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Journal of Pharmacy

Precision medicine using monoclonal antibodies in cancer therapy

Muhammad Taher[1,2*]

EDITORIAL

Cancer is among the most threatening diseases in the world resulting in 19.3 million new cases and causing 10 million deaths in 2020. Female breast cancer is the most diagnosed with 2.3 million new cases (11.7% of total new cancer cases), followed by lung (11.4%), colorectal (10%), prostate (7.3%) and stomach (5.6%). It was recorded that lung cancer is still the leading cause of cancer death with 1.8 million deaths (18% of total cancer deaths) followed by colorectal (9.4%), liver (8.3%), stomach (7.7%) and female breast (6.9%) (Sung et al., 2021).

Presently, the use of monoclonal antibodies (mAbs) or immunotherapy is considered one of the main components in cancer therapy together with chemotherapy, surgery and radiation. The monoclonal antibody is a biological macromolecule that is known as a therapeutic protein. The protein-based molecules have led the top selling drugs for a few consecutive years. At the end of 2023, the global total drug revenue was led by immunology ($35.23 billion in revenue), oncology ($64.41 billion), and infectious diseases ($39.95 billion) (Bunts, 2023). Keytruda® overtook the position of Cominarty® Covid-19 vaccines over the declaration of the lifting of Covid-19 on 5 May 2023 by the World Health Organization. Keytruda® uses pembrolizumab as the monoclonal antibody to treat melanoma, lung cancer, cervical cancer, head and neck cancer, stomach cancer, and breast cancer.

Antibodies are secreted by B lymphocyte cells in response to the introduction of foreign antigens. A monoclonal antibody is a Y-shaped immunoglobulin which possesses two antigen-binding fragments (Fb) and a crystallisable fragment (Fc). They specifically bind to specific antigens molecules and inactivate the invader by blocking signalling pathways or inducing cell death (Ebrahimi & Samanta, 2023). Therapeutic monoclonal antibodies are the immunoglobulins that have been engineered to recognise certain antigen targets.

The practical and feasible production of monoclonal antibodies via hybridoma technology was first described by Kohler and Milstein in 1975 by fusion of mouse myeloma and isolated mouse spleen cells after the introduction of antigen into the mouse donor (Köhler & Milstein, 1975). Since then, potential therapeutic applications of monoclonal antibodies have been apparent. Following active research and development, OKT3 was the first monoclonal antibody developed in a clinical trial in 1986. However, it has safety and efficacy concerns due to immunogenic reactions. As a strategy, recombinant DNA technology has been introduced to modify the structure of monoclonal antibodies to chimeric and humanised monoclonal antibodies. Later, a fully human monoclonal antibody was designed using a variety of technologies ranging from original (natural) or synthetic libraries of human antibodies (Guimaraes Koch et al., 2022).

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Pembrolizumab is an immunoglobulin G4κ (IgG4-kappa) isotype antibody, an FDA-approved monoclonal antibody that works against programmed cell death protein 1 (PDP-1) on T-cell lymphocytes. It was initially used to treat advanced melanoma in 2014. Subsequently, it has been approved for many types of cancers. Pembrolizumab itself is a humanised antibody. It can be recognised from its infix name "zu" is humanized. The suffix "mab" refers to monoclonal antibodies according to the International Nonproprietary Names (INN) Programme of the World Health Organisation. However, since 2021 INN discontinued using the suffix mab due to the abundance number of products with mab in the market. It will be replaced with *-tug*, *-bart*, *-mig*, and *-ment* (Guimaraes Koch et al., 2022).

The specific targeting of monoclonal antibodies against antigens that are uniquely overexpressed by tumour cells has opened the opportunity to develop many other antibodies against specific cancers. The general mechanism of antibodies is inducing cell death by blockade of growth factor receptor signalling (Zahavi & Weiner, 2020). Overexpression of epidermal growth factor receptor (EGFR) by many different cancers led to the discovery of cetuximab which is anti-EGFR and able to induce apoptosis in tumour cells. Ovarian and breast cancers overexpress human epidermal growth factor receptor 2 (HER2) and tyrosine kinase receptor has inspired the development of trastuzumab. Anti-CD20 monoclonal antibody, ofatumumab is postulated to destroy B cells via several pathways including complement-dependent cytotoxicity (CDC) which is used to treat multiple sclerosis along with rituximab (Hauser et al., 2023).

In conclusion, the success story of antibody-drug conjugation (ADC) has been achieved by brentuximab vedotin (Adcetris®), and trastuzumab emtansine (Kadcyla®) for the treatment of Hodgkin lymphoma and breast cancer, respectively. The magic bullet theory of Paul Erlich (1854-1915) on the use of monoclonal antibodies has been able to identify the target without any side effects on the body. It has a great opportunity to be used in delivering cytotoxic drugs to the tumour site and benefiting a synergistic effect of the monoclonal antibody action. This concept will be an interesting point regarding the use of mAbs in cancer therapy.

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Journal of Pharmacy

ORIGINAL ARTICLE Open Access

Terpenoid Profiling of Thai Strain Cannabis Leaves (*Cannabis sativa* L. subsp. *sativa*) by Headspace (HS) Couple with GC/MS

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ABSTRACT

Introduction: Cannabis terpenoids, especially volatile terpenes, were used for the classification of cannabis strains. The leaves of *Cannabis sativa* L. subsp. *sativa* Thai strain 'Hang Krarok' are used legally in traditional Thai medicines, cosmetics, and food ingredients in Thailand under the control of the tetrahydrocannabinol (if lower than 0.2% dry weight). One of the specific characteristics of this plant is the volatile oil which consists of mono-and the sesqui-terpenoids.

Materials and methods: Fresh cannabis leaves were ground and 1 g samples were kept in gas chromatography/mass spectrometry glass vials at 4 °C prior to measurement using headspace.

Results: More than 50 terpenoids were identified from the fresh leaves in the cannabis samples. The major compounds were *β*–ocimene, L–limonene, terpinolene, p–cymenene, *β*–(E)–caryophyllene, (Z,E)–*α*–farnesene, *β*–bisabolene, and (E)–*α*–bisabolene.

Conclusion: The variation in the unique terpenoids in the Thai strain could be used in novel medicines and food and cosmetic products.

KEYWORDS:

Cannabis sativa, terpenoids, Headspace-gas chromatography/mass spectrometry (HS-GC/MS)

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Introduction

A Thai strain of cannabis (*Cannabis sativa* L. Cannabaceae) known as 'Hang Krarok' (squirrel tail) is commonly grown in Sakon Nakhon province, northeast Thailand. The leaves have been used as a folk medicine for the treatment of epilepsy, paralysis, stomachache, nausea, lack of appetite, and as a sleeping aid. In traditional Thai medicines, leaves with a size equal to or bigger than a human hand are recommended for the recipes, such as for a sleeping aid, lower abdominal pain, and paralysis (Duangdamrong et al., 2022; Pussapaphan & Busabong, 2022).

Cannabis is an dioecious annual plant with an erect stem 0.2−6 m height (UNODC, 2019). Leaves are palmately compound with 5−11 pointed, serrate leaflets 5−15 cm long and 1−2 cm wide. The female inflorescence is 10−30 cm long and 4−8 cm in diameter. The nearly spherical achene seeds with a color range from light brown to dark gray, are about 2.5−4 mm in diameter and are 3−6 mm long (Ehrensing, 1998).

Terpenoids in *Cannabis sativa* L. play an important role in the biosynthesis of the cannabinoids that contribute to the much-appreciated aroma and flavor of cannabis seed oil (Booth and Buhlmann, 2019; Zager et al., 2019). More than 200 terpenoids have been identified in cannabis, with the main constituents being mono-and sesqui-terpenes (Gallily et al., 2018). Flower buds contain about 0.5−3.5% essential oil (Fischedick et al., 2017). However, the ratios differ between the mono-and sesqui-terpenes in cannabis inflorescence and leaves (Gaggiotti et al., 2020; Casano et al., 2010). In addition, the cannabinoid-terpenoid ratio from the herbal extract supports the synergistic action of cannabinoids and terpenes in pain management, analgesia, cancer, and severe epilepsy (Brousseau, et al., 2021). Dziok et al (2021) reported the positive effect of *Cannabis sativa* L. extract on skin care and there are cannabis-based products used for skin inflammatory disease (Martin et al, 2022).

Volatile cannabis products are usually analyzed using gas chromatography coupled with flame ionization (GC-FID) or a mass spectrometric detector (GC/MS) (Casano et al., 2010; Fischedick et al., 2010; Giese et al., 2015; Ibrahim et al., 2019; Jin et al., 2017; Choi & Verpoorte, 2019). In addition, headspace (HS) coupled with GC-FID, HS-GC/MS, and solid phase headspace micro-extraction GC/MS have been used to determine the volatile constituents in *Cannabis sativa* L. inflorescence (Omar et al., 2014; Porto et al., 2014). The advantages of using headspace GC/MS are fast and simple by directly injected samples without extraction plant materials as for GC/MS or GC-FID. But the fresh samples needed to keep in GC/MS vials under cold temperature before directly injection. Thus, the aim of the present study was to characterize the terpenoids in the fresh leaves of the Thai medicinal strain of cannabis based on HS-GC/MS analysis.

The biochemical profiles of terpenoids in cannabis are more influenced by genetic factors than by the environment (Casano et al., 2014; Gillily et al., 2018). Cannabis terpenoids, especially volatile terpenes, could be used for the classification of cannabis strains. Thus, the identification of terpenoids in Thai cannabis leaves can be used to compare these results with other cannabis varieties and to learn more about the characteristic compounds in the Thai strain that are related pharmaceutical, food, and cosmetic industrial use.

Materials and methods

Cannabis leaf collection

Cannabis sativa L. Thai strain ('Hang Krarok') plants were grown in a greenhouse at Kasetsart University, Chalermprakiat Campus, Chiang Khruea, Muang, Sakon Nakhon, Thailand under a growth license (License number 6/2562) issued by the Narcotics Control Division, Food and Drug Administration, Ministry of Public Health, Thailand. The cannabis leaves were collected during 5−6 AM after growing for 50 days. The leaves were 15–20 cm long, which was consistent with the recommendations for traditional Thai medicine use. The fresh leaves were ground and 1 g samples were stored in HS-GC/MS glass tubes (size 22 ml) and kept at 4 °C in darkness prior to HS-GC/MS analysis.

Turbomatrix headspace extraction

The cannabis leaf samples were analysed using HS-GC/MS (Perkin Elmer; GC type Clarus 680; and MS type Clarus SQ8C; USA) at the Faculty of Science, Kasetsart University Sriracha Campus, Thailand under production license number 21/2563 and occupancy license number 43/2563 issued by the Narcotics Control Division, Food and Drug Administration, Ministry of Public Health, Thailand. Static HS analysis was carried out using a model TurboMatrix 40 Headspace Sampler (PerkinElmer Ltd; USA) as shown in Fig.1. Each sample of cannabis leaves (1 g) was placed in a 20 ml HS bottle. The temperatures for extraction, the needle, and transfer line were 125, 130, and 135 °C, respectively. The times for extraction, pressurization, and injection were 25, 1.5, and 0.03 min, respectively.

Figure 1: Cannabis leaf samples for Headspace-GC/MS analysis.

GC/MS analysis for separation and identification of cannabis volatiles

GC/MS analysis was performed using an Clarus model 690 gas chromatography (PerkinElmer, MA, USA) coupled to a model SQ8 mass-selective detector. The specifications for the PerkinElmer Elite-5M capillary column (USA) were 5% phenylmethylpolysiloxane, 30 m × 320 µm ID × 0.25 µm film thickness. Initially the oven temperature was held at 60 ºC, then increased at a rate of 7 ºC/min to a final temperature of 200 ºC. The injector temperature was 200 ºC. Purified helium was used as the carrier gas at a flow rate of 1 ml/min. Electron Ionization mass spectra were collected at a 70 eV ionization voltage over the m/z range 45–550. The electron multiplier voltage was 1,400 V. The ion source and quadrupole temperatures were both set at 200 ºC. The identification of terpenoids was based on Kováts retention indices (Adams 2001), relative to C_8–C_{22} n-paraffin hydrocarbon mixtures and mass spectral data comparison with database libraries (NIST, 2019), supported by the linear temperature program retention indices data (LTPRI), which were calculated from retention times on the first column. Retention times of known standard compounds were also used to confirm identities.

Results and Discussion

This is the first reported of terpenoid profile from *Cannabis sativa* L. subsp. *sativa* Thai strain 'Hang Krarok' leaves. The identification of terpenoid compounds in the leaves of *C. sativa* Thai strain 'Hang Krarok' was performed using HS-GC/MS (Fig. 1), which showed the separation of 53 terpenoids components (Table 1 and Fig. 2). The 53 signals were classified as 16 monoterpenes, 19 oxygenated monoterpenes, 11 sesquiterpenes, and 7 oxygenated sesquiterpenes. The major monoterpenes were *β*−ocimene and L−limonene, with terpinolene and *p*−cymenene as the major oxygenated monoterpenes, *β*−(E)−caryophyllene and *β*−bisabolene as the major sesquiterpenes, and (Z,E)−*α*−farnesene and (E)−*α*−bisabolene as the major oxygenated sesquiterpenes. Gaggiotti et al. (2020) and Porto et al. (2014) found monoterpenes and sesquiterpenes in different amounts and caryophyllene was the most abundant in *Cannabis sativa* L. inflorescence based on their GC/MS analysis. However, Porto et al. (2014) found the major compound caryophyllene in both fresh inflorescence and inflorescence extracted with supercritical carbon dioxide using HS-SPME combined with GC/MS analysis, as well as detecting major terpenoids, such as *α*–pinene, myrcene, limonene, terpinolene, caryophyllene, and farnesene from *Cannabis sativa* (hemp) inflorescence that had been grown and produced in northern Italy (Carnia region). In the present experiment, the *Cannabis sativa* L. Thai variety, which was grown in northeastern Thailand had cymenene as the most abundance terpenoid, followed by terpinolene, caryophyllene, bisabolene, and limonene, respectively. Thus, volatile terpenoid profiling could be used for the classification of *Cannabis* strains. Not only was the strain different (genetic factors), but in addition, environmental factors (such as soil, temperature, and moisture) influenced the profile and the ratios of plant natural compounds (Li et al., 2020; Sommano et al., 2020; Borges et al., 2017).

The most abundant mono-and sesqui-terpenoids identified in the cannabis Thai strain ('Hang Krarok') were *p*–cymenene, followed by terpinolene, *β*–(E) –caryophyllene, *β*–bisabolene, L–limonene, and *β*–ocimene (Fig.3). *p*–Cymenene was only found in the leaves of this Thai cannabis strain. This compound, possibly in combination with other cannabis terpenoids, caused the specific odor of cannabis Thai strain ('Hang Krarok') leaves. Some terpenoids found in cannabis, such as bisabolene, are used as a food additive and have pharmaceutical properties (Jou et al., 2015; Jou et al., 2016; Yeo et al., 2016), which is consistent with some traditional uses of cannabis leaves in Thailand. Nevertheless, some other compounds (or the synergistic effect of many compounds) in cannabis can be the source of other therapeutic effects. Cymenene, the most abundance terpenoid in the cannabis Thai strain leaves, is an oxygenated monoterpene present in the essential oil of *Ageratina pentlandiana* leaves with antibacterial activity (Quispe et al., 2019) and is also found in the essential oil of *Limbarda crithmoides* with antioxidant activity (Andreani et al., 2013). Another oxygenated monoterpene, terpinolene, is not only found in cannabis, but also in various plant sources, such as pine and fir trees, sage, apple, tea tree, and lemon and is widely used as a flavoring agent in the industry (Menezes et al., 2021). A sesquiterpene, caryophyllene, has been reported as a prominent constituent in many cannabis varieties (including the Thai variety in the present experiment) and caryophyllene oxide has been reported as a main component for cannabis identification by drug-sniffing dogs (Booth & Buhlmann, 2019; Gaggiotti et al., 2020). Bisabolene is in a group sesquiterpenes that has been used as food additives, with both *β*−and *γ*−bisabolene having anti-cancer properties (Jou et al., 2015; Jou et al., 2016; Yeo et al., 2016). In addition, bisabolene was identified in hop, lemon, and oregano. Limonene, a monoterpene, has been identified in the inflorescence of *Cannabis sativa* cultivars in Canada and the Netherlands (Jin et al., 2017; Fischedick et al., 2010). However, normally, it is found in lemons and other *Citrus* sp. (Nuutinen, 2018). Limonene has reported to have significant antimicrobial activity (Thielmann and Muranyi, 2019). Limonene, ocemene, terpinolene, caryophyllene, and bisabolene have been identified in the non-psychoactive chemotypes of *Cannabis sativa* harvested in Slovenia (Gallily et al., 2018).

