then filtered using Whatman No.1 filter paper, and the solvent was evaporated using a vacuum rotary evaporator (Buchi). The crude extract was stored in a chiller until further use.

Qualitative Phytochemical Profiling

Phytochemical profiling was conducted to identify the presence of various phytochemicals in *D. linearis* leaves extract. The $FeCl_3$ test (Roghini & Vijayalakshmi, 2018) was used to detect phenolic compounds, while the alkaline test (Pant et al., 2017) was used for flavonoid detection. Tannins were identified using the Braymer's test (Saswade, 2019), and saponins were detected using the foam test (Shaikh & Patil, 2020).

Total Phenolic Content (TPC)

TPC of the methanol extract was determined using the Folin-Ciocalteu method (Zain & Omar, 2018)

with minor modifications. To each well, 10 µL of sample (2 mg/mL) was added, followed by 50 µL of Folin-Ciocalteu reagent (50% v/v). After a 5-minute incubation at room temperature, 40 µL of Na_2CO_3 solution was added, and the mixture was incubated in the dark for 1 hour. Absorbance was measured at 765 nm using a microplate reader. The TPC was calculated from a gallic acid standard curve, and the results were expressed as gallic acid equivalents per gram dry weight (mg GAE/g). The assay was performed in triplicate.

Total Flavonoid Content (TFC)

TFC of methanol extract was measured using the $AlCl_3$ colorimetric method (Zain & Omar, 2018). In this assay, 100 µL of sample (2 mg/mL) was mixed with 100 µL of 2% $AlCl_3$. The mixture was incubated in the dark for 30 minutes. Absorbance was recorded at 415 nm using a microplate reader. The TFC was determined using a quercetin standard curve, and results were expressed as quercetin equivalents per gram dry weight (mg QE/g). The assay was conducted in triplicate.

Fractionation by Vacuum Liquid Chromatography (VLC)

Silica gel 60 PF254 was activated overnight at 80°C. The activated silica gel was mixed with the sample solution (diluted crude extract in methanol) and heated on a hotplate at 70°C until the solvent evaporated. A VLC column was packed with silica gel to a height of 7 cm, then compressed to about 5 cm. Filter paper was placed on top of the silica gel to prevent direct force. Hexane was poured through the silica gel and eluted with the assistance of a vacuum pump. The process was monitored to ensure that no cracks or bubbles appeared in the packed silica gel. If any crack occurred, the packing process was repeated. After eluting with hexane, the column was left overnight to stabilize. Solvent systems, which are ethyl acetate, ethyl acetate: methanol (5:5) and methanol were used to separate fractions, F1, F2 and F3, accordingly. Each fraction was collected based on observed separation. Once separation was complete, the fractions were evaporated using a vacuum rotary evaporator at 40°C.

Kirby-Bauer Disc Diffusion Method

The disc diffusion assay was performed according to the Clinical and Laboratory Standards Institute (2019), with minor modifications. A 400 mg/mL stock solution was prepared by dissolving 200 mg of crude extract in 0.5 mL of distilled water. The mixture was stirred vigorously until the crude was completely dissolved. This stock solution was then serially diluted to obtained concentrations ranging from 400-50 mg/mL. A 20 µL aliquot of each sample was pipetted onto sterile 5 mm paper discs (Whatman AA, USA). The discs were dried and stored in a chiller.

E. coli and *S. aureus* strains, obtained from the BMS Laboratory, International Islamic University Malaysia, were used for testing. The bacterial suspension was adjusted to 0.5 McFarland (approximately 10^8 CFU/mL) and swabbed onto sterile Mueller-Hinton Agar (Oxoid, UK). After drying for 5 minutes, the sample discs were placed onto the agar. 10µg gentamycin discs (Oxoid, UK) were known to have broad-spectrum antimicrobial for testing both Gram-negative and Gram-positive organisms used as the positive control that will show inhibition activity in the assay. Distilled water served as the negative control with no inhibition activity in the assay, any inhibition indicates contamination or error. The plates were incubated at 35°C for 18 hours. The inhibition zone around the discs was measured to assess antimicrobial activity. This assay was performed in triplicate.

Broth Microdilution Assay

The broth microdilution assay was performed according to Veiga et al. (2019), with minor modifications. In a 96-well microtiter plate, 100 µL of a mixture containing inoculum, microbes, and Mueller-Hinton Broth was added to each well. Gentamicin was used as the positive control, and distilled water as the negative control. The plate was sealed and incubated at 35°C for 18 hours. The Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration at which no microbial growth was observed, as indicated by the absence of turbidity in the well. This assay was conducted in triplicate.

Minimum Bactericidal Concentration (MBC)

The MBC was determined according to Senhaji et al. (2020), with slight modifications. After identifying the MIC, the samples were streaked onto sterile

Mueller-Hinton Agar and incubated at 35°C for 18 hours. The MBC was defined as the lowest concentration where fewer than 3 colonies were present, indicating a 99.99% reduction in microbial growth.

Results and Discussion

Results

Extraction Yield

Out of 78.363 gram of dried leaves powder, 15.961 gram of extract were obtained after solvent separation. Therefore, the extraction yield of *D. linearis* leaves were 20.36 %.

Qualitative Phytochemical Profiling

The qualitative phytochemical profiling result as seen in **Table 1** showed that *D. linearis* leaves methanolic extract to contain phenolic compounds, flavonoid compounds, tannin, and saponin.

Table 1. Qualitative Profiling Result

Phytochemical	Result
Phenolic	+
Flavonoid	+
Tannin	+
Saponin	+

Quantitative Phytochemical Profiling-TPC and TFC

The TPC of the methanol extract was measured against the gallic acid standard curve with the equation y = 0.0488x +0.4593 (R2 = 0.9901) and the result was 225.43 ± 4.16 mg GAE/g. The TFC was measured against the quercetin standard curve with the equation y = 0.0548x + 0.5473 (R2 = 0.9809) and the result was 50.20 ± 4.78 QE/g.