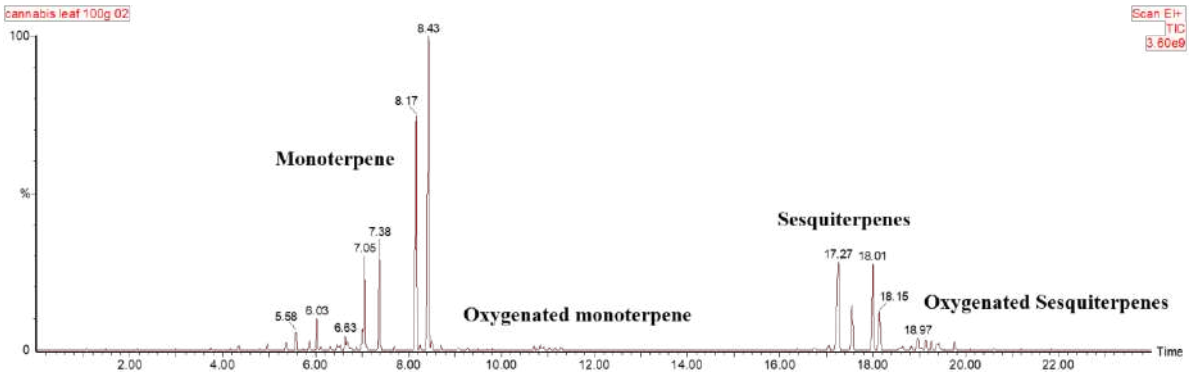

Figure 2: Headspace GC/MS chromatogram of terpenoid compounds in leaves of Cannabis sativa L. Thai strain ('Hang Krarok').

Table 1: Percentages of identified terpenoid compounds in leaves of Cannabis sativa L. Thai strain ('Hang Krarok') using HS-GC/MS.

Retention Time (min)	Assigned compound	Ri	Percentage relative abundance (%)±SD
Monoterpenes			
4.34	Sabinene	897	0.3453 ± 0.03
4.96	Camphene	943	0.4253 ± 0.03
5.36	*β*–Pinene	979	0.5981 ± 0.03
5.58	*α*–Pinene	937	1.3889 ± 0.06
5.87	(+)–Camphene	952	0.7545 ± 0.03
6.03	*β*–Myrcene	958	2.1678 ± 0.07
6.11	*α*–Phellandrene	969	0.2918 ± 0.01
6.31	Ocimene	976	0.2513 ± 0.01
6.63	3,7,7–Trimethyl–1,3,5–Cycloheptatriene	971	1.1975 ± 0.01
6.69	*γ*–Terpinene	1060	0.9139 ± 0.02
6.74	*p*–Cymene	1022	0.1541 ± 0.01
6.87	*o*–Cymene	1025	0.2643 ± 0.01
7.01	Limonene	1030	1.2034 ± 0.06
7.07	*β*–Ocimene	1037	6.4091 ± 0.30
7.38	L–Limonene	1031	7.3742 ± 0.11
7.69	*γ*–Terpinene	1060	0.2671 ± 0.01
Oxygenated monoterpenes			
8.19	Terpinolene	1088	17.3488 ± 0.82
8.25	*p*–Cymenene	1090	21.7511 ± 1.03
8.44	Isoterpinolene	1089	0.5967 ± 0.03
8.50	Cineole	1089	0.3073 ± 0.30
8.69	Linalool	1099	0.3217 ± 0.01
8.86	Fenchone	1121	0.0238 ± 0.01
9.05	Camphor	1121	0.1071 ± 0.01

Retention Time (min)	Assigned compound	Ri	Percentage relative abundance (%)±SD
9.25	exo–Fenchol	1116	0.1128 ± 0.01
9.48	Isoborneol	1138	0.0463 ± 0.01
9.61	Borneol	1138	0.0188 ± 0.01
9.67	*α*–Terpineol	1143	0.0331 ± 0.01
9.78	*γ*–Terpineol	1143	0.0391 ± 0.01
9.89	Isopulegol	1196	0.0203 ± 0.01
10.76	Carveol	1219	0.2578 ± 0.03
10.76	Pulegone	1212	0.0295 ± 0.01
10.82	Carveol	1219	0.3083 ± 0.03
10.89	Nerol	1228	0.1981 ± 0.01
11.02	Geraniol	1228	0.1986 ± 0.01
11.14	Geranyl acetate	1352	0.1559 ± 0.01
Sesquiterpenes			
16.05	*α*–Cubebene	1351	0.0611 ± 0.01
16.71	7–epi–Sesquithujene	1391	0.1294 ± 0.01
16.81	(Z) –*β*–Caryophyllene	1405	0.0159 ± 0.01
16.99	(E) –*α*–Bergamotene	1435	0.5373 ± 0.03
17.21	*β*–(E)–Caryophyllene	1419	11.2503 ± 0.81
17.31	(Z)–*β*–Farnesene	1444	0.0054 ± 0.01
17.68	Z,E)–*α*–Farnesene	1491	4.3015 ± 0.25
17.96	*β*–Bisabolene	1509	7.5583 ± 0.44
18.09	(E)–*α*–Bisabolene	1512	4.1021 ± 0.23
18.78	Germacrene B	1527	0.3473 ± 0.01
18.92	Aromandendrene	1530	1.1464 ± 0.06
Oxygenated sesquiterpenes			
19.09	Caryophyllene Oxide	1537	0.9545 ± 0.05
19.22	Cedrol	1543	0.7272 ± 0.06
19.34	Cis–Nerolidol	1564	1.0488 ± 0.04
19.37	(E)–Nerolidol	1564	1.0488 ± 0.04
19.49	*α*–Humulene	1579	0.0531 ± 0.01
19.72	Guaiol	1614	0.7604 ± 0.03
20.12	*α*–Bisabolol	1625	0.0682 ± 0.03

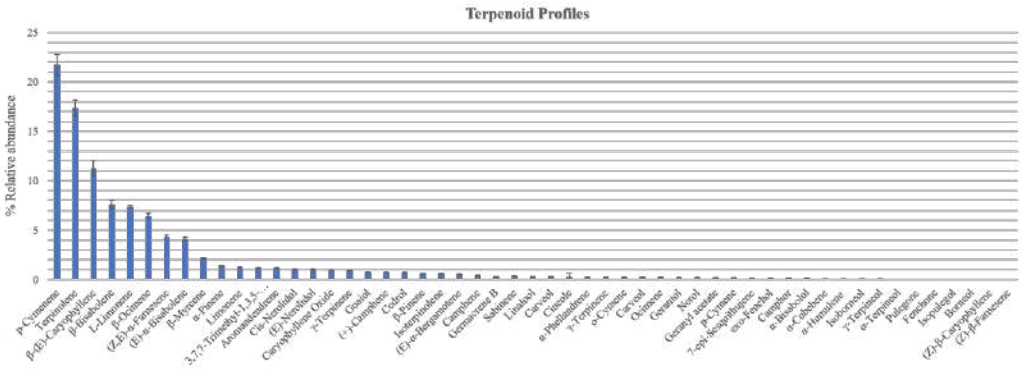

Figure 3:Profile of terpenoid compounds in leaves of Cannabis sativa L. Thai strain ('Hang Krarok') based on HS-GC/MS analysis

However, the absolute quantitative analysis for identification of terpenoids in the Thai strain of *Cannabis sativa* L. using internal standard could be further studied to identify precious major bioactive terpenoids.

Cannabis extracts can be a valuable source of biologically active substances that reduce oxidative stress, inhibit skin aging processes, and positively affect the viability of skin cells. Hydrogels based on cannabis extracts have a positive effect on skin hydration (Dziok et al., 2021). *Abies sibirica* terpenes had potential anti-aging and anti-cancer effects on senescent and cancer cell lines in human cells (Kydryavtseva et al., 2016). Thus, further study could focus on cannabis terpenes for skin treatment and therapy.

The green material left over from the production of medicinal cannabis could be an interesting source for various novel products. For pharmaceutical applications, the traditional uses could provide good indicators for producing interesting medicines that might also have a positive connection to cosmetic usage. In addition, the essential oil could be tested for various applications, such as an insect repellent, for food conservation, or as a taste enhancer. Further studies on these activities are needed as well as a proper analysis of the variability of the metabolome of the Thai strain and a comparison with other preparations on the market. The level of cannabinoids in the essential oil will be an important factor in obtaining licensed approval for the sale and use of such cannabis products.

Conclusion

Monoterpenes and sesquiterpenes were the main groups of volatile compounds from *Cannabis sativa* L. subsp. *sativa* Thai strain 'Hang Krarok' fresh leaves using Headspace-Gas Chromatography/Mass Spectroscopy. The major compounds were *β*–ocimene, L–limonene, terpinolene, *p*–cymenene, *β*–(E)–caryophyllene, (Z,E)–*α*–farnesene, *β*–bisabolene, and (E)–*α*–bisabolene. This terpenoid profile was different from other strains which has been reported in other country (Gaggiotti et al., 2020 and Porto et al., 2014). Thus, the volatile terpenes could be used for the classification of *Cannabis* strains in all over the world. Moreover, the variation in the unique terpenoids of cannabis Thai strain could be used to produce novel medicines, food ingredients and cosmetic products.

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Conflict of Interest

The authors declare no conflicts of interest.

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Team Experiential Learning Through Community Services Delivery at Private Drug Rehabilitation Centres by University Students: Focus Group Discussion

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ABSTRACT

Introduction: Substance abuse problem necessitates comprehensive community responsibility including university students to assist with the recovery of the marginalised population. This study aimed to investigate perception and experiences addressing team experiential learning as well as barriers to learning perceived by university students who provided community services at private drug rehabilitation centres.

Materials and methods: Six focus group discussions were conducted with graduated and undergraduate pharmacy students who provided community services in private drug rehabilitation centres. A guide was used to explore students' experiences, challenges encountered, and perceived learnings. Data were extracted from interview transcripts, sorted, and coded using Atlas.ti® version 9 and subjected to thematic analysis.

Results: The themes identified according to the scope of learning experience were (1) contribution of knowledge, (2) positive interaction, (3) application of interprofessional learning, and (4) appreciation of team experiential practice. Themes emerged under the scope of gaps and barriers were (1) initial negative perception, (2) communication barriers, (3) technical problems and (4) difficulties in coping with behaviours. For perceived learnings, themes identified were (1) enhanced confidence and skills, (2) contact with reality, and (3) increased empathy.

Conclusion: Team experiential learning in community service for marginalised population provided students with opportunities to directly contribute to the community and improved their learning.

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Introduction

Substance use disorder is a chronic brain illness that requires improved access to health care services and education (McLellan et al., 2000). The empowerment of the affected population is important towards preventing new drug users and sustaining the recovery of former drug users. The dynamics of substance use problems in Malaysia over the past decades were partly attributable to drug relapse, community rejection, peer pressure and familial issues (Mustapha et. al., 2023; Chie, 2016).

University students are valuable resources to be involved in providing services for the marginalised population of recovering drug users. Students from different disciplines can provide inter-disciplinary collaboration, specific communication, and care in addressing substance abuse. However, teamwork is frequently faced with resistance, and students may face difficulties in developing the skills necessary for collaboration. Teamwork has enabled students to synthesise knowledge, assess their accomplishment in the task, and encourage innovation and the creation of a support system (Brusa, 2019). To increase students' competency, experiential education method which involves close interaction between students and patients in clinical and public healthcare settings is crucial (Legal, 2019).

Students' engagement in experiential learning in the community allows the discovery of actual issues. These encourage them to create solutions to the problems (Ssekamatte et. al., 2022). Community engagement as a team enables them to learn from each other. The concepts of team experiential learning are different from individual experiential learning. Learning within teams is important because they are the fundamental learning unit in modern organisations (Boak, 2016). This aligns with the Kolb Team Learning Experience theory where it addresses all six aspects of team development which include purpose, membership, role leadership, context, process, and action through a structured written simulation. After completing it, the team generally possesses an understanding of team functions, specific experience related to its own team's functions, awareness of learning and progress facilitated by the learning cycle modes (Kayes, Kayes & Kolb, 2005).

The interprofessional team is focused on communication, mutual respect, interaction, and participation which involves a community of health professionals (Chatalalsingh & Reeves, 2014). It has been demonstrated that interprofessional team-based treatment enhanced patient outcomes, system effectiveness, and value (Sakr, 2022). Students' learning experience in the setting of drug rehabilitation centre is worth exploring because drug addiction problem is complex that calls for rich understanding. The struggle of this marginalised group to sustain their recovery from drug addiction is indirectly affected by the interprofessional team who provides the treatment services. This study aimed to explore perceptions and experiences addressing team experiential learning among university students who provided community services at private drug rehabilitation centres, including the gaps and barriers perceived by university students and the impact on their learning.

Materials and methods

Study design

This was a qualitative study employing a phenomenological approach to explore the experience of students who provided community service at private drug rehabilitation centres. The research question aligned well with this approach because it could grasp the essence of social phenomena hence allowing us to gain students' perception on insight, experience and difficulties (Ataro, 2020). This qualitative research utilised online focus group discussion (FGD) method. The interactional, synergistic aspect of the focus group enabled members to clarify or improve their discussion contributions considering issues raised by other participants, that could be undeveloped from an in-depth interview (Powell & Single, 1996). The FGDs were conducted using an online platform via Zoom to ensure flexibility, convenience, and anonymity. A fully virtual focus group facilitation had been reported to have a low dropout rate, low operational cost, and low time investment over conventional techniques (Halliday, 2021).

This study was reported based on the Consolidated Criteria for Reporting Qualitative Research (COREQ) checklist (Tong et al., 2007). Data were collected between November and December 2022 via semi-structured FGDs. Ethical approval was granted by the International Islamic University Malaysia (IIUM) Research Ethics Committee (ID No. IREC 2022-148). All participants provided written informed consent.

Participant recruitment

The participants were recruited using purposive sampling. Students who were involved with community service activities at the Drug Intervention Community (DIC) were invited to participate in this research. A total of 67 students who were eligible to participate were approached by email. The eligibility criteria for participants were IIUM graduating and undergraduate students who provided community services at DIC between January and April 2022. The potential participants confirmed their schedules to ensure they were available for the FGDs. The participant information sheet and informed consent form were sent via email to participants who confirmed their availability. Those who consented were divided into a group of four to six, irrespective of service type, in six heterogenous FGDs.

The heterogenous FGDs in this study referred to the

mix of students who provided different services. Heterogenous (dissimilar) teams frequently produce more original ideas, and research on learning style variety showed that diverse teams outperform homogenous teams in terms of performance (Kayes, Kayes & Kolb, 2005). The sessions were notified from 10 to 14 days in advance to enable participants to plan their schedule.

Interview topic guide and procedure

The semi-structured interviews of FGDs were led by the first author who was a final year pharmacy student at IIUM, using an FGD guide that was developed through the collaboration of the authors based on Tates et al. (2009). The moderator was trained by a professional and experienced moderator for FGD. The first two sessions were attended by two senior moderators and the necessary improvements were suggested and made. The list of questions focused on individual experiences of being a participant, including any challenges or learnings of their services contribution; gaps and barriers in interaction; and their perception. The question domains are provided in Table 1.

The duration of FGDs ranged from 76 to 94 minutes. All participants preferred to speak in Malay and the interviews were recorded, transcribed verbatim, and translated into English. The transcripts were compared and checked by two other researchers to ensure accurate interpretation. Field notes were also taken with the help of an assistant moderator, and participant names were kept confidential for privacy reasons. The transcripts were returned to the participants through email for comment or correction.

Data analysis

Data analysis was conducted using qualitative research software for qualitative study Atlas.ti® version 9. A thematic analysis was done using an inductive approach. The data were analysed by using qualitative themes identified in the FGDs. The meanings of accounts were identified, and a list of codes was constructed for major themes following repeated and close reading of the FGD transcripts. Three researchers independently constructed, compared, and cross checked to produce a final list theme codes. The coding decisions were agreed upon and the coded data were aggregated into code files. The summary of the data was done by manually scrutinising and generalising the data sets as they were relatively small. All participants were coded as Participant 1 until Participant 27 for data analysis and reporting.

Results

A total of 27 students consented to this study and participated in six FGDs. Each group comprised of four to six participants (Table 2). Data saturation occurred after the fifth meeting, from which no new information on the concept was gained during data analysis. All data were used in the analysis. The themes that emerged were by the scope of the themes: learning experience, students' gaps or barriers and perceived learning. Each theme was explained based on the relevant concepts and related quotes. The coding tree is provided in Figure 1.

Student's experience

There were four themes identified which were the contribution of knowledge, positive interaction, application of interprofessional learning (IPL) and appreciation of team experiential practice. Table S1 presented the summary of students' responses.

Contribution of knowledge

The students shared their contributions for the respective services covering spiritual and general knowledge sharing. These included knowledge of Quranic recitation, home medication review, academic lessons, social service, data management and smoking cessation. Despite feeling nervous during their first encounter, they managed to contribute what they had to offer throughout the services.

Positive interaction

Most participants have expressed their worries and nervousness about meeting the residents at DIC. Contrary to their expectation, they generally received warm responses from the clients and residents. They were respected and able to interact properly with the residents.

Application of IPL

Some students were able to experience IPL and managed to recognise its importance and benefits. Students who did not experience IPL also acknowledged that its application is important, and they were looking forward to it in the future programme. Majority also highlighted that IPL would be necessary only in certain services which require respective expertise.

Appreciation of team experiential practice

All students were aware of the benefits and drawbacks of working in a team. They discussed the positive and negative values which affect their service provision. They did not deny the significance of working in a team. Most of them were satisfied with their team members in terms of grouping size, communication, and work delegation. Although several pointed out the presence of uncooperative members in the group, they provided suggestions for future improvement.

Barriers and gaps

The most frequently mentioned barriers and gaps were initial negative perception, difficulties in coping with behaviours, communication barriers and technical problems (Table S2).

Table 1: Domain in Focus Group Discussion Guide

No.	Domain in Focus Group Discussion Guide
1.	Demographic data
2.	Student's experience, challenges and learning perceived during the provision of the community services.
3.	Students' gaps or barriers in the interaction and communication with Drug Intervention Community (DIC) residents and among team members.
4.	Student's perceptions on the effectiveness of the team experiential learning in providing services to marginalised population.
5.	Student's opinion to improve the integration of multidisciplinary approach in community service to the people affected with drug abuse setting.

Table 2: Demographic characteristics of participants

Characteristics	Number, n=27
Age (years)	
20-25	27
Gender	
Male	8
Female	19
Kulliyyah/Faculty	
Pharmacy	23
Science	3
Allied Health Science	1
Education	
First year	1
Second year	0
Third year	2

Characteristics	Number, n=27
Fourth year	18
Graduated	6
Elective drug abuse course	
Yes	14
No	13
Type of services provided	
Smoking Cessation Education & Carbon Monoxide Screening to Male Adults' Shelter homes (Casa Villa)	4
Data Management Workshop with DIC	5
Smoking Prevention Among Children and Adolescents at Children Shelter Home (Casa Harapan)	6
Home Medication Review Services at Women's Shelter Home (Casa Femina)	2
Weekly Quranic Recitation Class (Casa Femina)	4
Home Medication Review Services at shelter for palliative care (Casa Palliativo)	3
Social Services at Female Drop-in Centre (Persona Grata Wanita)	2
Children's Activities at Children Shelter Homes (Casa Harapan)	1

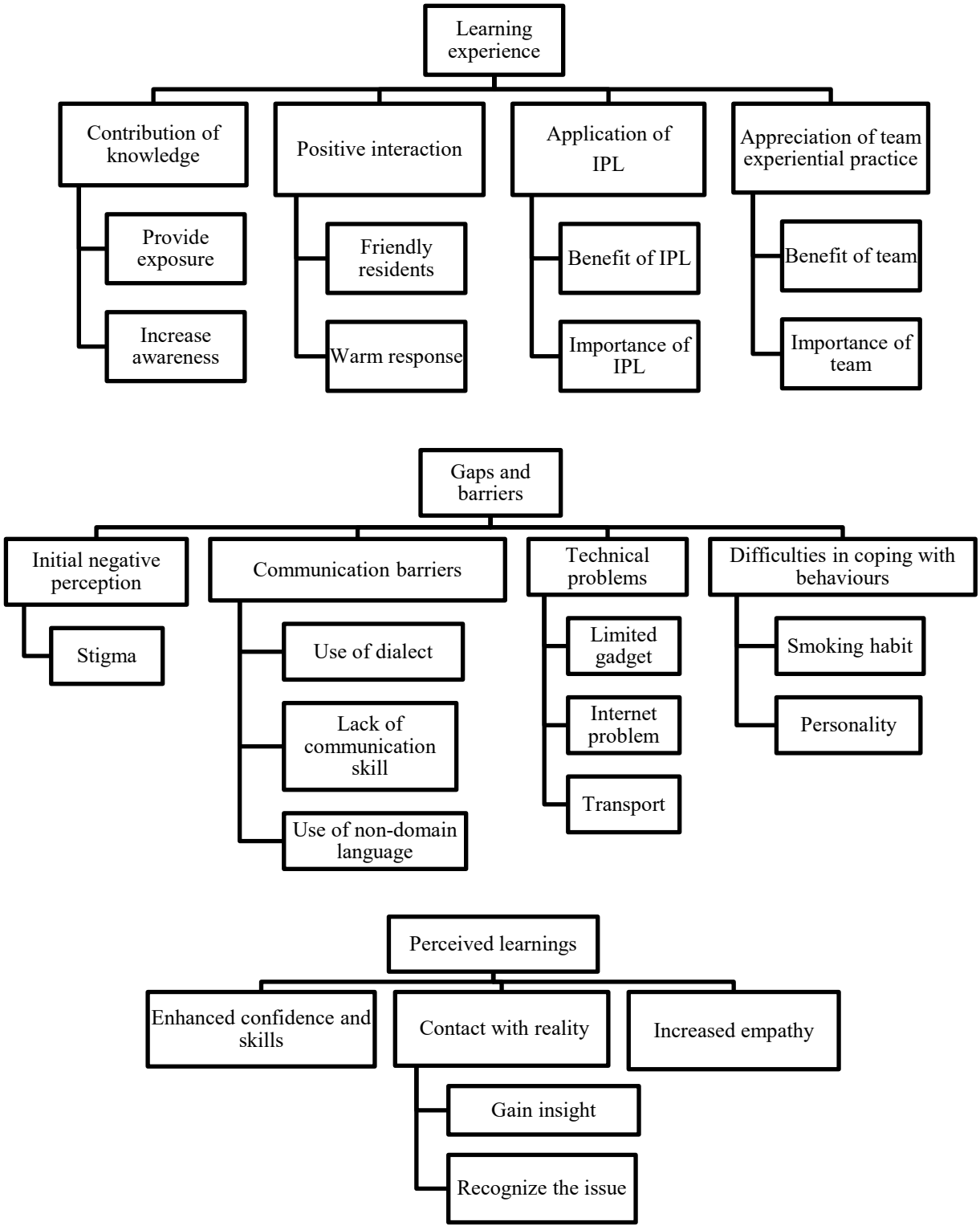

Figure 1: Coding tree diagram of focus group discussion coding.

Initial negative perception

Students admitted to having an initial stigma towards this population. They revealed on hesitancy and fear in regard to human immunodeficiency virus, expected aggressive behaviour and negative personality. However, students were able to adapt with the unfamiliar environment. They stated that the stigma progressively subsided through more interaction with the population. They managed to self-reflect their attitude for improvement.

Difficulties in coping with behaviours

Students stated about the residents' behaviour which impeded their service contribution. These include lack of attention, personality issues, and smoking habits in which they initially felt uncomfortable and conflicted. Some of them acknowledged that they faced difficulties due to their lack of knowledge and understanding of the related behaviours.

Communication barriers

The students elaborated about not having a common language comprehension between patients and providers that affected the communication. One student stated about the usage of dialect in the communication with the resident. Some also highlighted the gap in communication due to diverse audiences. They realised that communication barriers could result in poor service outcomes.

Technical problems

Technical problem was frequently mentioned during the discussion, especially among participants who provided the service at Casa Harapan through an online platform. They stated that they did not anticipate this challenge before the program, reflecting their high expectation towards the services. Communication barriers also happened from the technical problems.

Learnings perceived

Themes that emerged under learnings perceived include contact with reality, enhanced confidence and skills, and increased empathy (Table S3).

Contact with reality

Students were able to gain a deeper understanding about the residents who struggled with substance use. They managed to acknowledge the reality of the situation which was not as they had expected. The realisation was gained through the interaction with the residents. Most of the students were able to incorporate this learning with their self-awareness and personal growth.

Enhanced confidence and skills

Most participants initially found that communicating and interacting with the residents were difficult due to their lack of exposure and familiarity. They realised that consistent involvement in real practice could help them to overcome their fear and improve their self-confidence. They agreed that they became more confident throughout the services provided.

Increased empathy

The students acknowledged that the residents confronted difficulties to survive in the society. They managed to put themselves in the residents' place through a better understanding about trying to have a restart in their lives. Students' feeling of compassion helped them to reduce their stigma towards this population which is important as future healthcare providers.

Discussions

The three scopes covered in this study were learning experience, gaps and barriers as well as perceived learnings to explore team experiential learning among university students. This study discovered four major themes under learning experience which were contribution of knowledge, positive interaction, application of IPL, and appreciation of team experiential practice. Students' ability and willingness to provide exposure and action to increase awareness were in line with studies that emphasised on the need for this contribution (Ramli et al., 2009; Bratberg, 2019). Team experiential practice was helpful when students confronted new challenges through shared knowledge (Boak, 2016; Muzyk, 2020). The variety of benefits gained from teamwork efforts such as shared learning, expertise and motivation reflect the effectiveness of incorporating team practice in educational experiences. Majority of students highlighted that IPL serves as an added value and the need would depend on the setting. This perception mirrors the findings that stated the results of IPL could be influenced by the type of experience provided (Hudson et al., 2016).

Under the scope of gaps and barriers, the themes were initial negative perception, communication barriers, technical problems, and difficulties in coping with behaviours. Some students regardless of prior education on substance expressed the presence of stigma during their initial encounter with the population which led to hesitancy to interact. This was quite inconsistent with Stein (2003) who noted that those with education on substance abuse exhibited more favourable attitudes and behaviours in comparison to those who have not received any training. Nevertheless, the findings were aligned with a previous study which highlighted the stigma associated with illicit drug use (Tommasello, 2004). This suggests that university students rarely get the opportunities to interact with this marginalised population. The hesitation and fear

to approach them are usually caused by the lack of experience, contact and knowledge about this population.

The language barriers in communication were highlighted on the clients' incapability to understand non-domain language (English), students' accent proficiency and students' competence in interacting with diverse age participants. Students expressing their weaknesses can be regarded as self-knowledge which is commonly thought to be the initial stage of personal growth. The ability to effectively interact, communicate, and work with people from diverse cultural backgrounds is crucial for effective services. One of the best ways to teach cultural competency is through service learning (Trotter & Dunnivan-Mitchell, 2019). Difficulties in coping with behaviours have also been reported as one of the barriers (Galvani, 2007; Reid et. al., 2008).

It is understandable to anticipate technical problems especially when any programmes are conducted virtually via online platforms (Tallent-Runnels et al., 2006). The problems mentioned comprised of limited gadgets, internet problems, and difficulties for interaction and engagement. This further supports that maintaining a learner's belongingness was challenging due to technology (Baasanjav, 2013).

There were three themes that emerged under the perceived learning scope. These were enhanced confidence and skills, contact with reality, and increased empathy. Students have a restricted capacity to evaluate their learning (Dunning, Heath& Suls, 2004). Not many were able to point out self-reflection of their own personal weakness or lack of knowledge or skills in addressing the challenges. However, students perceived themselves as more confident after doing the community service, consistent with a report by Hendry et al. (2016). Although continuous exposure is one of the factors for the improved confidence level, it is undeniable that confidence level is closely related to the knowledge and skills of students (Zieber & Sedgewick, 2018).

Our study revealed that students' contact with reality regarding the negative perception surrounding an illness or rehabilitation treatment could prevent affected individuals from seeking treatment in a timely manner. This is similar to the findings reported by Rusdi et al. (2008). Moreover, students commented on the presence of stigma among healthcare workers. Many researchers have reported high rates of negative attitudes among social workers towards those who abuse substances. Van Boekel et al., (2013), Mattila et al., (2022) and Ford (2011) reported that health professionals perceived violence, manipulation, and lack of motivation as obstacles that hinder the provision of healthcare to these patients. This realisation led to a deeper understanding of the emotions involved in learning and can help students to become more aware of their feelings, actions, and values.

Another perceived learning was increased empathy. Items involving empathy received a more positive response given the value-based and principles of social work practice (Galvani & Hughes, 2010; Mohd Taufek et al., 2021). Additionally, attitudes towards substance abuse can be positively influenced using simulations and listening to the experiences of those who have recovered (Stein, 2003). Students were able to instil an understanding towards this population by trying to engage with their feelings.

Enabling small groups of students to engage in discussions about their experiences can facilitate the integration of reflective thinking, contributing to their ongoing professional growth and the development of problem-solving (Beck, Thomas & Janer, 1996). It enhanced the benefits of early and ongoing experiential education by fostering active learning (Turner, 2018). It was depicted in this FGD, that students were inclined to engage in discussions about their practical experiences within small groups due to the personal relevance of these experiences (Gordon et. al., 2010). It does not only allow students to naturally acknowledge the learning exposure gained from community service, but the mutual interaction from the FGD also naturally offered an additional benefit of enhanced self-esteem through the process of expressing and articulating their opinions (Anwar, 2016).

While most prior research discussed IPL in experiential learning, our results expanded the studies on team experiential learning. It provided better opportunities for IPL in learning new skills and knowledge from other disciplines. It enables pharmacy students to teach and learn from other students' experiences including non-pharmacy students. The findings of this study will be used to guide the development or improvement of current curricular design of how to ensure an effective learning experience for university students when doing community service towards marginalised populations. It may also open more opportunities for future expansion of impactful collaboration to obtain more comprehensive evidence and strengthen community action by overcoming the barriers in the management of drug problems. The positive impact of team experiential learning among university students could also influence policy changes to encourage more collaboration between educational organisations and private drug rehabilitation centres while providing more resources or incentives. The major limitation of this study was its limited sample size and the homogeneity in the demographics of the participants. The distribution of the sample was skewed in favour of Kulliyyah/Faculty of Pharmacy, so it might not reflect the actual setting among university students. Further research with a more diverse group of participants will be necessary to establish the generalisability of the results. There is a chance that the interview responses were biased as the interviewer and participants had prior knowledge of each other, but the

interviewer was not involved in the community services. There was no evaluation or assessment to reduce the risk of biased outcomes.

Future studies may consider data triangulation to ensure the validity and reliability of data. For example, multiple focus groups, interviews and surveys can be used to gather data from different groups of people. Further studies are required to evaluate the effectiveness of team experiential learning in community service in improving the design of volunteering programmes and curriculum.

Conclusion

In conclusion, team experiential learning in community service exposed university students to valuable insights addressing marginalised populations. The study identified positive learning experiences, knowledge sharing and interprofessional learning. Gaps and barriers, including initial negative perceptions and communication challenges, prompted students to address their limitations. The findings emphasise the importance of community involvement in raising students' awareness towards sensitive issues. Through small group discussions, students could actively reflect on their experiences, fostering personal and professional growth while enhancing problem-solving skills.

Authors Contributions

AAMF & NHMT designed the study and collected data. AAMF, NHMT & NSAR analysed the data. NIMN, IER, CSZ & CJT supervised, reviewed and edited the writing. All authors have read and reviewed the manuscript.

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Ethical Approval Statement

This study was approved by the Ethics Committee of International Islamic University Malaysia (IIUM) (ID No.: IREC 2022-148, 21 September 2022).