Kirby-Bauer Disc Diffusion Agar

The obtained inhibition zones from Kirby-Bauer disc diffusion agar was shown in **Table 2**. The extract and its fractions demonstrated antimicrobial activity against both tested microorganisms, with *S. aureus* showing greater susceptibility than *E. coli*. The largest inhibition zone against *S. aureus* was observed with the F1 400 mg/mL disc, yielding an inhibition zone of 10.40 mm. Similarly, for *E. coli*, the highest inhibition zone was also produced by the F1 400 mg/mL disc, although the zone was slightly smaller at 9.80 mm. The results indicated that some of the lower concentration inhibition zones exceeded 6 mm, suggesting potential bacteriostatic effects. As a result, the Minimum Inhibitory Concentration (MIC) test was conducted. The F1 50 mg/mL disc produced a 6.40 mm inhibition zone against *S. aureus*, while the CE 50 mg/mL F3 50 mg/mL discs produced inhibition zones of 6.80 mm and 6.60 mm, respectively, against *E. coli*.

MIC and MBC Results

The broth microdilution assay of the extract showed that the MIC for *E. coli* was undetermined while for *S. aureus*, the MIC was 1.56 mg/mL. This showed that *S. aureus* was more susceptible than *E. coli*. As for the MBC assay, all of the tested concentrations still have more than three colonies. Therefore, it can be concluded that the MBC was more than 50 mg/mL and the extract is a bacteriostatic antimicrobial agent.

Table 2. D. *linearis* Leaves Extract and VLC Fractions Antimicrobial Result

Sample Name	Concentration (mg/mL)	Inhibition Zone (mm)	
		E. coli	*S. aureus*
Methanol Crude	400	8.2 ± 0.84	9.2 ± 0.84
	200	7.2 ± 0.84	-
	100	7.0 ± 0.71	-
	50	6.8 ± 0.45	-
F1 (Ethyl acetate (EA))	400	9.8 ± 1.30	10.4 ± 0.55
	200	7.4 ± 0.55	8.2 ± 0.45
	100	4.2 ± 3.90	7.2 ± 0.84
	50	2.6 ± 3.58	6.4 ± 0.55
F2 (EA: Methanol, 5:5)	400	6.8 ± 0.45	4.0 ± 3.67
	200	6.4 ± 0.55	2.0 ± 2.83
	100	2.8 ± 3.83	-
	50	1.4 ± 3.13	-
F3 (Methanol)	400	7.6 ± 0.55	7.6 ± 0.55
	200	7.4 ± 0.55	6.6 ± 0.55
	100	6.8 ± 0.45	3.8 ± 3.49
	50	6.6 ± 0.55	2.2 ± 3.03
Controls			
Gentamycin (+)		23.0	27.4
Distilled water (-)		-	-

(-) means no inhibition zone was formed; Inhibition zone are depicted in four categories: Sensitive (≥15 mm), Intermediate (10-14), Potential Inhibition (6-10), Resistant (≤5 mm). Results are depicted as mean of five replicates ± standard deviation.

Discussion

Extraction is a crucial step in isolating bioactive compounds from plant materials. Solvent extraction is the most common method used, though alternative techniques such as distillation, pressing, and sublimation are also employed. Factors such as solvent choice, temperature stability, and extraction duration must be considered (Abubakar & Haque, 2020). The clinical significance of using ethyl acetate and methanol as extracting solvents lies in their distinct physicochemical properties, which influence the types of bioactive compounds they can extract. This is particularly important in phytochemical research, drug discovery, and clinical pharmacology. The profiling result of the phytochemical concedes with the study conducted by Aboshoufa & Elgubbi (2019), who reported that *D. linearis* leaves methanolic extract contains various phytochemicals such as tannins, saponins, flavonoids, steroids, phenols, steroids, terpenoids, glycosides, and reducing sugars. These metabolites were the cause of *D. linearis* leaves possessing significant antioxidative properties and other medicinal benefits, such as antimicrobial activity, gastroprotective, anti-inflammatory, and antipyretic effects (Rajesh et al., 2016).

In terms of TPC and TFC, the results from this study align with previous research by Aboshoufa & Elgubbi (2019), which reported high TPC and TFC levels in *D. linearis* methanolic extracts. The variation in results may be due to differences in extraction solvents. The TPC value of *D. linearis* leaves, roots, and stems ranging from 193.50 – 266.39 mg GAE/g. The most notable difference is between TPC obtained from extraction with different solvent selection, as shown by Zakaria et al., (2019) that used aqueous as solvent obtained less TPC than this study that used methanol as solvent, with TPC value of 193.50 ± 14.80 mg GAE/g and 239.63 ± 3.91mg GAE/g, respectively.

The plant part and solvent that were used in all three studies vary among each other, indicating these factors that influence the number of phenolic compounds that can be extracted from the plant. Compound extraction can be done if the solvent can separate the target compound from its matrix. Solvent polarity plays a crucial role in the extraction process, as solvents with polarity similar to the target compounds are more effective in extracting them. Phenolic compounds, for instance, are generally polar and are more efficiently extracted with high-polarity solvents like methanol (Gil-Martín et al., 2022). This study showed a higher extraction yield compared to the study by Ismail et al. (2014), which used distilled water as the solvent. As noted by Rasul (2018), polyphenols are best extracted with higher-polarity solvents like methanol, which may explain the difference in yield between the two studies. For future studies, non-conventional extraction methods, such as those utilizing additional energy sources, could enhance extraction efficiency (Farooq et al., 2022).

Fractionation was done with 100% ethyl acetate (F1), 50% ethyl acetate and 50% methanol (F2), and 100% methanol (F3). Ethyl acetate is suitable solvent to be used for fractionating the medium-polarity compounds from the crude extract. Meanwhile, methanol suitable to be used for fraction high-polarity compounds, such as phenols, flavonoids, tannins, and saponins. F1 shows that medium-polarity compounds inhibit *S. aureus* better than *E. coli* and effective on Gram-positive bacteria. F2 as a combination of two solvents, acts as a flush for medium polar compounds that were left behind. It didn't show any significance inhibition activity to *S. aureus* and *E. coli.* F3 shows that high-polarity compounds inhibit *E. coli* better than *S. aureus* with more consistent inhibition zone and effective on Gram-negative bacteria.

The antimicrobial activity test demonstrated that the methanolic extract and fractions of *D. linearis* leaves possess antimicrobial properties against the tested microorganisms, with a more pronounced inhibition zone observed against *S. aureus*. This finding is consistent with the study by Breijyeh et al., (2020), which suggested that the difference in inhibition zones can be attributed to the structural differences between *S. aureus* and *E. coli*. Gram-negative bacteria are generally more resistant to antimicrobial agents due to their outer membrane, composed of phospholipids and lipopolysaccharides, which acts as a barrier to certain antimicrobial compounds, especially those that disrupt peptidoglycan structure.

The antimicrobial activity of the crude extract and its fractions can be attributed to the phytochemicals present, including phenolic compounds, flavonoids, saponins, and tannins. The varied inhibition zones observed against *E. coli* and

S. aureus, with smaller zones at lower concentrations, suggest that higher extract concentrations contain more active phytochemicals. As noted by Behbahani et al. (2019), higher concentrations of extracts typically result in greater antimicrobial effects due to the higher content of bioactive compounds.

Phenolic compounds, present in the extract, are known to protect plants from pathogens by altering microbial cell membrane permeability, leading to irreversible damage (Bouarab-Chibane et al., 2019). Flavonoids, another class of compounds found in the extract, can penetrate microbial cell membranes, disrupting their integrity and leading to bacterial inhibition or even death (Kumar & Pandey, 2013). Tannins inhibit microbial growth by several mechanisms, including metal chelation, membrane and protein interaction, destabilization, and enzyme inhibition (Molino et al., 2020). Saponins, identified in this study, possess surfactant properties that interact with cell membranes, reducing surface tension and leading to microbial death (Dong et al., 2020).

A notable limitation of this study is the lack of minimum inhibitory concentration (MIC) data for *E. coli*. While the extract showed inhibitory effects in diffusion assays, the absence of a defined MIC restricts the ability to characterize whether the activity is bacteriostatic or bactericidal for this organism. The data for *S. aureus* suggest a bacteriostatic activity, as evidenced by the minimum bactericidal concentration (MBC) being higher than the MIC. This distinction is crucial because bacteriostatic agents inhibit bacterial growth without killing the organism (Loree & Lappin, 2023), necessitating an intact immune system to clear the infection. Therefore, the potential therapeutic application of *D. linearis* extract may be limited in immunocompromised individuals or in systemic infections without co-treatment. Moreover, the lack of MIC data against *E. coli* precludes meaningful comparisons between the antimicrobial potency of the extract across different bacterial species.

While the findings support the antimicrobial potential of *D. linearis* extracts, especially against Gram-positive bacteria, further research is needed to detail the phytochemical profile from the fractions, establish MIC values for Gram-negative organisms, assess the extract's bactericidal efficacy, and exploring the full antimicrobial potential of the extract.

Conclusion

The methanolic extract of *D. linearis* leaves was found to contain several phytochemicals with known antimicrobial properties, including phenols, flavonoids, tannins, and saponins. The TPC and TFC assays indicated that the extract contains 225.43 ± 4.16 mg GAE/g of phenolic compounds and 50.20 ± 4.78 mg QE/g of flavonoids. The antimicrobial activity of the extract was confirmed through disc diffusion and broth microdilution assays, where it showed greater activity against *S. aureus*, with an inhibition zone of 10.4 mm. The inhibition zone increased with higher extract concentrations, suggesting a dose-dependent effect. The MIC for *S. aureus* was 1.563 mg/mL for F1, while no MIC was determined for *E. coli* due to inconsistent results (growth observed in all wells). The MBC was not determined for *S. aureus*, as no bactericidal activity was observed at concentrations of 50 mg/mL or lower. These results indicate bacteriostatic potential, but further research is needed to determine the bactericidal properties of the extract.

For future studies, non-conventional extraction methods and factors such as agitation could be explored to improve extraction efficiency and yield while preserving bioactive compounds. Additionally, isolating and identifying the specific bioactive compounds responsible for antimicrobial activity is essential for understanding the mechanism of action and assessing the toxicity of these compounds. MIC and MBC determinations for a broader spectrum of pathogens to better understand the extract's full antimicrobial profile and potential clinical relevance must be prioritized.

Authors contribution

Study design, S.Z.M.S and G.R.C.R. Direction and Coordination, S.Z.M.S. Investigation, G.R.C.R. Resources, S.Z.M.S. Writing-Original Draft, G.R.C.R. Writing-Review, S.Z.M.S. Writing-Editing, G.R.C.R. and S.Z.M.S. Supervision, S.Z.M.S., and E.Z. Project Administration, S.Z.M.S. and G.R.C.R., Botanical identification, S. K.

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Ethical statement

This study did not involve any research with human participants or animals. No ethical approval was required for this work, as it did not include experiments, data collection, or procedures involving humans or animals in any form.

Conflict of interest

The authors declare there were no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author declared the use of ChatGPT in order to improve readability and language.

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Medical Cannabis Regulation in East and Southeast Asia: A Scoping Review and Policy Insights for Malaysia

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Abstract

Introduction: A global shift towards legalising cannabis for therapeutic use has sparked significant debate in East and Southeast Asia, a region historically defined by stringent anti-narcotics laws. As nations navigate the tension between therapeutic evidence and public health concerns, regulatory responses have diverged, ranging from progressive legalisation to the continuation of strict prohibition. **Methods**: This scoping review examines the regulatory frameworks across Indonesia, Malaysia, Thailand, the Philippines, South Korea, Singapore, and Japan to identify divergent models, persistent challenges, and potential policy insights for Malaysia. **Results**: The findings reveal a fragmented landscape. Thailand is a regional outlier, having legalised medical cannabis through a controlled system integrating pharmaceutical and traditional medicine. Japan permits only cannabidiol products with negligible tetrahydrocannabinol. Conversely, countries like Indonesia, Singapore, and the Philippines maintain strict prohibition with severe penalties, despite ongoing debates and legislative proposals. This regulatory diversity highlights the tension between public health concerns, economic opportunities, and treaty obligations. For Malaysia, a cautious, incremental policy reform guided by scientific evidence is recommended. **Conclusion**: Adopting a regulated CBD-only framework could offer Malaysia a low-risk entry point, balancing therapeutic potential with strict controls. The study underscores the need for evidence-based strategies and stakeholder engagement to facilitate safe patient access while minimising risks of misuse.