Informed Consent Statement

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

Conflict of Interest

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

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Layer-By-Layer Coating of Sesame Oil in Alginate-Chitosan Beads for Enteric Coating and Sustained Release

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ABSTRACT

Introduction: Medical uses of alginate-chitosan beads have been growing widely in recent years due to their varied applications in pharmaceutical and biomedical technology. Moreover, a variety of research have used drug encapsulation in the alginate-chitosan matrix to facilitate the enteric coating and sustained release of therapeutic molecules. Sesame oil has various medical applications as it contains a significant amount of lignans, which enhance its antioxidant function and anti-inflammatory effects for external or internal medical uses. In addition, it has contributed to the treatment of several inflammatory bowel diseases. Layer-by-layer assembly provides an effective coating for drugs, improving the oil instability in the gastric media, preventing drug leakage, and elongating the release time for sesame oil. This study aims to encapsulate sesame oil in alginate-chitosan beads and to optimize the formulation for enteric coating.

Method: Consuming sesame oil directly will not enable gastrointestinal tract to obtain the desired quantity of active ingredients in the oil due to the early degradation of oil. Therefore, the beads were prepared by using the external gelation method with layer-by-layer technique to provide multicoated layers. To illustrate, the usage of layer-by-layer assembly for the encapsulated alginate-sesame oil beads was accomplished by alginate and chitosan polysaccharides. A stability test was held to ensure the formulation stability during the study. In addition, the beads were characterized for particle size, roundness, and in-vitro drug release in different simulated buffers.

Results: This study revealed that the layer-by-layer approach is a viable method to obtain a sesame oil alginate-chitosan bead formulation for enteric coating and sustained release. Formulation coated layer-by-layer provided a successful pass of the stomach system whereas 68% of cumulative drug release occurred in the intestine within 5 h. To illustrate, during 135 min uncoated beads showed a cumulative drug release of 65% while the same percentage was achieved in 255 min for coated beads.

Conclusion: Sesame oil alginate-chitosan beads could be introduced as a promising carrier for encapsulating essential oils with favourable features.

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JOP

Introduction

The interest in natural products has been revitalized as they are considered as drug leads (Atanasov et al., 2021). Sesame oil (SO) is an example of natural compound that has been widely used for a long time in both pharmaceutical and food industries (Esmaeilzadeh Kenari & Razavi, 2022), and its efficiency has been documented in different diseases treatment in both aspects of traditional medicine and preventive medicine. Sesamin and sesamolin are the main phenolic components with high content compared to other oil ingredients (Akhila & Beevy, 2015; Esmaeilzadeh Kenari & Razavi, 2022), and several pharmacological properties, including antioxidant, anti-inflammation, and anticancer action, were related to them (Mekky, Abdel-Sattar, Segura-Carretero, & Contreras, 2019; Ostovan, Fazljou, Khazraei, Araj Khodaei, & Torbati, 2020). Several studies have revealed the potency of SO on a variety of health problems, such as colon cancer, cardiovascular diseases (CVD), osteoarthritis (OA), ulcerative colitis (UC), and oxidation stress inhibition (Anilakumar, Pal, Khanum, & Bawa, 2010; Hsu & Parthasarathy, 2017). One study revealed an inhibition effect of free radical formation that works against oxidative stress (Kheirati Rounizi et al., 2021). Another research has reported that it provides a valuable protective property for CVD (Jayaraj, Narasimhulu, Rajagopalan, Parthasarathy, & Desikan, 2020). In addition, the ability to suppress carcinogenesis will enable it to have a wide effect on the prevention and treatment of colon cancer (Valaei et al., 2022). Moreover, ulcerative colitis (UC) is a chronic inflammatory bowel disorder that infects the colon; a medical trial on rats has reported the decline in ulcerative colitis inflammation by using mastic and sesame (Ostovan et al., 2020).

According to the sesame oil medical potency, the enteric coating was the goal of this study which would enhance the therapy availability and effectiveness throughout long-term exposure and specific delivery. Reducing the drug side effect, increasing drug effectiveness, and enhancing oral delivery are all advantages of enteric coating. The intestinal sites have unique chemical properties, including a high pH value and increased enzyme activities compared to other gastrointestinal tract (GIT) parts. Therefore, enteric coating increases drug protection from unsuitable environments in the GIT. It was attained in our research by alginate and chitosan polysaccharides that had an opposite pH property.

Recently, the microencapsulation of alginate and chitosan polysaccharide has shown great promise in large clinical studies due to their biocompatibility, biodegradability, and nontoxicity properties that enable them to be important biopolymers in medicinal and therapeutic aspects (Bakshi, Selvakumar, Kadirvelu, & Kumar, 2020; Hariyadi & Islam, 2020). Alginate is an anionic polymer with high solubility in basic pH media (H. A.-O. Choukaife, Doolaanea, & Alfatama) while chitosan is a cationic polymer with high solubility in acidic pH media (Bakshi et al., 2020). They are strong alternatives due to their high availability, low cost, and environmentally friendly aspect (Bakshi et al., 2020; H. A.-O. Choukaife et al.). Besides that, the alginate "egg-box" structure, which is formed by ion exchange between calcium and sodium ions, provides the gelation property, and hydrophilic feature that will maintain its structure while absorbing a large amount of water (H. A.-O. Choukaife et al.; Hariyadi & Islam, 2020). Furthermore, enteric coating has been improved with the usage of layer-by-layer (LbL) technique, which is presented as a multi-layered structure for the desired drug, and it is demonstrated for a wide spectrum of pharmaceutics by using chemical bonds, such as electrostatic interactions between opposite charges that have been used in this study (Shende, Patil, & Prabhakar, 2020; Zhang et al., 2019). The ability of LbL assembly to coat large surface areas without changing or damaging the active ingredients is one of its advantages that broaden its applications (Alkekhia, Hammond, & Shukla, 2020). Eventually, using external gelation and LbL techniques for enteric coating has provided a successful enteric coating for SO, and improved the drug bioavailability in the targeted site with sustained release properties.

The study aimed to optimize alginate-SO formulation for enteric coating, and to estimate the cumulative drug release percentage throughout the stomach and intestine by using simulated buffers.

Methodology

Sodium alginate with low molecular weight and viscosity at 20°C (grade IL-6G, high G, 30-60 mPa.S) was procured from Kimica (Tokyo, Japan). Chitosan (plant-based) source: aspergillus niger with deacetylation ≥90 was attained from Modernist Pantry LLC (Eliot, USA). Calcium chloride anhydrous (fused), $CaCl_2$ was obtained from Techno Pharmchem (India). Citric acid 5% was produced in Saudi Arabia by Al Faris Food Industries LTD (Riyadh, SA). SO was purchased from Soybean Crushing CO. & Derivatives LTD (Yanbu, Saudi Arabia).

Preparation of Alginate SO Emulsion

Three different emulsions with different concentrations of alginate (0.5%, 1%, 2% w/v) were prepared. A homogenizer was used to mix SO (10% w/v relative to the total emulsion volume) with three different concentration of sodium alginate (0.5%, 1%, 2% w/v) consecutively for 5 min The outcome was three emulsions with a variation in alginate concentration and the total volume was 100 ml for each.

Alginate SO Emulsion Stability Test

A sample of 10 ml from the resulting emulsion was dispersed into a centrifuge tube (n = 3) that was held for 6 h to check for the sesame oil alginate emulsion separation. Every 30 min, a visual inspection was performed to evaluate the detachment during the experiment time of 6 h.

Preparation of Alginate SO Beads

The SO alginate beads were prepared by using an electrospray technique with an external gelation method. Alginate SO emulsion of 2% sodium alginate was selected for beads preparation as weight by volume (w/v). The emulsion was pumped through a 22G needle using a syringe pump (Shenchen Lab2015, Baoding, China) to provide a flow rate of 15 ml/min. At the same time, a beaker for a gelling bath of 100 ml calcium chloride (2% w/v) was prepared to provide the cross-linking of beads. SO beads were kept in calcium chloride solution while stirring for 30 min to confirm the complete gelation. Then, stainless steel sieve was used to collect the beads from the solution, and they were washed with distilled water. An oven of 60°C was used to dry out the beads for 24 hours.

Characterization of the Beads

MS2 digital microscope was used to capture images for wet and dry beads. Afterward, ImageJ program was used to determine the beads' size and shape. The beads roundness was evaluated using sphericity factor (SF). The SF was calculated using the following equation (Almurisi et al., 2020):

$$SF = \frac{D_{max} - D_{per}}{D_{max} - D_{per}}$$

where D_{max} is the maximum diameter passing through a bead centroid (mm), and is the diameter perpendicular to the Dmax passing through the bead centroid (mm). A perfect sphere is presented by zero SF while a higher SF directs to a higher shape distortion. Furthermore, all bead with SF ≤ 0.05 is considered as a spherical bead

LbL Assembly of Alginate SO Beads

The dried SO beads were encapsulated in a matrix comprised of two alternating layers of 1% w/v chitosan and 1% w/v alginate. The beads were poured in 100 ml of 1% w/v chitosan solution that was prepared by adding 1 g of chitosan (powder) to 74 ml of DW, and 25 ml of 5% citric acid was added to confirm the complete solubility of chitosan. The beads were transferred to 100 ml of DW to prevent beads' adhesions, then 100 ml of 2% w/v alginate was added. These two layers were obtained by electrostatic LbL technology, and it was held for 15 min for each. Lastly, the beads immersed in 2% w/v CaCl2 gelling bath for 5 min to ensure the alginate crosslinking and micro-gel formation. The beads were dried out on the laboratory bench for 6 h.

In-Vitro Drug Release profile of Coated Beads Vs Uncoated Beads

Initially, an experiment was held to ensure the efficiency of the coated beads vis uncoated beads in the basic 6.8 pH media. Briefly, 0.5 g of coated beads and 0.5 g of uncoated beads (control sample) were added to 100 ml of simulated intestine fluid (SIF; pH 6.8). During three hours of experiment, 3 ml aliquot of samples were withdrawn every 10 min. with a plastic dropper, and it was returned to the dissolution media. The spectrophotometer (Jenway 7305, Bibby Scientific Ltd Stone, Staffs, UK) at 600 nm was used to measure the absorbance while the calculation of cumulated drug release was accomplished by the following equation:

$$\text{Drug Release (DR\%)} = \frac{\text{Buffer volume (100)} \times \text{Actual oil conc.} \left(\frac{\text{mg}}{\text{ml}}\right)}{\text{Theoretical oil conc.} \left(\frac{\text{mg}}{\text{ml}}\right)}$$

value in counts per second (cps). The system software was then used to translate the intensities for various isotopes in the tested samples into concentrations and compared them to those obtained from calibration standard solutions. Each sample underwent analysis for a total of one minute and forty seconds.

To investigate the sequence in-vitro drug release (DR%) of SO beads, the dissolution was carried out at 37 ± 1°C and 60 rpm using an orbital incubator shaker under continuous stirring (Innova™ 4000 Benchtop Orbital Shakers, New Brunswick Scientific™, Edison, NJ, USA). Then, 0.5 g for each coated and uncoated SO beads was weighed and poured into 100 ml of simulated gastric fluid (SGF; pH 1.2) for 2 hours, separately. Afterwards, the beads were removed and filtered using a metal mesh, and they were dispersed in 100 ml of pH 6.8 SIF for 3 hours. Then, 3 ml aliquot of samples were withdrawn every 10 min. with a plastic dropper from each aliquot, and it was returned to the dissolution media. The spectrophotometer (Jenway 7305, Bibby Scientific Ltd Stone, Staffs, UK) at 600 nm was used to measure the absorbance whereas cumulated drug release was calculated through the following equation:

$$\text{Drug Release (DR\%)} = \frac{\text{Buffer volume (100)} \times \text{Actual oil conc.} \left(\frac{\text{mg}}{\text{ml}}\right)}{\text{Theoretical oil conc.} \left(\frac{\text{mg}}{\text{ml}}\right)}$$

Linearity, LOD, LOQ, Accuracy and Repeatability

The validation parameters that were assessed and calculated included linearity, LOD, LOQ, accuracy and repeatability. To assess the linearity of the method, calibration graphs for each element were generated by the system software at final concentrations of 0.02 g/mL, 0.04 g/mL, 0.06 g/mL, 0.08 g/mL, 0.1 g/mL, and 0.12 g/mL.

Statistical Analysis

Minitab and imageJ programs were used to perform statistical optimization. Each measurement was done in triplicate (n = 3) and the measured data were expressed as the mean ± standard deviation (SD). T-test and one-way ANOVA were used to compare the data, while considering $p < 0.05$ as indication of significant difference.

Results

Characterization of Alginate-SO Emulsion

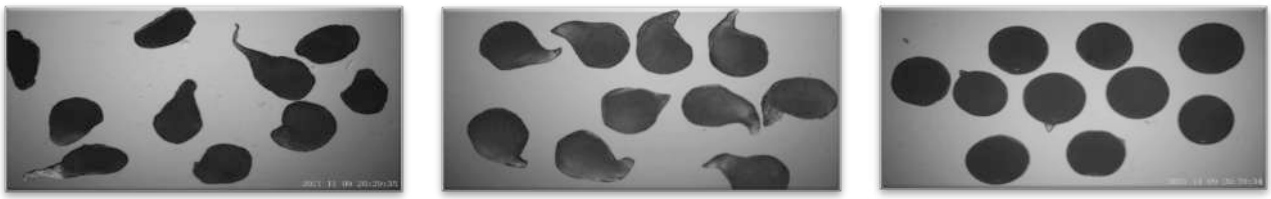
In the present study, a homogenizer was used to confirm the complete mix of the alginate and SO emulsion for 5 min. Therefore, the emulsion stability was checked to ensure its stability during the experiment. It was shown by visual inspection that the emulsion was not separated or detached for 6 hours, meaning that the emulsion was stable for more than the time needed for preparation as shown in Figure 1. Therefore, the result during the testing period has indicated no change in the emulsion.

Characterization of Alginate-SO Beads

The emulsion was optimized by variation of alginate (0.5%, 1%, 2% w/v) concentrations to ensure the optimal spherical shape. To illustrate, we found that as alginate concentration increased, the shape distortion of hydrogel beads declined as shown in Figure 2. Moreover, the beads' roundness increased with the increase of alginate as shown in Figure 3. According to the statistical analysis of ANOVA test, 0.5% alginate concentration was significantly different from 1% and 2% while no significant difference was shown between 1% and 2% concentrations. A research paper (Wong et al., 2021) mentioned the usage of 2% alginate to form hydrogel beads that had spherical shape with higher stability against structural deformation. Therefore, 2% alginate emulsion was chosen for external gelation method which was applied for SO beads encapsulation with the incorporation of 2% w/v CaCl2 for 30 min. alongside a continued stirring to facilitate alginate crosslinking and micro-gel formation.

A)10% SO w/v 2%Alg. B) 10% SO w/v 2%Alg. C) 10% SO w/v 2%Alg. D) 10% SO w/v 2%Alg.

Figure 1: Alginate SO emulsion mixed with homogenizer for 5 min. (a) at zero time, (b) after 2 hours, (C) after 4 hours, and (d) after 6 hours.



a)10% SO w/v, 0.5% Alg. B) 10% SO w/v, 1% Alg. C) 10% SO w/v, 2% Alg.

Figure 2: Microscopic image of alginate SO beads. (a) Alginate conc. of 0.5% w/v, (b) Alginate conc. of 1% w/v, and (c) Alginate conc. of 2% w/v. Magnification at 10×.