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Introduction

Medical cannabis, which involves the therapeutic use of cannabis and its derivatives, has garnered increasing attention for managing a variety of medical conditions (NSDUH Annual National Report, 2024). The primary bioactive compounds in cannabis are tetrahydrocannabinol (THC) and cannabidiol (CBD). THC is responsible for the psychoactive effects associated with cannabis, whereas CBD is non-psychoactive and has been extensively studied for its potential anti-inflammatory, analgesic, and anxiolytic benefits (Expert Committee on Drug Dependence, 2023). In recent years, there has been a global trend towards the legalisation and regulation of medical cannabis, as countries seek to harness the potential therapeutic benefits while mitigating the risks associated with uncontrolled access and use.

The term medical cannabis encompasses a broad array of products derived from the *Cannabis sativa* plant or synthesised to mimic its bioactive compounds. These include the raw plant material, extracts, and isolated or synthetic cannabinoids used for therapeutic purposes. Regulatory agencies in various jurisdictions have approved specific cannabinoid-based medications such as dronabinol and nabiximols for conditions including chronic pain, multiple sclerosis-related spasticity, epilepsy, and chemotherapy-induced nausea (Silva & Carvalho, 2022). However, the definitions and legal status of medical cannabis vary significantly across countries, contributing to regulatory complexity and inconsistent research methodologies (Solís Sánchez et al., 2024).

In the Asia-Pacific region, the regulatory landscape for medical cannabis is diverse and rapidly evolving. Thailand was the first country in Southeast Asia to legalise medical cannabis in 2019, marking a significant milestone for the region. Australia and New Zealand have also approved the medical use of cannabis, while other countries in the region, such as South Korea and Singapore, have more limited or restrictive policies (Areesantichai et al., 2020). The regulation of medical cannabis typically involves a range of considerations, including the types of cannabis products allowed, the conditions for which they can be prescribed, the process for obtaining a prescription, and the oversight and quality control measures in place.

The regulatory approaches employed by different countries can be broadly categorised into two models: the medicalised model and the commercial model (Rehm et al., 2019). The medicalised model emphasises the treatment of specific medical conditions using cannabis-based products, with strict controls and oversight from healthcare professionals and regulatory authorities. In contrast, the commercial model aims to create a more open market for cannabis products, often with less stringent requirements for medical conditions and prescriptions, as seen in some North American jurisdictions (Souza et al., 2022; Rehm et al., 2019).

Malaysia, however, maintains one of the region's most stringent stances. Under the Dangerous Drugs Act 1952, cannabis is classified as a Schedule I drug with severe penalties, including capital punishment for trafficking (Dangerous Drugs Act 1952, 2012). Although the country currently permits cannabis only for government-sanctioned research, recent debates on the potential medical benefits of cannabis have spurred discussions on regulatory reforms (Dapari et al., 2022). This review seeks to compare medical cannabis policies across selected East Asia and Southeast Asia countries with Malaysia's policies, aiming to identify opportunities and challenges for reform. The study is justified by the global momentum toward medical cannabis legalisation and the need for Malaysia to re-examine its policies in light of emerging scientific and economic opportunities. The existing policy tension in Malaysia, between its research-only allowance and the broader regional and global shifts towards medical cannabis access, forms a central motivation for this comparative review.

Methods

This scoping review follows the methodology outlined by the Joanna Briggs Institute (JBI) for conducting systematic evidence syntheses (Santos et al., 2018). The review process consisted of

formulating the research question, systematically identifying relevant literature, selecting studies based on eligibility criteria, and extracting and synthesising key findings to map existing knowledge on medical cannabis regulations in East Asia and Southeast Asia.

The study conducted a systematic literature search using PubMed and Google Scholar to identify relevant peer-reviewed studies, policy documents, and government reports. This scoping review employed a broad search strategy that allowed for reproducibility, transparency, and reliability in mapping the current state of the literature. The PubMed search was performed using a structured query with MeSH terms and Boolean operators to enhance precision. The following search string was used in PubMed:

("Cannabis"[MeSH] OR "Cannabis"[TIAB] OR "medical marijuana"[TIAB] OR "medicinal cannabis"[TIAB] OR "cannabinoid therapy"[TIAB] OR "cannabis-based medicine"[TIAB] OR "THC"[TIAB] OR "CBD"[TIAB] OR "cannabidiol"[TIAB] OR "hemp"[TIAB] OR "cannabis-derived products"[TIAB])

AND

("Legislation as Topic"[MeSH] OR "Health Policy"[MeSH] OR "regulation"[TIAB] OR "law"[TIAB] OR "policy"[TIAB] OR "drug control"[TIAB] OR "regulatory framework"[TIAB] OR "government strategy"[TIAB] OR "legalisation"[TIAB] OR "controlled substances"[TIAB])

AND

("East Asia"[MeSH] OR "Southeast Asia"[MeSH] OR "East Asia"[TIAB] OR "Southeast Asia"[TIAB] OR "Far East"[TIAB] OR "Asia-Pacific"[TIAB] OR "China"[TIAB] OR "Japan"[TIAB] OR "South Korea"[TIAB] OR "Mongolia"[TIAB] OR "Thailand"[TIAB] OR "Vietnam"[TIAB] OR "Indonesia"[TIAB] OR "Malaysia"[TIAB] OR "Philippines"[TIAB] OR "Singapore"[TIAB] OR "Myanmar"[TIAB] OR "Cambodia"[TIAB] OR "Laos"[TIAB] OR "Brunei"[TIAB] OR "Timor-Leste"[TIAB])

Additionally, Google Scholar was used to manually hand-pick relevant articles, allowing for the inclusion of grey literature, government policy papers, and regulatory reports that may not be indexed in traditional academic databases. The Google Scholar search used a broader set of keyword terms, including "medical cannabis", "cannabis regulation", and country names of East Asia and Southeast Asia. This dual approach ensured a comprehensive and diverse dataset covering both academic and policy perspectives.

Identification of Relevant Studies

The search was restricted to peer-reviewed articles published in English within the past decade. All identified studies were transferred to a reference management tool, and duplicates as well as titles in other languages were removed. Two authors independently screened the titles and abstracts to assess relevance and then conducted full-text reviews to ensure alignment with the inclusion criteria. Any discrepancies in the selection process were resolved through discussion. Articles were included if they explored the regulatory frameworks, policies, and governance structures surrounding medical cannabis in East Asia and Southeast Asia. Studies were excluded if they focused solely on recreational cannabis or discussed regulations outside the target regions.

Data Extraction and Synthesis

Key information was extracted and synthesised into a narrative format, focusing on country-specific regulatory frameworks, enforcement mechanisms, and stakeholder perspectives. The thematic synthesis aimed to identify common patterns, regulatory challenges, and policy gaps across different jurisdictions. The findings were then critically analysed in relation to existing policies and emerging trends in medical cannabis regulation within the region.

Results and Discussion

Characteristics of Included Studies

The initial search identified 351 articles. Following the screening of titles and abstracts, 118 articles were

deemed eligible for full-text review. After a comprehensive full-text assessment, 34 articles met the inclusion criteria and were included in this scoping review. These studies examine the regulatory environments for medical cannabis in seven selected countries across East and Southeast Asia: Indonesia, Malaysia, Thailand, the Philippines, South Korea, Singapore, and Japan. The study selection process is illustrated in the PRISMA flow diagram (Figure 1).

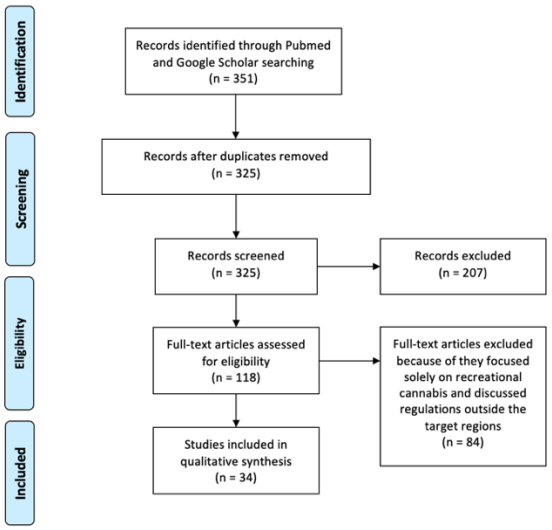

Figure 1: Prisma flow diagram of study selection

The geographical distribution of the 34 included studies indicates a notable concentration of research on specific countries. The majority of studies focused on Indonesia (n=17) and Thailand (n=15). Malaysia (n=6), Japan (n=4), and South Korea (n=3) were also represented. Fewer studies focused primarily on the Philippines (n=2) and Singapore (n=2) within the final selection, although these countries were often discussed in broader regional reviews. It is important to note that several studies addressed the regulatory landscape in multiple countries from this list; consequently, the sum of these country-specific mentions exceeds the total number of unique articles (n=34) included in this review. This concentration suggests that academic and policy discourse may be heavily influenced by the experiences of Indonesia, with its strict prohibition and ongoing debate, and Thailand, a regional pioneer in legalisation. Such a focus might inadvertently create knowledge gaps for other nations, including Malaysia, which is the primary focus for policy insights in this review.

A diverse range of methodological approaches was employed across the included studies, with many researchers utilising multiple research methods. Normative legal research was particularly prevalent in Indonesia-focused studies, reflecting a strong emphasis on analysing legal frameworks and their implications for medical cannabis regulation. The studies examined key policy issues such as legal classification, enforcement mechanisms, medical access models, and the potential socio-economic impact of cannabis reform. Table 1 summarises the key characteristics of the included studies.

Definition of Medical Cannabis

A clear and consistent definition of "medical cannabis" is often elusive in the diverse regulatory and research landscape. The articles included in this review demonstrate this variability. Generally, medical cannabis refers to the use of the *Cannabis sativa* plant, its components (cannabinoids like THC and CBD), or derived products for therapeutic purposes to treat or alleviate symptoms of medical conditions, under some form of regulatory oversight or medical guidance. This use is typically distinguished from recreational consumption, which lacks a therapeutic intent and regulatory control for medical application.

The scope of products considered medical cannabis varies significantly. Some frameworks, like Japan's, are highly restrictive, focusing almost exclusively on imported, pharmaceutical-grade CBD products with negligible THC content (less than 0.3%). Other contexts, such as Thailand, encompass a broader array of products. These can include pharmaceutical-grade cannabis-based medicines, which are standardised products, often with defined THC/CBD ratios like Sativex or Epidiolex, that have undergone clinical trials and received regulatory approval in various countries. Additionally, some regulatory systems or research studies consider the use of whole-plant cannabis or cannabis extracts, such as dried cannabis flower or broader extracts containing a wider spectrum of cannabinoids and terpenes.