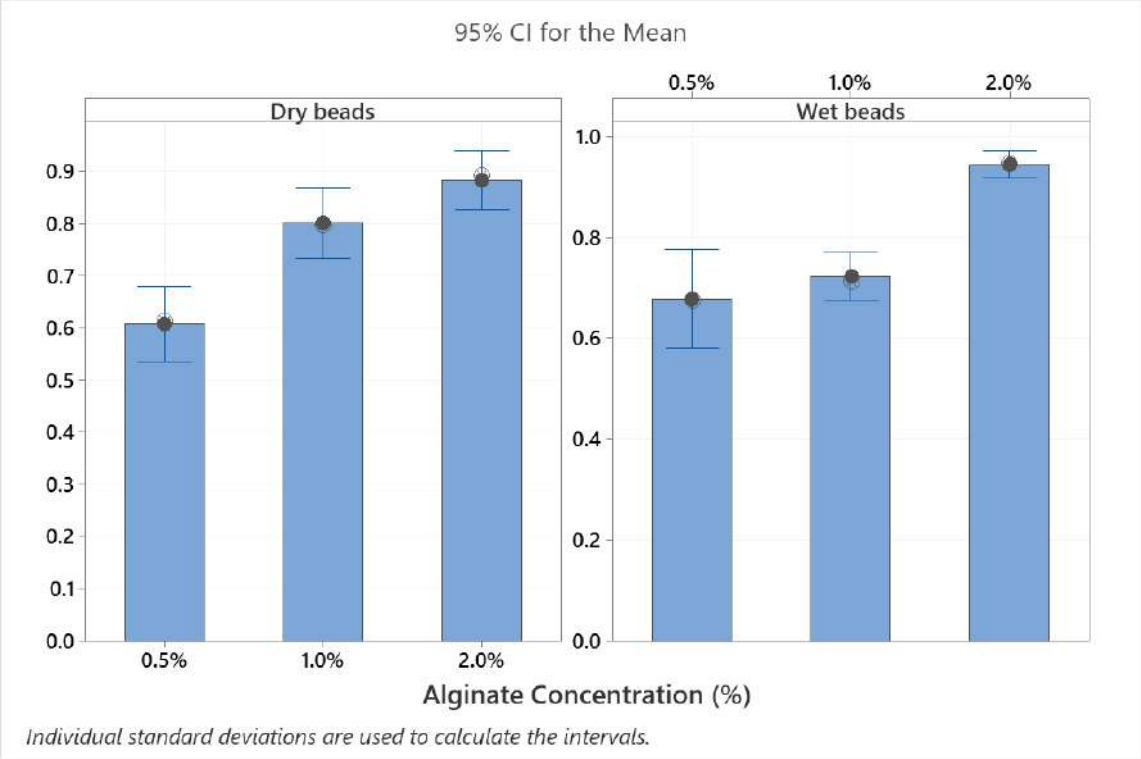


Figure 3: Dry and wet beads' roundness measurements correlation with alginate concentration. On the left side are the dry beads bars and on the right side are the wet beads bars (mean ± SD; n = 3).

A) Wet SO-alginate beads B) Dry SO-alginate beads

Figure 4: (A) The wet SO-alginate beads, and (b) dried beads.

Afterward, the beads were dried out by oven to provide dry alginate SO beads that were ready for LbL-encapsulation. Bead size and roundness measurements were checked by using the imageJ program for wet and dry beads, and the result showed a significant difference between the wet and dry beads' area while no significant difference was shown in roundness property (t-test $P < 0.05$) as shown in Table 1. Thus, we achieved a successful SO microencapsulation as shown in Figure 4.

In vitro drug release measurements in SGF and SIF of LbL coated and uncoated beads

SO-alginate beads were LbL coated with chitosan and alginate. To investigate the effectiveness of LbL coated beads vis-uncoated beads, the beads were dispersed in pH 6.8 SIF for three hours. The free beads showed 61% drug release in SIF for 3 hours, while zero percent was released

Table 1: The difference between wet and dry beads were observed based on the roundness property. Wet beads had a roundness mean value of 0.94 with low SD of 0.035 compared to dry beads (mean ± SD; n = 3).

	Wet Beads		Dry Beads	
Beads No.	Area (mm^2)	Roundness	Area (mm^2)	Roundness
1	1.74 ± .099	0.952 ± .035	0.518 ± .032	0.778 ± .078
2	1.715 ± .099	0.892 ± .035	0.493 ± .032	0.926 ± .078
3	1.78 ± .099	0.978 ± .035	0.436 ± .032	0.985 ± .078
4	1.785 ± .099	0.969 ± .035	0.496 ± .032	0.931 ± .078
5	1.896 ± .099	0.974 ± .035	0.455 ± .032	0.855 ± .078
6	1.623 ± .099	0.932 ± .035	0.538 ± .032	0.744 ± .078
7	1.56 ± .099	0.888 ± .035	0.508 ± .032	0.858 ± .078
8	1.79 ± .099	0.962 ± .035	0.474 ± .032	0.86 ± .078
9	1.651 ± .099	0.966 ± .035	0.489 ± .032	0.946 ± .078
10	1.647 ± .099	0.9 ± .035	0.449 ± .032	0.952 ± .078
Mean value	1.7187	0.9413	0.4856	0.8835

from coated beads as shown in Figure 5. According to the t-test statistical analysis, there was a significant difference between the two different beads ($p < 0.05$). This increased viability of coated beads was contributed to the LbL efficiency of the beads' outer surface.

Regarding to oral administration, the uncoated beads of SO would be degraded and digested in a short time because of the low pH in the stomach and the high absorbance in the intestine due to enzyme actions. Therefore, maintaining an adequate quantity of SO beads until they reach the last part of the intestine would be a key factor in improving the therapeutic impact. To investigate the in-vitro drug release (DR%) of SO beads, the method by Samah (Almurisi et al., 2020) was used with slight modifications. The beads were poured into pH 1.2 SGF for 2 hours, followed by pH 6.8 SIF for 3 hours. The cumulative drug release of 65% was reached in 135 min for uncoated beads whereas the same percentage was achieved in 255 min for coated beads. After 300 min, the dry coated beads showed a cumulative drug release of 68% while uncoated beads showed 83.7% of cumulative drug release as shown in Figure 6. In addition, a significant difference (t-test $P < 0.05$) was shown between both beads (coated and uncoated).

Discussion

The preparation of alginate-SO emulsion was the first step in our study. A recent study indicated the possibility of using sesame oil as the fat base of an emulsion, and the long-term stable emulsion by mixing SO with lecithin and carboxymethylcellulose (Kowalska & Żbikowska, 2016). According to oil encapsulation, several polysaccharides were used for oil encapsulation, such as sodium alginate which was successfully used to control the release of black seed oil (Azad et al., 2020). The ability of alginate to form hydrogels supports its uses as a gelling agent, stabilizer, thickener, and emulsifier in several studies (Ahmad Raus, Wan Nawawi, & Nasaruddin, 2021; Gheorghita Puscaselu, Lobiuc, Dimian, & Covasa, 2020). In addition, thyme oil encapsulated with sodium alginate was achieved effectively in another study (Volić et al., 2018).

Alginate concentration had a significant influence on SO beads formation as it plays the most important role regarding shape and size (H. Choukaife, Doolaanea, & Alfatama, 2020; Lotfipour, Mirzaeei, & Maghsoodi, 2012; Narin, Ertugrul, Tas, Sahin, & Oztop, 2020). Therefore, the emulsion in this study was optimized by variation of alginate (0.5%, 1%, 2% w/v) concentrations to ensure the optimal spherical shape. A similar study used alginate in different concentrations and reported that the shape of the beads was deformed when they decreased the alginate concentrations to 0.5% and 1% (Wong et al., 2021). This deformation happened when water consisted in the wet hydrogel beads evaporated throughout the drying process, causing volume shrinkage of hydrogel beads. Therefore,

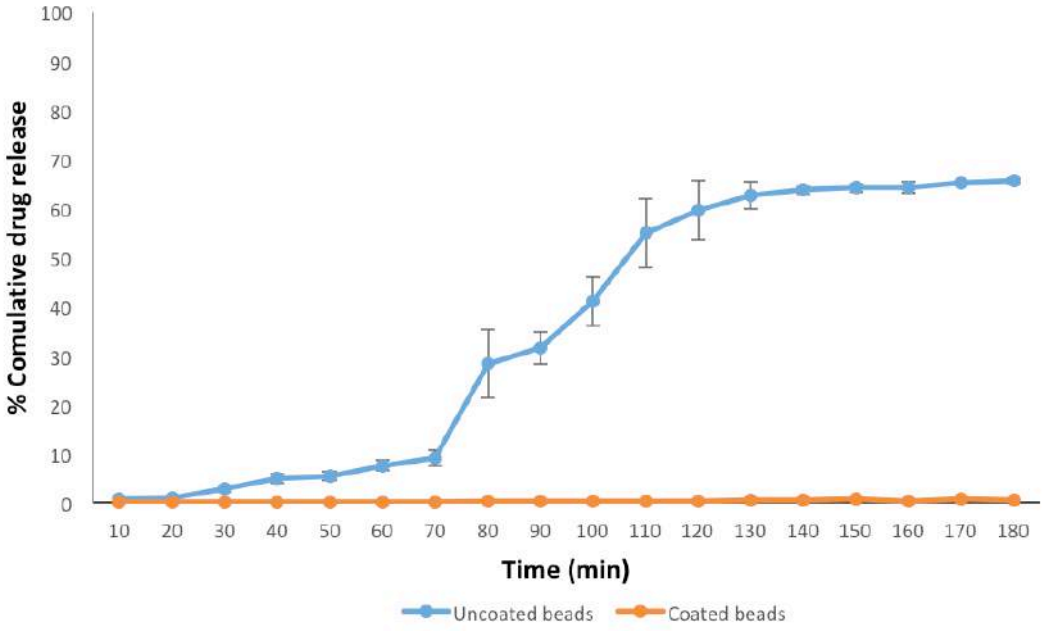


Figure 5: In vitro drug-release profile of LbL coated and uncoated alginate-SO dry beads in simulated intestinal fluid pH 6.8 (mean ± SD; n = 3).

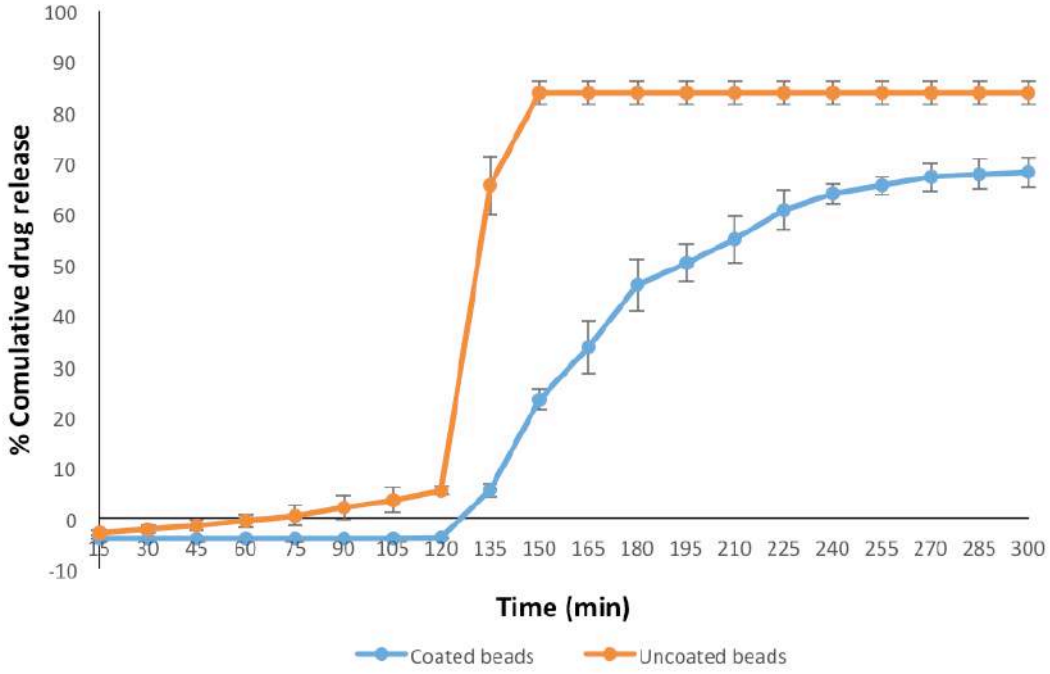


Figure 6: In vitro drug-release profile of LbL coated and uncoated alginate-SO dry beads in simulated gastric fluid pH 1.2 and simulated intestinal fluid pH 6.8 (mean ± SD; n = 3).

the presence of high alginate concentration will minimize the loss of water due to the formation of larger wet beads (Wong et al., 2021).

Multifunctional controlled drug release has been improved with the usage of LbL encapsulation. Alginate and chitosan were the chosen polymers for SO delivery as alginate is an anionic polymer and chitosan is a cationic polymer. Besides that, both hydrophilic and hydrophobic drugs can be delivered by using chitosan (Shafabakhsh et al., 2020). Doxorubicin was successfully delivered in the study by Chai et al. who prepared alternative multilayer of chitosan and alginate to control doxorubicin release for antitumor activity (Chai et al., 2017). Another study has synthesized an EcN encapsulated in a chitosan-alginate matrix with LbL assembly and CaCl2 cross-linking, and suggested an improvement of probiotic viability in the stomach environment (Luo et al., 2020). According to the control drug delivery via oral administration, chitosan and alginate polysaccharides have shown a promising efficiency in the drug delivery as mentioned in a study of emodin-encapsulated micelles into alginate and chitosan matrix. The beads showed sustained-release properties, and they could be used for site-specific drug delivery systems for hydrophobic drugs (Cong et al., 2018).

Conclusion

To enhance the oral administration and prevent drug leakage in the stomach, this study represented a double protection for active ingredients, the external gelation of SO and LbL coating of alginate-SO beads. The outcome of the study showed that LbL coated beads had zero release of SO in SIF compared to uncoated beads because it did not pass through the SGF. On the other hand, when beads passed through both buffers of pH 1.2 and 6.8, the coated beads showed a very low cumulative drug release in SGF while a higher drug release occurred in SIF. Uncoated beads showed a high drug release in both media, SGF and SIF. The previous results showed that the enteric coating designed in this study has the potential to be applied in intestinal treatments and colon delivery if it is inside the capsules. Furthermore, we are looking for further studies that will verify the drug biodistribution on animal models to show in-vivo mechanism of action.

Author Contribution

All authors made a great contribution to design the study, collect, and analysis data. In addition, the corresponding author revised the draft critically and gave a final approval for the paper to be published.

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Conflict of Interest

The authors declare no conflict of interest.

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Evaluation of Okra Pectin from Different Genotypes as Effective Suspending Agents in Pharmaceutical Formulations

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ABSTRACT

Introduction: Natural suspending agents are increasingly being investigated because of their relative non-toxicity, lesser cost, availability and biocompatibility compared to the currently utilised synthetic and semi-synthetic suspending agents. Pectin, a biopolymer found naturally in plants is gaining increased application in the pharmaceutical and biotechnology industry following its successful functional application as gelling agents, emulsifying agents and fat substitutes in the food industry. This study aimed at evaluating the suspending properties of pectin obtained from five okra (*Abelmoschus esculentus* L.) genotypes; PL1 (Penkrumah), PL2 (Agbagoma), PL3 (Asha), PL4 (Sengavi) and PL5 (Balabi).

Materials and methods: The pectin was extracted using standard protocols and characterised by investigating properties such as degree of esterification. A 5% $^{w}/_{v}$ paracetamol suspension was formulated utilising okra pectin as a suspending agent at concentrations of 0.5%, 1% and 2%$^{w}/_{v}$ and compared to Tragacanth gum suspensions at the same concentrations (0.5%, 1% and 2%$^{w}/_{v}$).

Results: All the extracted pectins had low degrees of esterification (<50 %). The pH, redispersibility, apparent viscosity, sedimentation rate and sedimentation volume of the formulated suspensions were investigated over a 4-week period. The suspensions were stable as evidenced by no significant ($p \geq 0.05$) fluctuations in pH during the period of study. Compared to when tragacanth was used as a suspending agent, the sedimentation rates, the flow rates of suspensions and redispersibility of the paracetamol suspensions utilising okra pectin were lower while the sedimentation volumes were higher at all the concentrations utilized and met standard requirements.

Conclusion: The evidence suggests that all five okra genotypes exhibit better suspending properties when compared to tragacanth gum and thus may be used as an alternative suspending agent.

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Introduction

Suspension as a pharmaceutical liquid dosage form is a coarse dispersion in which the internal phase is uniformly dispersed throughout the external phase (Alalor & Obunezie, 2020). Suspensions are thermodynamically unstable and thus require the addition of suspending agents to mitigate caking, sedimentation, and permit resuspension which are the major challenges facing suspension formulations (Owusu et al., 2022; Woldu et al., 2021a). Suspending agents can be categorised into three; natural suspending agents such as acacia, tragacanth, and pectin; semi-synthetic agents such as methylcellulose; and synthetic suspending agents such as carbopol (Doye et al., 2017; Woldu et al., 2021a). Nevertheless, the cost, availability, sustainability and compatibility issues of synthetic and inorganic suspending agents, render the natural agents preferable alternatives. Natural suspending agents have the attribute of being non-toxic, biodegradable, less expensive, and readily available (Kaushik et al., 2022; Onyishi et al., 2014).

Pectin, a biopolymer found naturally in plants is gaining increased application in the pharmaceutical and biotechnology industry as binders, disintegrants and carriers of drugs purposed for controlled release particularly following its successful functional application as gelling agents, emulsifying agents and fat substitutes in the food industry (Boakye-Gyasi et al., 2021; Malviya & Kulkarni, 2012; Owusu et al., 2021). Presently, the commercially available pectin is predominantly obtained from citrus peels or apple pomace. However, despite both being by-products of the juice or cider manufacturing industry, their long maturation periods and increased susceptibility to diseases pose a major challenge (Boakye-Gyasi et al., 2021; Chen et al., 2014; Malviya & Kulkarni, 2012; Owusu et al., 2021). Therefore, efforts are being made to obtain pectin from plants with relatively shorter maturity periods, which can also produce a substantial yield. One of the promising plant leads is okra (*Abelmoschus esculentus* L.).

Okra (*Abelmoschus esculentus* L.) is an essential vegetable crop which is primarily cultivated worldwide for its immature pods (Boakye-Gyasi et al., 2021; FAO et al., 2013; Kpodo et al., 2017a). There are over 50 species of okra that have been investigated as possible excipients in pharmaceutical formulations (Al-Shawi et al., 2021; Chen et al., 2014; Elkhalifa et al., 2021; Naveen et al., 2017; Prajapati et al., 2013; Srivastava & Malviya, 2011) Nevertheless, compared to other natural polymers, like alginate and gums, translation into routine pharmaceutical industrial application is limited. Several reported literature reveals the potential of okra as a binder, disintegrant and other excipients in pharmaceutical formulations (Al-Shawi et al., 2021; Elkhalifa et al., 2021; Sonawane et al., 2021).

Rhamnogalacturonan-I (RG-I) segments with varying molecular weights (10-767 × 10^3g/mol) and compositions of side chains have been reported to be abundant in isolated okra pectins (Kpodo et al., 2017a). Alba & Kontogiorgos posit that, several factors particularly the genotype of the okra plants can cause extracted pectins to exhibit heterogeneity in macromolecular characteristics such as the degree of esterification, chemical composition and molecular weight, which can subsequently affect their functional properties (Alba & Kontogiorgos, 2017).

Previous works have established that the genotypes; Penkrumah, Agbagoma, Asha, Sengavi, and Balabi are commonly cultivated and consumed in Ghana while preliminary characterisation also highlights the potential for pharmaceutical application (Afotey et al., 2023; Agbenorhevi et al., 2020; Kpodo et al., 2017b). Moreover, the high burden of Ghana's drug importation (70-90%) contributes significantly to Ghana's annual healthcare expenditure resulting in the high cost of medicines and impeding the attainment of the World Health Organisation (WHO) Universal Health Coverage (WHC) (Adebisi et al., 2022; Conway et al., 2019).

Consequently, this study aims to investigate the potential and comparative suspending properties of pectin obtained from five okra genotypes cultivated in Ghana using paracetamol suspensions. Understanding the effects genetic variations have on the quality of pectin produced will impact the commercialization of cultivated okra pectin as a pharmaceutical excipient (suspending agent) while providing the needed literature to address its potential as a pharmaceutical suspending agent. Moreover, the utilization of locally sourced pectin has the potential to reduce the country's healthcare expenditure and attain Universal Health Coverage.

Materials and methods

Materials

Paracetamol BP (Biotech Co., China), tragacanth (Sigma Aldrich, Damsta Germany), 0.1%w/v benzoic acid (BDH England), and laboratory grades of concentrated hydrochloric acid and ethanol (96%) (Department of Pharmaceutics, Kwame Nkrumah University of Science and Technology (KNUST), Ghana). Okra (*Abelmoschus esculentus* L.) genotypes: PL1 (Penkrumah), PL2 (Agbagoma), PL3 (Asha), PL4 (Sengavi), and PL5 (Balabi) were obtained from several markets in Ghana and authenticated at the department of Herbal Medicine by Mr. Clifford Asare. The specimen voucher numbers KNUST/HMI/2023/F002-F006 were assigned to the okra pods respectively after they were deposited at the herbarium. All reagents employed were of analytical grade.

Method

Extraction of Pectin

Okra pectin was extracted and isolated using reported protocols (Alba et al., 2015; Kpodo et al., 2017a). The seeds were removed after the pods were cut open and then sun-dried and milled into powder. An amount of 20 g of the dried okra powder was subsequently defatted for 4 hours with petroleum ether (1 g:10 mL). Employing the extraction conditions of pH 6.0 and 80°C, an aqueous extraction of the defatted okra powder was carried out using 0.1 M phosphate buffer (1 g powder:30 mL buffer solution) for 1 hour. Subsequently, the soluble polymer was isolated from the insoluble polymer using centrifugation (3000 rpm for 10 min at 25 °C). The solubilized pectin in the supernatant was then concentrated using evaporation at 80 °C for 30 min and then precipitated with 96% ($^{v}/_{v}$) aqueous ethanol and then washed in isopropanol and freeze-dried. To prevent absorption of moisture while pending further analysis, the freeze-dried pectins were stored in airtight containers in desiccators.

Characterization of Pectin

Pectin yield

The pectin yield was determined using reported protocols (Kpodo et al., 2017; Samavati, 2013).

Determination of the degree of esterification of extracted pectin using Fourier Transformed Infrared (FTIR) Spectroscopy

FTIR analysis of okra pectin from the different genotypes was performed with the aid of a Bruker Alpha II FTIR spectrophotometer (400 to 2000 cm^{-1}). The degree of esterification (DE) of the samples was subsequently calculated by determining the peak area values of the free carboxyl groups and the esterified groups by following the equation (Pappas et al., 2004).

$$DE = 124.7 \times R + 2.2013 \quad (1)$$

$$R = \frac{A_{1722.24}}{A_{1722.24} + A_{1606.75}} \times 100 \quad (2)$$

Where DE is the degree of esterification and $A_{1722.24}$ and $A_{1606.75}$ are the absorbance densities at 1722.24 and 1606.75 cm^{-1} respectively (Güzel & Akpınar, 2019).

Formulation of Paracetamol Suspension

The paracetamol suspensions (5.0 % $^{w}/_{v}$) were prepared using okra pectin concentrations of 0.5, 1.0 and 2.0 $\%^{w}/_{v}$. Benzoic acid (0.1 $\%^{w}/_{v}$) was employed as a preservative in each formulation. The direct incorporation and levigation techniques as described by (Owusu et al., 2022) were used. Table 1 depicts the formula used in formulating the paracetamol suspensions using tragacanth as the suspending agent. The same formula was used for the pectin suspensions. The concentrations of tragacanth and okra pectin (suspending agent) were the only variables altered in the formula (Oppong et al., 2016).

Table 1. Composition of paracetamol suspensions using tragacanth as a suspending agent

Ingredient	Quantities
Paracetamol powder	5.0 g
Tragacanth (0.5,1,2 % $^{w}/_{v}$)	0.5,1.0,2.0 g
Benzoic acid (0.1 % $^{w}/_{v}$)	0.1 g
Distilled water to	100.0 mL

Quality Control Evaluations on Formulated Suspensions

pH

The pH of the formulated suspensions was determined after they were freshly prepared and then at weekly intervals using the Hanna pH meter (HI 2215). The measurements were carried out in triplicates and the means and standard deviations were noted after ensuring the electrodes were fully immersed (Mahmud et al., 2010; Nep & Conway, 2011; Oppong et al., 2016).

Flow Rate

The flow rate was determined by measuring the time it took for the formulated suspensions to flow through a 20 mL pipette with a stopwatch. The mean values and standard deviations were calculated after the triplicate readings were recorded (Mahmud et al., 2010; Nep & Conway, 2011; Oppong et al., 2016).

Redispersibility

The redispersibility was measured by recording the number of strokes (inversions) needed to resuspend the formulations completely. This was carried out by turning 50 mL of the formulated suspensions in a measuring cylinder through a 180° cycle. This determination was done at weekly intervals for 4 weeks (Mahmud et al., 2010; Nep & Conway, 2011; Oppong et al., 2016).

Sedimentation Rate and Volume

The sedimentation rates of the formulated suspensions were measured by determining the sediment level in the measuring cylinder at intervals of 10, 20, 30, 40, 50 and 60 minutes. During 4 weeks, the sedimentation volume of the suspension was determined by measuring the sediment volume in 50 mL of the formulated suspensions, weekly. The sedimentation volume (F) was calculated using Equation 3. Triplicate measurements were recorded and the mean value was calculated (Mahmud et al., 2010; Nep & Conway, 2011; Oppong et al., 2016).

$$F = \frac{Vu}{Vo} \quad (3)$$

Where Vu is the ultimate volume of sediment in mL and Vo is the original volume of sediment in mL before settling occurred.

Results

Characterization of Extracted Okra Pectin of Different Genotypes

Percentage yield and physicochemical evaluation of okra pectin

The percentage yields from all the five different okra (*Abelmoschus esculentus* L.) genotypes ranged from 6 to 19 $\%^{w}/_{w}$ in the following order PL2 > PL5 > PL3 > PL4 > PL1 as shown in Figure 1.

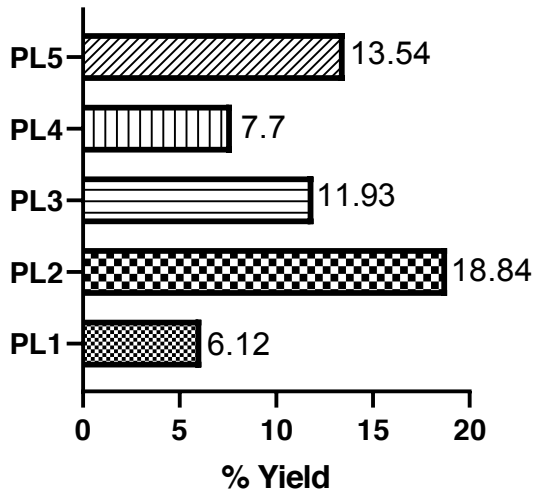

Figure 1: Percentage of pectin yields from different okra genotypes

FTIR Spectroscopy

The FTIR spectra of pectin from all the different genotypes depicted a high level of similarity when peak positions were compared (Table 2 and Figure 2)). Prominent absorption bands were observed between 1730 and 1700 cm^{-1} and 1600-1630 cm^{-1}. The carbohydrate fingerprint regions (1000-1200 cm^{-1}) were also similar which could be assigned to the stretching vibrations of glycosidic bonds (C-O) and pyranoid rings (C-C). The absorption bands between 1730 and 1700 cm^{-1} could be assigned to the C=0 stretching vibrations of the esterified methyl and free carboxylic acid groups. In the region of 1600-1630, the stretching vibration of the carboxylate anion (COO^{-}) was assigned (Manrique & Lajolo, 2002).

Figure 3 shows the degree of esterification (DE) of all the okra genotypes using the two critical peaks of the free carboxyl groups and the esterified groups. The DE of all the genotypes were between 43.56 and 49.00% with PL5 recording the highest DE.

Quality Control Evaluations of Formulated Suspensions

pH, Redispersibility and Flow Rate of Formulated Suspensions

Table 3 shows the pH of the formulated suspensions during the 4-week observation period. The pH of all the formulated suspensions was weakly acidic during the evaluation period and showed no visible signs of incompatibility such as caking, aggregation and crystal growth. When compared to the initial pH of the freshly prepared suspensions, a non-significant difference ($p \geq 0.05$) was observed for all suspensions during the 4-week study period.

The redispersibility of suspensions, an essential qualitative test which assures uniformity of doses is presented in Figure 5 (a-c) (in Appendix). The results indicate that the okra pectin (PL) irrespective of the concentrations or genotype employed was readily re-dispersible when compared to tragacanth (ST).

When the flow rates or apparent viscosities of the suspensions were investigated, it was observed from the time course curves that at all concentrations, when tragacanth was used as a suspending agent, the flowrates were significantly ($p < 0.0001$) higher than when PL was used. The area under the time course curves which highlight cumulative effect also supported this by highlighting a significant difference in flow rates ($p < 0.0001$) as shown in figures 6 (a-c) (in appendix).

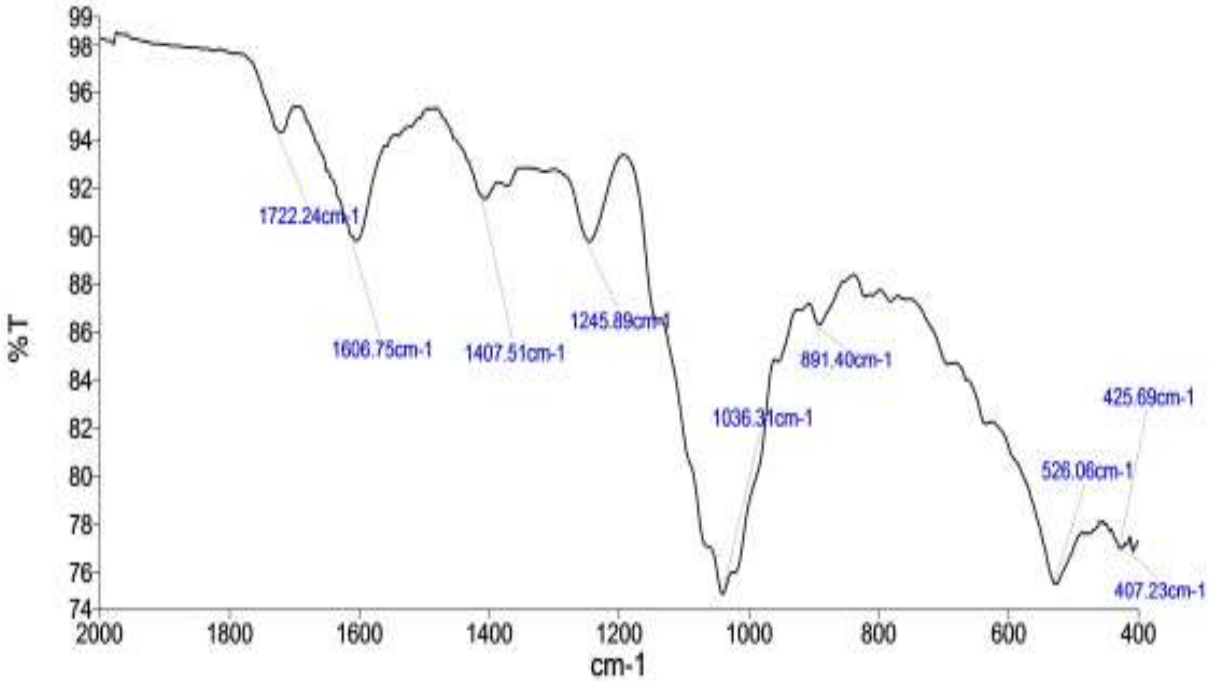

Figure 2: FTIR spectra of different okra genotypes PL1

Sedimentation Rate and Volume

The sedimentation rates of the different concentrations of suspending agents are presented in Figure 7 (a-c) (in the appendix). An increase in concentration from 0.5 to 2% resulted in a decrease in sedimentation rate for all suspensions. Moreover, when compared to tragacanth, PL recorded lower sedimentation rates over the study period at all concentrations which were proportional to the concentrations of PL used.

Figures 8 (a-c) (in the appendix) show the sedimentation volumes (SV) of suspension during the 4-week evaluation period. At 0.5%, SV values were very close to 0 when compared to the higher concentrations (1% and 2%) and were non-significantly different ($p > 0.05$) compared to tragacanth. At 2%, significant differences ($p < 0.0001$) were observed for all PL genotypes when compared to tragacanth while only PL1, PL4 and PL5 were significantly different ($p < 0.0001$) at 1%. PL4 comparatively had the highest SV however, cumulatively, the SV of tragacanth was lower than all PL genotypes at all concentrations.