Table 1: Key characteristics of the included studies

No	Author	Year Published	Country Focus	Study Type
1	Joni et al.	2023	Malaysia	Thematic review
2	Aditya & Al-Fatih	2022	Indonesia	Policy analysis
3	Areesantichai et al.	2020	Thailand, South Korea, Singapore, Asia-Pacific*	Systematic review
4	Pribowo et al.	2024	Indonesia	Normative legal research
5	Dalmacion et al.	2021	Philippines	Policy analysis
6	Fauziah et al.	2023	Indonesia	Narrative review, policy analysis
7	Fransiska	2022	Indonesia	Normative legal research
8	Guntara et al.	2024	Indonesia	Normative legal research
9	Han et al.	2016	South Korea	Narrative review
10	Indriani and Madjid	2022	Thailand, Indonesia	Normative legal research
11	Kalayasiri et al.	2019	Thailand	Narrative review, policy analysis
12	Kartika et al.	2024	Indonesia, Canada*, Italy*, Australia*	Normative legal research
13	Lestari	2024	Indonesia	Normative legal research
14	Matsushita	2020	Japan, Thailand, Germany*, USA*, Canada*	Policy analysis, systematic review
15	McGregor et al.	2020	Japan, USA*, Canada*, Germany*, Ireland*, UK*, Switzerland*, Australia* & New Zealand*	Policy analysis, systematic review
16	Nasir	2024	Indonesia	Normative legal research
17	Mohamed et al.	2022	Malaysia	Narrative review
18	Ransing et al.	2021	Thailand, Malaysia	Narrative review
19	Rehm et al.	2019	Thailand, Canada, Germany	Policy analysis
20	Risano & Ningtias	2023	Indonesia	Normative legal research, policy analysis
21	Aristiani & MH	2024	Indonesia	Normative legal research
22	Tomiyama and Funada	2020	USA, Japan	Narrative review, policy analysis
23	Triyatna et al.	2024	Indonesia, Netherlands, Thailand	Normative legal research
24	Vorapani et al.	2024	Thailand	Qualitative research
25	Widjaja	2018	Indonesia, global comparison*	Normative legal research, policy analysis
26	Yustina et al.	2023	Indonesia, Thailand, Malaysia, Singapore	Normative legal research, comparative method
27	Dapari et al.	2022	Malaysia	Cross-sectional study
28	Deng et al.	2023	Thailand	Media narrative analysis
29	Ehambaranathan et al.	2023	Thailand, Southeast Asia	Conceptual analysis
30	Jensema	2025	Thailand	Policy analysis
31	Karen	2022	Global including Southeast Asia	Narrative review
32	Mokwena	2019	Global including Southeast Asia	Narrative review
33	Razali et al.	2019	Malaysia	Normative legal research
34	Zinboonyahgoon et al.	2020	Thailand	Narrative review

*Countries or regions not included in the analysis

Thailand's model also uniquely incorporates traditional and folk medicine formulations, reflecting an integration of cultural practices by utilising cannabis in traditional Thai medicine (TTM) and folk remedies. Furthermore, beyond CBD-only products, research and some regulatory discussions involve cannabinoid-specific products, including THC-containing products or those with other minor cannabinoids, for conditions where THC's psychoactive or therapeutic properties are deemed necessary. Table 2 summarises common themes and variations in how "medical cannabis" is defined within the reviewed articles.

Table 2: Definitions and Scope of "Medical Cannabis" in Reviewed Literature

Definition/ Scope	Description
Pharmaceutical Preparations	Approved, standardised medicines containing specific cannabinoids (natural or synthetic) subject to rigorous clinical trials and regulatory approval.
CBD-Dominant Products	Products primarily containing cannabidiol (CBD) with very low or non-detectable levels of THC (e.g., <0.3% as per Japanese regulation). Often derived from hemp.
Whole-Plant/ Broad-Spectrum Extracts	Use of the cannabis plant flower or extracts containing a wider range of cannabinoids and terpenes, potentially offering an "entourage effect."
Traditional/ Folk Medicine Formulations	Cannabis preparations based on traditional medical systems or folk practices, often with less standardisation than pharmaceutical products.
General Therapeutic Use of Cannabis and its Derivatives	Broad term referring to any use of cannabis or its chemical components for managing medical conditions or symptoms, often without specifying product type.

The lack of a uniform definition of medical cannabis across the reviewed literature complicates direct cross-country comparisons of regulatory frameworks and their outcomes. This definitional variance means that legalisation of medical cannabis can imply very different practical realities regarding patient access, economic opportunities, and public health risks. This nuance is critical for Malaysian policymakers, as the choice of definition and product scope will fundamentally shape any future regulatory framework.

Legal Classification and Control Frameworks

The regulatory frameworks governing medical cannabis in East and Southeast Asia vary significantly, reflecting the region's diverse political, legal, and socio-cultural contexts. According to the findings of this review, medical cannabis laws in the region can be broadly categorised into three groups: prohibited (Singapore, Malaysia, the Philippines, Indonesia, and South Korea), limited approval (Japan), and legalised for medical use (Thailand). Table 3 summarises the legal status of medical cannabis in each country.

Table 3: Legal status of medical cannabis

Country	Legal Status	Recent Changes
Indonesia	Prohibited	No significant changes; ongoing debates
Malaysia	Prohibited	Discussions on potential authorisation for therapeutic purposes
Thailand	Legalised	Legalised in 2019; further liberalisation in 2022
Philippines	Prohibited	Proposal for a new bill
South Korea	Prohibited	Discussions on potential authorisation for therapeutic purposes
Singapore	Prohibited	No significant changes; ongoing debates
Japan	Limited approval	Discussions on potential authorisation for therapeutic purposes

Most countries maintain strict prohibitionist policies, particularly Singapore, Malaysia, the Philippines, Indonesia, and South Korea, where zero-tolerance drug laws impose severe criminal penalties for cannabis-related offences. However, pharmaceuticals derived from cannabis, such as Epidiolex or Sativex, may be allowed in some cases if they comply with national drug regulatory frameworks governing psychotropic substances. Even so, domestic research and development of cannabis-based treatments remain highly restricted. Japan represents a middle-ground approach, permitting CBD-only products with THC content below 0.3%, but maintaining strict bans on all other cannabis-related substances. This reflects a highly cautious, pharmaceutical-driven approach.

Thailand is the only country in the region to have fully legalised medical cannabis, implementing a structured framework for production, distribution, and patient access (Jensema, 2025; Areesantichai et al., 2020). The Thai

model categorises medical cannabis into three product classes: pharmaceutical-grade cannabis-based medicines (CBMs), Traditional Thai Medicine (TTM) formulations, and folk medicine products. Thailand's state-controlled system aims to prevent misuse while expanding legal access, with cannabis increasingly integrated into public healthcare services.

Divergent Legal Approaches: Public Health, Criminalisation, and Sociocultural Context in Malaysia

The stark contrast in regulatory approaches across East and Southeast Asia raises critical legal and ethical questions regarding the justification of strict cannabis prohibition in light of growing evidence supporting its medical use. Countries such as Indonesia, Malaysia, the Philippines, and historically South Korea and Singapore, largely uphold stringent drug laws, often classifying cannabis as a dangerous narcotic. While the dominant legal interpretation in these jurisdictions has historically minimised or denied its legitimate medical value, it is noteworthy that some of the reviewed literature, even when discussing these prohibitionist regimes, acknowledges the growing body of international scientific evidence for cannabis's therapeutic applications. This classification has been increasingly contested by medical research and policy shifts in other regions, where cannabis is now recognised for therapeutic applications in managing conditions such as epilepsy, multiple sclerosis, and chemotherapy-induced nausea (Freeman et al., 2019).