Discussion

The study aimed to investigate the potential and comparative suspending properties of pectin obtained from five okra genotypes cultivated in Ghana using paracetamol suspensions. The yields obtained were comparable to those reported by Alba and colleagues who reported values between 13 and 15.9% (Alba et al., 2015). The differences in pectin yields attest to the fact that the source of pectin (genetic variations) has a significant impact on the amount of pectin extracted (Chan & Choo, 2013). The results were consistent with results reported by Owusu *et al.* (6-19%) who also discussed extensively the physicochemical properties of pectin from different okra genotypes (Owusu et al., 2021). The knowledge from their background informed the investigation into the suitability of okra pectin as a pharmaceutical suspending agent.

Suspending agents are increasingly utilized to enhance the viscosity and slow sedimentation rates of suspensions to ensure the administration of accurate doses (Ayesu Djakari et al., 2022). One of the essential determinants of suspension stability is the pH which is usually impacted by the nature of polymeric chains in suspending agents used (Reyes-Ortega, 2014). The slightly acidic pH values obtained were comparable with other published literature (The Lubrizol Corporation, 2020; Vázquez-Blanco et al., 2018). Slightly acidic pH (<5.0) is reported to enhance the adsorption of pectin and decrease sedimentation (Lam et al., 2007; Maroziene & De Kruif, 2000; Nakamura et al., 2006). Lower pH ranges tend to decrease the dissociation of carboxyl residues in the galacturonic acid chains of pectin. This culminates in reduced electrostatic repulsion, increased hydrogen bonds and hydrophobic interactions, all of which are essential in the stabilization of pectin gels (Gawkowska et al., 2018). Furthermore, the slightly acidic pH can prevent microbial degradation of suspensions upon storage which was evidenced by the lack of stability issues throughout the study period.

The redispersibility of suspensions is an essential quality which assures accuracy in dosing by ensuring that suspended particle do not aggregate but are uniformly distributed (Nutan & Reddy, 2010; Piriyaprasarth & Sriamornsak, 2011) Higher number of cycles represent poor redispersibility and this could be accounted for by the tighter packing of particles which can be resolved by the addition of a deflocculating agent (Allen & Ansel, 2013; Nutan & Reddy, 2010). The results indicate that the okra pectin irrespective of the concentrations or genotype employed was readily redispersible when compared to the standard (tragacanth) indicating loose aggregates which are easily redispersed by small agitation (Alalor & Obunezie, 2020).

The formulated okra suspensions were pourable from their containers with minimal agitation and thus exhibited pseudoplastic flow, a desired property of suspensions (Allen & Ansel, 2013; Nutan & Reddy, 2010). Higher concentrations of the suspending agent result in decreased viscosity and increased pourability, a key attribute for maintaining pseudoplastic flow (Bamigbola et al., 2017). An increase in concentration is associated with overlapping of coils and an increase in entanglements which increases viscosity but reduces the flow rate (Piriyaprasarth & Sriamornsak, 2011). This property also ensures increased stability and pourability of suspensions (Bamigbola et al., 2017; Larsson et al., 2012; Nutan & Reddy, 2010; Woldu et al., 2021b). At all concentrations, PL demonstrated a significant ($p < 0.0001$) difference in flow rate which suggests better suspending properties compared to tragacanth. The molecules in tragacanth are highly branched compared to pectin and this affects its ability to form gels (Vaclavik & Christian, 2003). Furthermore, both carbohydrates contain different monosaccharide compositions and molecular weights which have been reported to impact their gelling properties in addition to other physicochemical properties such as hydrogen bonding and Van der Waal forces(Mikušová et al., 2022).

An ideal property of a good suspension is the ability of suspended particles to settle slowly for an accurate dose to be administered. A high sedimentation volume (closer to 1) and a slow sedimentation rate are therefore recommended. PL possess good stability indexes and though the dispersed phase settled, the inter-particulate attraction and bonding were not strong enough to form a hard cake during

Table 2. Relevant peaks and assigned groups of other okra genotypes

Okra genotype	Relevant peaks (cm^{-1})	Assigned functional groups
PL2	1037.83	C-O stretching of alcohol (-OH) group
	1407.32	O-H bending of carboxylic acid (-COOH) group
	1603.30	the stretching vibration of the carboxylate anion (COO^-)
	1721.12	C=O stretching vibrations of the esterified methyl and free carboxylic acid groups
PL3	1032.36	C-O stretching of alcohol (-OH) group
	1408.17	O-H bending of carboxylic acid (-COOH) group
	1602.18	the stretching vibration of the carboxylate anion (COO^-)
	1723.62	C=O stretching vibrations of the esterified methyl and free carboxylic acid groups
PL4	1032.29	C-O stretching of alcohol (-OH) group
	1400.22	O-H bending of carboxylic acid (-COOH) group
	1604.32	the stretching vibration of the carboxylate anion (COO^-)
	1719.52	C=O stretching vibrations of the esterified methyl and free carboxylic acid groups
PL5	1037.40	C-O stretching of alcohol (-OH) group
	1407.07	O-H bending of carboxylic acid (-COOH) group
	1606.67	the stretching vibration of the carboxylate anion (COO^-)
	1720.12	C=O stretching vibrations of the esterified methyl and free carboxylic acid groups

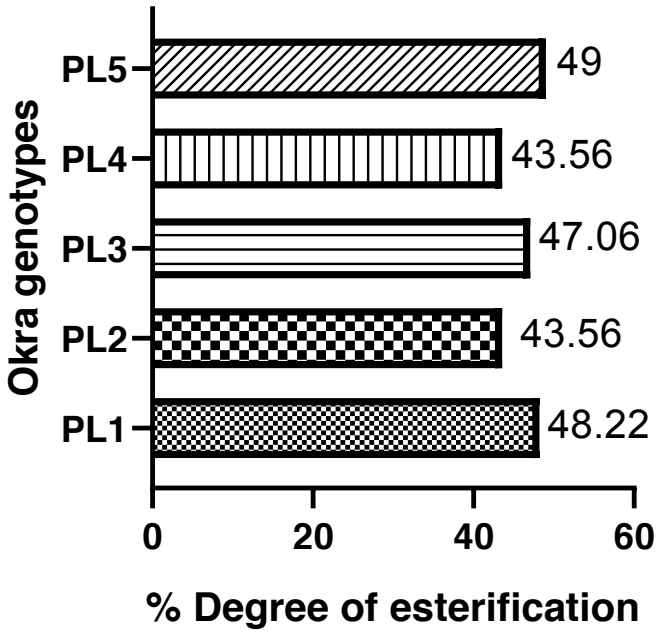

Figure 3. The Degree of Esterification of Pectin from Different Okra Genotypes

Table 3. pH of the Formulated Suspensions over a 4-Week Period

Formulation	pH				
	Freshly prepared	Week 1	Week 2	Week 3	Week 4
ST 0.5 %	4.60±0.04	4.60±0.03 [a]	4.61±0.06 [a]	4.61±0.04 [a]	4.62±0.02 [a]
ST 1.0 %	4.80±0.05	4.80±0.06 [a]	4.81±0.03 [a]	4.81±0.08 [a]	4.81±0.02 [a]
ST 2.0 %	4.81±0.03	4.81±0.01 [a]	4.82±0.04 [a]	4.82±0.08 [a]	4.82±0.06 [a]
PL1 0.5 %	4.82±0.06	4.83±0.06 [a]	4.83±0.09 [a]	4.83±0.03 [a]	4.83±0.08
PL1 1.0 %	4.83±0.04	4.82±0.04 [a]	4.82±0.09 [a]	4.83±0.06 [a]	4.83±0.07 [a]
PL1 2.0 %	4.83±0.07	4.82±0.03 [a]	4.82±0.08 [a]	4.82±0.03 [a]	4.82±0.05 [a]
PL2 0.5 %	4.81±0.04	4.81±0.06 [a]	4.83±0.02 [a]	4.83±0.02 [a]	4.83±0.02 [a]
PL2 1.0 %	4.82±0.02	4.83±0.09 [a]	4.83±0.06 [a]	4.83±0.05 [a]	4.83±0.06 [a]
PL2 2.0 %	4.84±0.03	4.84±0.03 [a]	4.83±0.05 [a]	4.84±0.07 [a]	4.84±0.07 [a]
PL3 0.5 %	4.77±0.07	4.77±0.01 [a]	4.83±0.02 [a]	4.83±0.02 [a]	4.83±0.02 [a]
PL3 1.0%	4.78±0.08	4.78±0.04 [a]	4.79±0.06 [a]	4.78±0.03 [a]	4.78±0.05 [a]
PL3 2.0 %	4.79±0.04	4.79±0.04 [a]	4.79±0.08 [a]	4.79±0.06 [a]	4.79±0.06 [a]
PL4 0.5 %	4.79±0.04	4.78±0.05 [a]	4.78±0.04 [a]	4.78±0.05 [a]	4.79±0.06 [a]
PL4 1.0 %	4.79±0.06	4.79±0.03 [a]	4.79±0.05 [a]	4.79±0.04 [a]	4.79±0.05 [a]
PL4 2.0 %	4.80±0.08	4.79±0.03 [a]	4.79±0.05 [a]	4.80±0.02 [a]	4.80±0.04 [a]
PL5 0.5 %	4.88±0.03	4.88±0.04 [a]	4.88±0.04 [a]	4.88±0.05 [a]	4.88±0.02 [a]
PL5 1.0 %	4.90±0.05	4.90±0.06 [a]	4.89±0.05 [a]	4.89±0.06 [a]	4.89±0.04 [a]
PL5 2.0 %	4.91±0.07	4.91±0.06 [a]	4.91±0.03 [a]	4.90±0.04 [a]	4.91±0.06 [a]

Values are means ± SD (n = 3). [a] $p \geq 0.05$ non-significant difference in pH between freshly prepared suspensions and during the evaluation period using one-way ANOVA. (ST, standard).

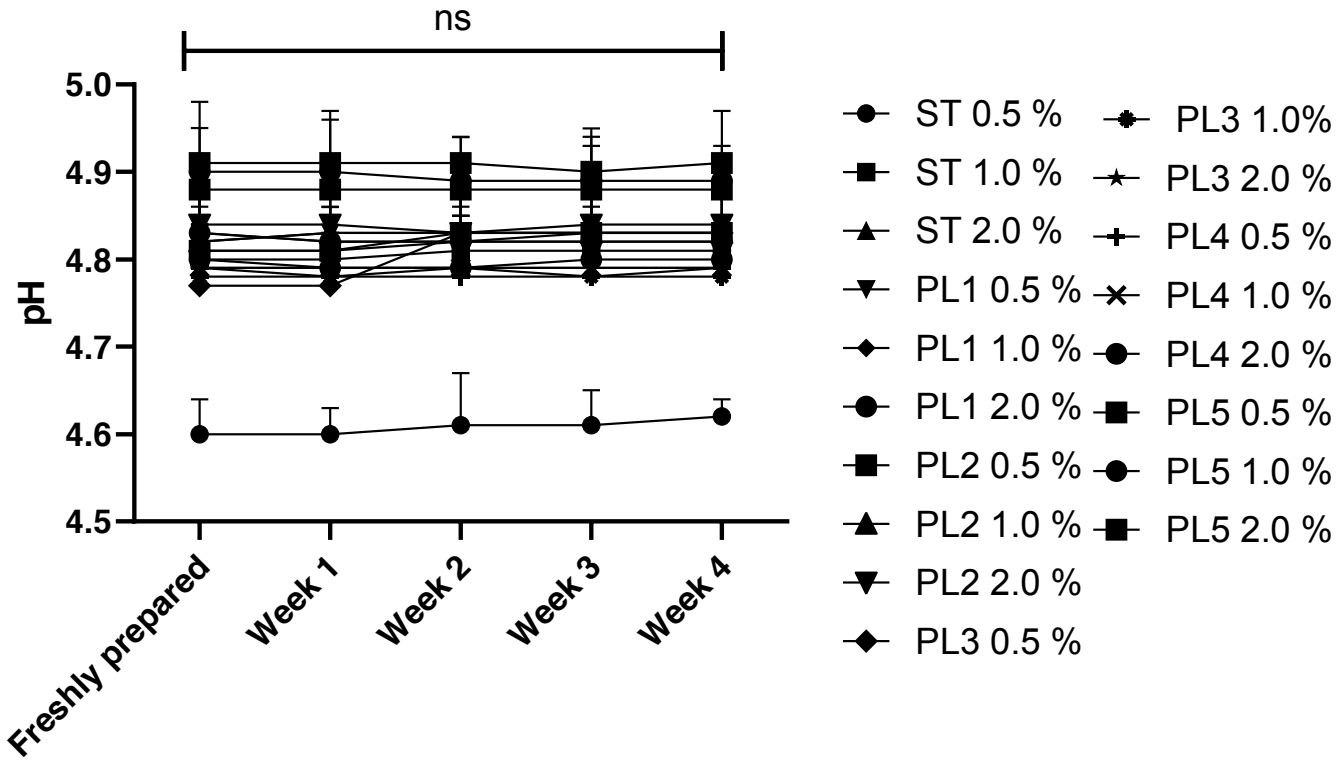

Figure 4. pH of the Formulated Suspensions over a 4-Week Period.

the evaluation period. The sedimentation rates of the suspensions were inversely proportional to the concentrations of PL used in the formulation which indicates that, as the concentration increases, the suspending ability of pectin also increases and supports the findings in the flow rate.

Conclusion

Okra pectin demonstrated good suspending properties when compared to routinely used natural suspending agent, tragacanth. At all concentrations, okra pectin exhibited superior pH, flow rate, and redispersibility, as well as sedimentation volume and rate. The abundance, cost-effectiveness and non-toxicity of okra pectin positions it as a good raw material for the pharmaceutical industry in Ghana with the potential to reduce overhead costs in formulating oral dosage forms.

Authors Contributions

Conceptualization, MEBG, MTB, KOK. And FWAO.; methodology, FWAO, PGJA, JA, EAQ and BAA.; data curation, PGJA, PKT; writing—original draft preparation, FWAO, PJGA, MEBG; writing—review and editing, KOK, MTB. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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Woldu, G., Baymot, B., Tesfay, D., & Demoz, G. T. (2021b). Evaluation of Aloe elegans Mucilage as a Suspending Agent in Paracetamol Suspension. *BioMed Research International*, *2021*, 1–12. https://doi.org/10.1155/2021/5058372