The rationale for maintaining prohibitionist policies often stems from concerns over public health risks, the potential for abuse, and the lack of standardised dosing guidelines. However, emerging global trends suggest that a more nuanced approach, differentiating between recreational and medical cannabis use, is becoming increasingly viable. The World Health Organisation (WHO) has recommended rescheduling cannabis in international drug treaties to reflect its therapeutic potential while maintaining controls to prevent misuse (Bennett, 2017). Thailand's decision to legalise medical cannabis demonstrates that strict prohibition is not the only viable approach; by implementing a government-controlled programme, Thailand has expanded patient access while maintaining oversight to prevent recreational misuse (Jensema, 2025). This suggests that carefully regulated medical cannabis programmes, supported by scientific research and legal safeguards, may offer a more balanced approach.

In Malaysia, any reform must navigate complex sociocultural terrain. Islamic principles heavily influence law and policy, particularly regarding intoxicants. However, the concept of *darurah* (necessity) in Islamic jurisprudence permits the use of otherwise prohibited substances for essential medical purposes if prescribed by a qualified Muslim physician. Supporting this, a 2022 fatwa by Malaysia's National Fatwa Council allowed medical cannabis use under strict conditions: expert medical recommendation, official authorisation, and exclusive medical application (Ismail et al., 2023).

Cultural attitudes also play a significant role. Cannabis is strongly stigmatised and associated with criminality and social decay. This perception stems from decades of framing drug abuse as a national security threat, with severe legal penalties. Nonetheless, there are signs of change. A 2022 survey by Dapari et al. found that 64.7% of adults in Selangor conditionally accepted medical cannabis, particularly when presented with educational materials or reassured of low risk (Dapari et al., 2022).

Institutionally, Malaysia remains cautious but is gradually engaging with the issue of medical cannabis. While the Ministry of Health (MOH) has not formally adopted a policy, it has expressed openness to clinical trials for cannabis-derived products, emphasising the need for scientific validation and adherence to regulatory frameworks to prevent misuse (Astro Awani, 2022). The National Pharmaceutical Regulatory Agency (NPRA) continues to stress the importance of rigorous, evidence-based assessments before permitting any medical cannabis applications. Simultaneously, national security bodies like the Majlis Keselamatan Negara (MKN) remain critical stakeholders, as Malaysia's longstanding anti-drug stance frames narcotics, including cannabis, as threats to national security and societal stability.

The Role of Government Control in Medical Cannabis Regulation

The degree of government oversight is a defining feature of medical cannabis regulation across East and Southeast Asia. Even in countries where some form of cannabis legalisation exists, state control remains highly centralised, ensuring that access is tightly regulated, and misuse is minimised. In Thailand and Japan, governments have direct

control over cannabis production, distribution, and prescribing practices, demonstrating that strict regulation and legalisation are not mutually exclusive (Areesantichai et al., 2020; Indriani & Madjid, 2022). However, the effectiveness of these models varies, as excessive state control may restrict access, delay licensing processes, and limit patient eligibility.

Thailand's centralised model is notable for its emphasis on state supervision over cultivation and distribution (Zinboonyahgoon et al., 2020). The government has retained full authority over licensing, ensuring that only registered entities can legally grow and supply medical cannabis. This approach minimises the risk of illicit diversion, maintaining strict oversight while gradually expanding legal access. However, early reports suggest that high regulatory barriers and slow licensing processes have limited patient access, indicating that over-regulation may undermine the intended public health benefits of medical cannabis legalisation (Rehm et al., 2019; Zinboonyahgoon et al., 2020). Policymakers considering medical cannabis reform must therefore balance tight regulatory controls with patient accessibility, ensuring that legal pathways to medical cannabis use are not overly restrictive.

Japan's CBD-only approach offers another example of strict regulatory oversight, where only cannabis products containing less than 0.3% THC are legally permitted (Areesantichai et al., 2020; McGregor et al., 2020). This 0.3% THC threshold is a widely adopted international standard to differentiate industrial hemp (with negligible psychoactive properties) from marijuana varieties with higher THC concentrations (Sgro et al., 2021). Japan's adoption of this limit aligns with a cautious drug policy focused on minimising psychoactive risks while allowing potential therapeutic applications of non-psychoactive cannabinoids. The government allows imported pharmaceutical-grade CBD, ensuring that no domestic cannabis cultivation occurs. This policy framework is extremely restrictive, limiting the availability and affordability of CBD products while preventing any progression toward full medical cannabis legalisation.

While Japan's model effectively minimises regulatory risks, it also restricts medical cannabis's therapeutic potential, as many conditions require THC-containing formulations for effective treatment (Martin et al., 2020; McGregor et al., 2020). This highlights an important regulatory dilemma. Overly restrictive policies can hinder the development of medical cannabis programmes, reducing their effectiveness for patients who need them most.

Both Thailand and Japan illustrate different approaches to government control, but their experiences highlight a common challenge of balancing regulatory oversight with accessibility. Strict state regulation is necessary to prevent misuse, but excessive control can undermine the very purpose of legalisation which is to enable patients to safely and effectively access medical cannabis. Future cannabis policies should focus on streamlining regulatory processes to make medical cannabis more accessible without compromising public health and legal safeguards.

Economic and Public Health Considerations in Cannabis Policy Reform

The economic and public health implications of medical cannabis legalisation have been a key driving force behind policy changes in several countries. Thailand's decision to legalise cannabis was not solely based on medical necessity, but also on its potential economic benefits (Deng et al., 2023; Ehambaranathan et al., 2023). By establishing a domestic cultivation and production industry, Thailand has positioned itself as a regional leader in medical cannabis exports, aiming to supply international markets while also developing its domestic medical cannabis industry. This economic motivation underscores an important reality. Medical cannabis legalisation is not just a public health issue, but it is also a significant economic opportunity.

For countries considering cannabis reform, economic benefits must be weighed against public health concerns. One major concern is that legalising medical cannabis could lead to increased recreational use, especially in markets where regulatory enforcement is weak (Karen, 2022). Thailand has implemented strict safeguards to mitigate this risk, requiring prescriptions from licensed medical professionals and restricting cultivation licenses to government-approved entities. However, even with these safeguards, some critics argue that the commercialisation of medical cannabis could pave the way for a broader push towards recreational legalisation, potentially leading to higher rates of misuse (Mohamed et al., 2022; Zinboonyahgoon et al., 2020).

In contrast, countries that maintain strict prohibitionist policies argue that the potential risks of cannabis use outweigh any economic benefit. Governments in Malaysia, South Korea, and Singapore have repeatedly emphasised public health risks associated with cannabis legalisation, particularly regarding adolescent use, dependence, and impaired cognitive function (Mohamed et al., 2022; Mokwena, 2019; Ransing et al., 2021; Razali et al., 2019). However, emerging research suggests that well-regulated medical cannabis programmes do not necessarily increase rates of recreational use, provided that strict age restrictions, licensing systems, and enforcement measures are in place (Švrakić et al., 2012; Tomar et al., 2024). These findings suggest that economic incentives and public health concerns are not mutually exclusive. Governments can pursue a controlled medical cannabis industry while maintaining strong safeguards against misuse.

Malaysia's established pharmaceutical manufacturing sector, coupled with its agro-biotech capabilities and tropical climate, provides a strong foundation for domestic cultivation and production. Legalisation could stimulate local job creation, particularly in agriculture, processing, logistics, and research and development sectors. A 2023 regional market analysis projected that Southeast Asia's medical cannabis industry could be worth over USD 2 billion by 2027 (Deng et al., 2023). Moreover, export potential to regulated international markets offers an opportunity for foreign revenue and international partnerships.

A Cautious Path Forward for Malaysia

The debate over medical cannabis reform in Malaysia remains highly contentious, with strong opposing views from policymakers, law enforcement, and public health officials. While there is growing discourse on cannabis-derived pharmaceuticals, full-scale legalisation remains a distant prospect. Given Malaysia's historical stance on drug enforcement and its commitment to international drug control treaties, any move toward medical cannabis legalisation must be approached cautiously, incrementally, and with strong regulatory oversight.

Recent developments indicate a carefully measured willingness from the Malaysian government to explore avenues for cannabis-derived products for medical use. The former Health Minister announced in 2022 intentions to develop a framework for the registration of prescription drugs containing Cannabidiol (CBD) (Malay Mail, 2022). This sentiment has been echoed by the current Health Minister, who has invited applications for the registration of cannabis-based products with the Drug Control Authority (DCA), contingent upon sufficient scientific evidence of safety and efficacy (New Straits Times, 2025). The National Pharmaceutical Regulatory Agency (NPRA) would be the key body overseeing such registrations, applying existing drug approval pathways, although specific guidelines tailored to CBD or cannabis-derived medicines are not yet publicly detailed beyond these general statements of intent. This "policy in progress" situation suggests that while the door is ajar for pharmaceutical-standard CBD products, the translation of this intent into a fully functional and transparent regulatory pathway is a complex and ongoing process.

One possible pathway is for Malaysia to adopt a limited CBD-only framework, similar to Japan's, as a first step toward broader cannabis reform. CBD has minimal abuse potential and moderate therapeutic benefits, making it a low-risk entry point for medical cannabis regulation. However, regulating CBD alone may not fully address patient needs, as many medical conditions require THC-containing formulations for effective treatment. To avoid regulatory loopholes and enforcement difficulties, Malaysia must implement clear quality control measures, ensuring that CBD products contain THC below a legally defined threshold.

A research-based approach should guide Malaysia's policy decisions, ensuring that any regulatory changes are grounded in scientific evidence rather than political or economic motivations. Establishing pilot medical cannabis programmes, supporting clinical trials, and collaborating with international regulatory bodies could help Malaysia develop a controlled, evidence-based medical cannabis framework. Ultimately, a cautious, research-driven approach would allow Malaysia to evaluate the risks and benefits of medical cannabis before making any major policy shifts.

Limitations

This scoping review has several notable limitations that must be considered when interpreting its findings. Firstly, the review's focus on English-language publications potentially excludes relevant literature published in local languages, introducing a language bias. Important policy documents or academic studies written in regional languages may

have been overlooked, limiting the comprehensiveness of the findings. Second, the scope was restricted to East and Southeast Asian countries, focusing on only seven nations: Indonesia, Malaysia, Thailand, the Philippines, South Korea, Singapore, and Japan.

Consequently, the review may not fully capture the broader regulatory landscape or reflect nuanced regional variations and policy developments in countries outside this selection. Third, a significant portion of the included literature comprised normative legal analyses and policy documents, with fewer empirical studies assessing the practical impacts or effectiveness of medical cannabis policies. The limited empirical data available in the included studies restricts the ability to draw firm conclusions regarding the real-world outcomes and the overall effectiveness of regulatory models. Additionally, the rapidly evolving nature of medical cannabis regulation poses a challenge to the temporal validity of this review, as the findings presented may quickly become outdated due to ongoing legislative changes and shifting sociopolitical attitudes towards cannabis legalisation.

Conclusion

The regulatory landscape for medical cannabis in East and Southeast Asia remains highly fragmented, with a clear divide between prohibitionist and reformist policies. For Malaysia, the most prudent approach would be to proceed cautiously, focusing on CBD regulation, research-based policymaking, and strict enforcement measures to balance public health concerns with economic opportunities. A measured, evidence-based approach will be crucial in shaping Malaysia's medical cannabis policy in the years ahead.

Authors Contributions

Conceptualisation, FH; methodology, FH and RMZ; data curation, FH and RMZ; writing—original draft preparation, FH; writing—review and editing, RMZ; visualisation, FH; project administration, FH. All authors contributed equally to data extraction, analysis, manuscript preparation, and have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, ChatGPT (OpenAI) was used to enhance readability and language. The authors reviewed, edited, and validated all content generated by the AI tool and take full responsibility for the final manuscript.

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