APPENDIX

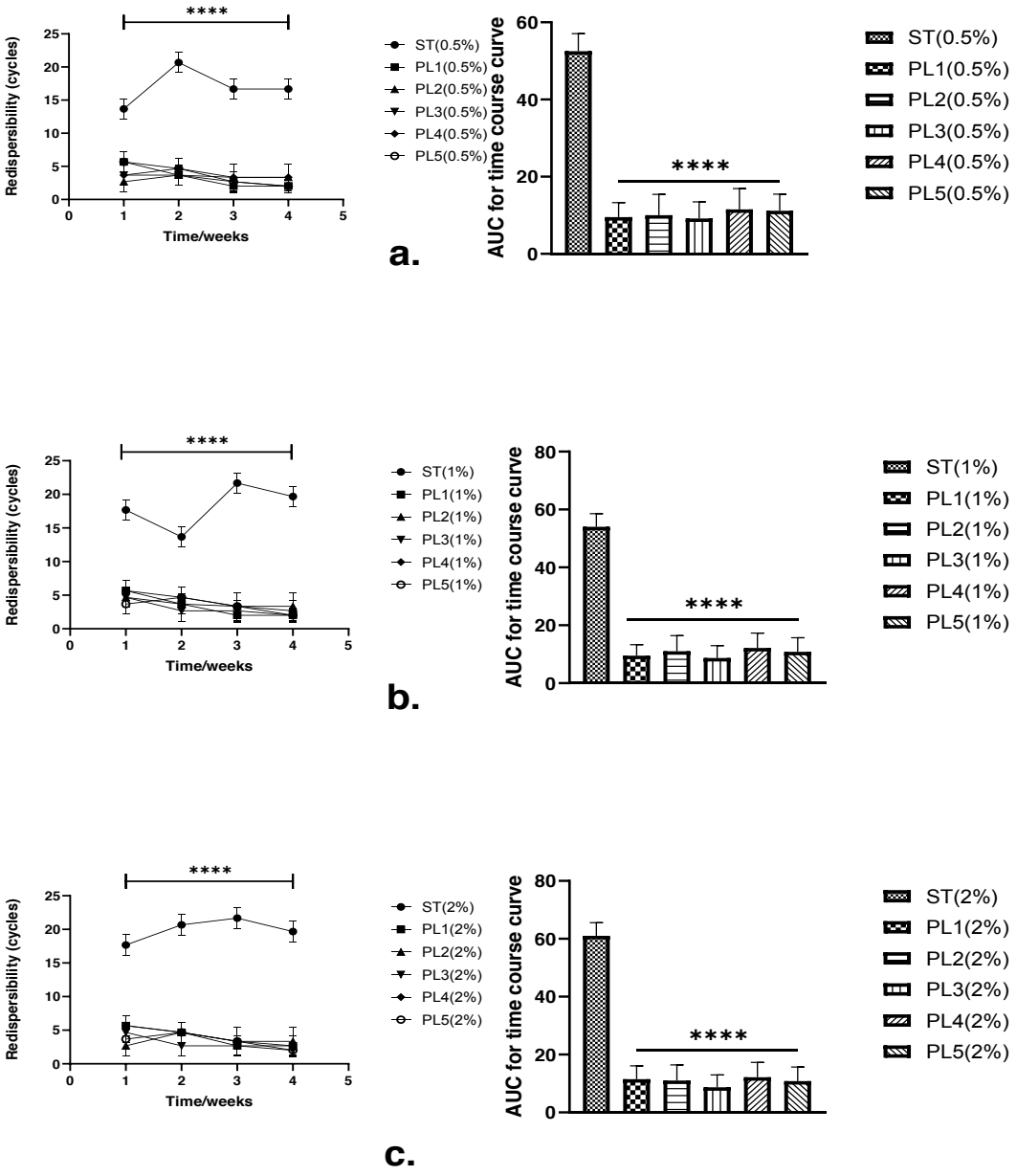

*Figure 5: Redispersibility (cycles) of suspensions formulated with different suspending agents (a) PL (0.5%w/v) and ST (0.5%w/v), (b) PL (1%w/v) and ST (1%w/v) and (c) PL (2%w/v) and ST (2%w/v). **** $p < 0.0001$ significance difference between (ST) Tragacanth and PL.*

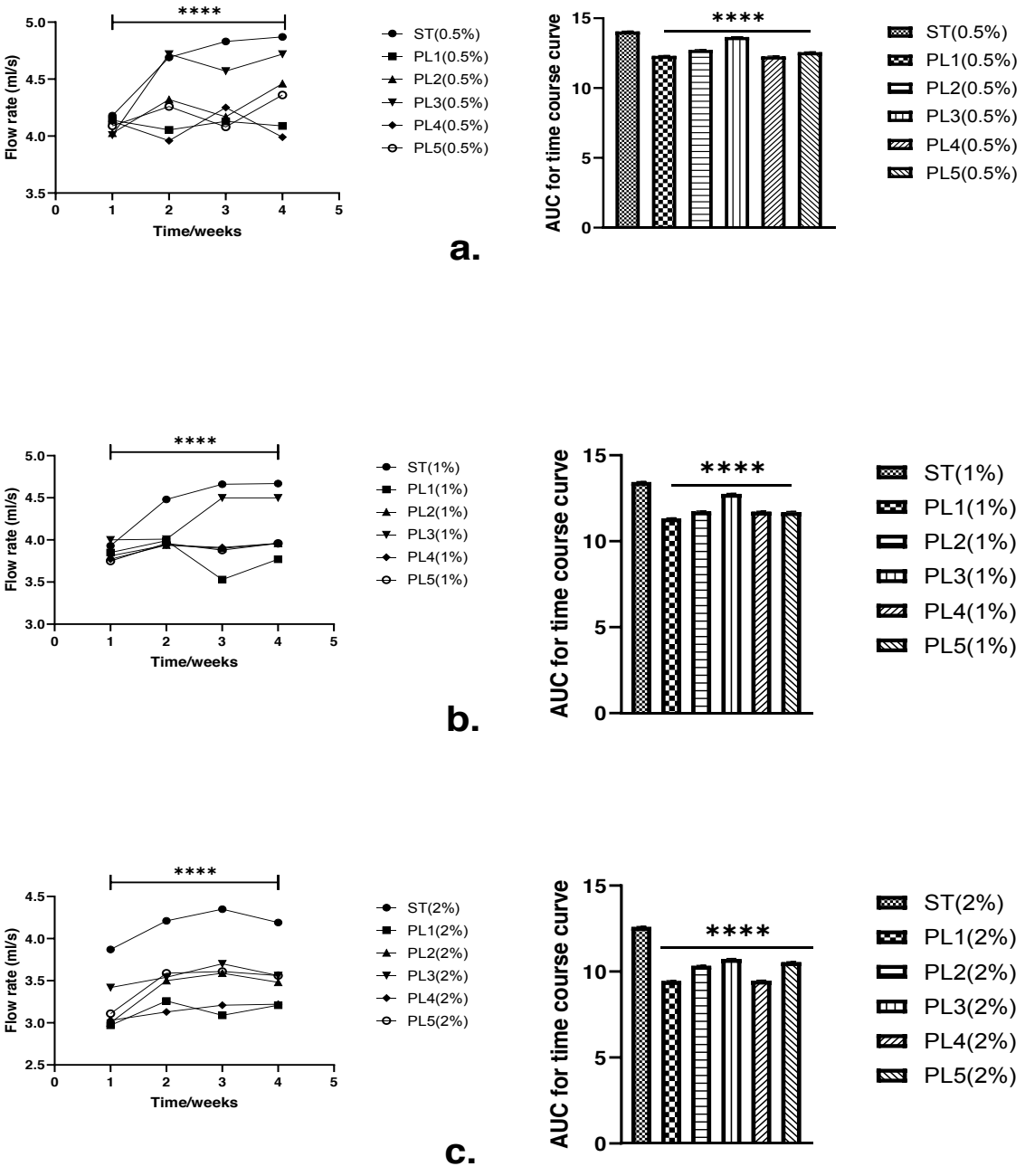

*Figure 6: Flow rate of suspensions (ml/s) of suspensions formulated with different suspending agents (a) PL (0.5%w/v) and ST (0.5%w/v), (b) PL (1%w/v) and ST (1%w/v) and (c) PL (2%w/v) and ST (2%w/v). **** p < 0.0001 significance difference between (ST) Tragacanth and PL.*

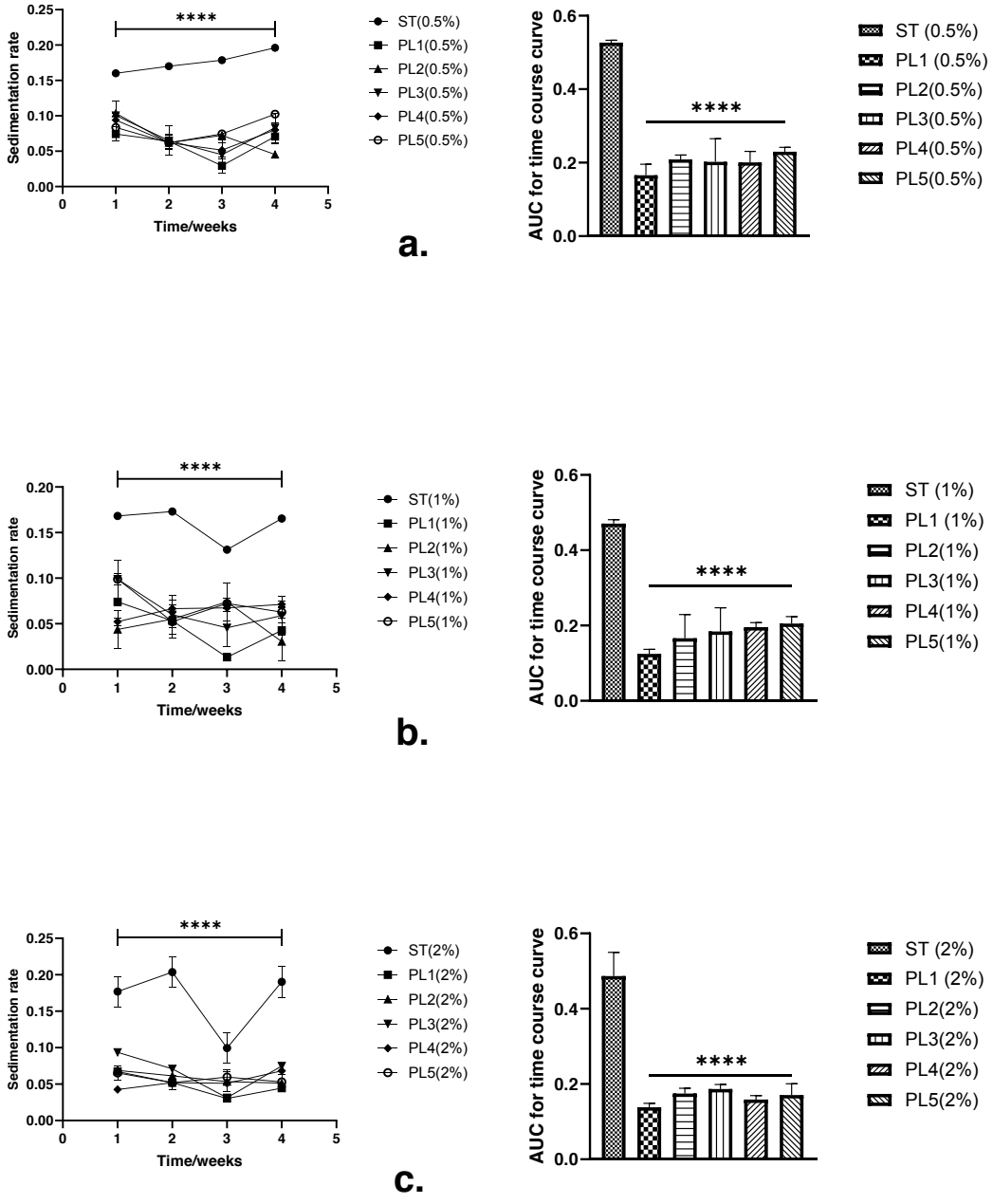

*Figure 7: Sedimentation rate of suspensions formulated with different suspending agents (a) PL (0.5%w/v) and ST (0.5%w/v), (b) PL (1%w/v) and ST (1%w/v) and (c) PL (2%w/v) and ST (2%w/v).). **** $p < 0.0001$ significance difference between (ST) Tragacanth and PL.*

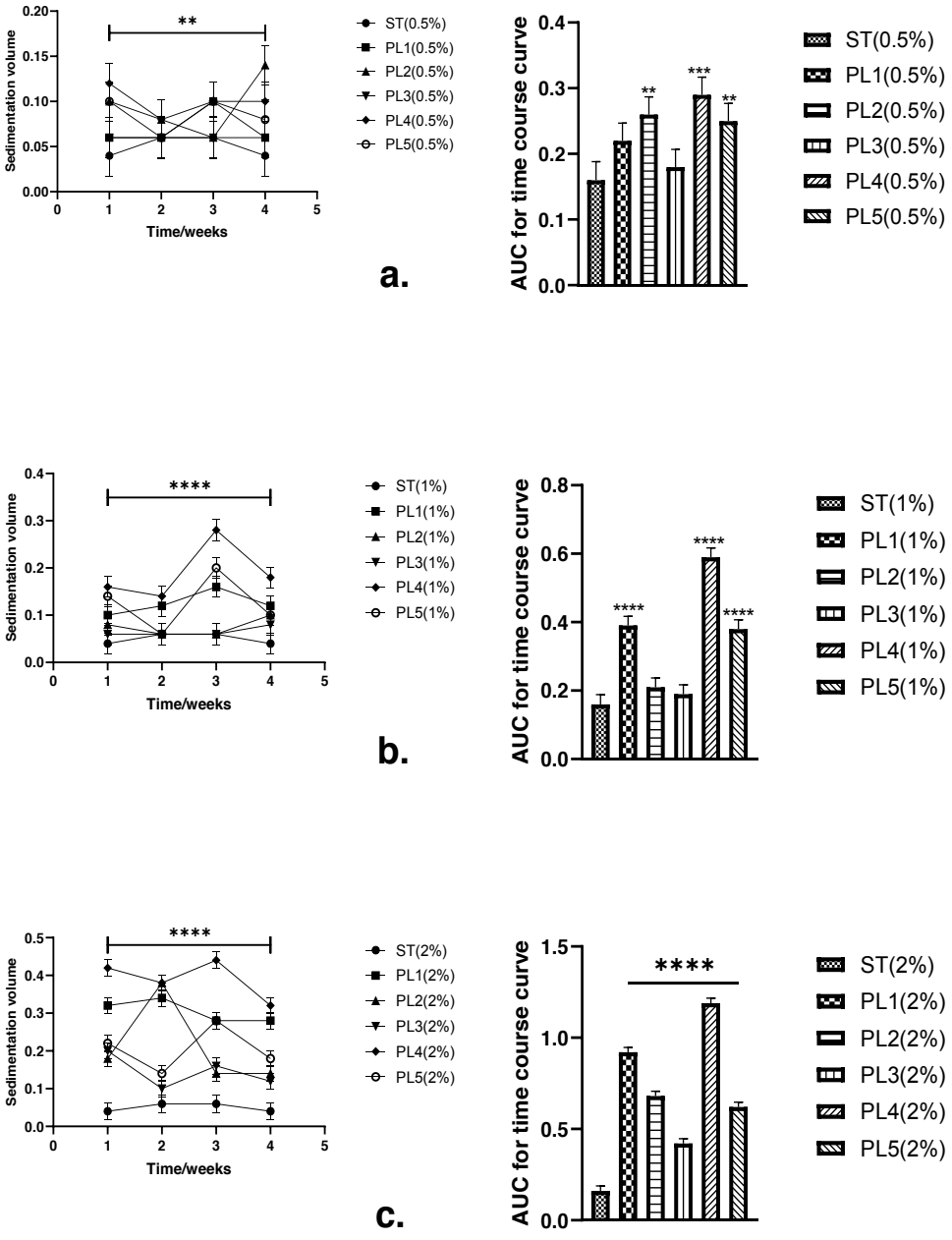

*Figure 8: Sedimentation volume of suspensions formulated with different suspending agents (a) PL (0.5%w/v) and ST (0.5%w/v), (b) PL (1%w/v) and ST (1%w/v) and (c) PL (2%w/v) and ST (2%w/v).). **p <0.01, ***p<0.001,**** p<0.0001 significance difference between (ST) Tragacanth and PL.*

Journal of Pharmacy

ORIGINAL ARTICLE Open Access

Antibacterial Potential of *Ximenia americana* L. Olacaceae: Molecular Docking, Molecular Dynamics, and ADMET Prediction

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ABSTRACT

Introduction: The devastating effect of persistent and recurrent bacterial infections coupled with antibiotic resistance is a driving force for prospects into alternative antibacterial therapeutics to achieve treatment. This study investigates the antibacterial potential of *Ximenia americana* (XA) *via* molecular docking, molecular dynamics, and ADMET approach.

Materials and methods: The ligands and target were downloaded from respective databases and docked using PyRx software followed by molecular dynamics simulation (MDS) with iMOD and CABflex 2.0 online servers then ADMET, drug likeness, lead likeness, and medicinal chemistry predictions of the top docked ligands using pkCSM and SwissADME online servers.

Results: Stigmasterol exhibited the lowest binding affinity and inhibition constant respectively with all the targets; enoyl-acyl-carrier-protein reductase (-7.1 kcal/mol and 6.16 µM), Penicillin-binding Protein 2X (-8.8 kcal/mol and 0.35 µM), dihydrofolate reductase (-9.6 kcal/mol and 0.09 µM), dihydropteroate Synthase (-7.8 kcal/mol and 1.89 µM), UDP-N-acetylglucosamine enolpyruvyl transferase (-7.1 kcal/mol and 6.16 µM), and topoisomerase IV (-7.8 kcal/mol and 1.89 µM). The MDS showed several cluster displacements and residue fluctuations with the docked targets with higher residue fluctuations observed for enoyl-acyl-carrier-protein reductase (11.33 Å), Penicillin-binding Protein 2X (4.67 Å), dihydrofolate reductase (3.61 Å), dihydropteroate Synthase (4.97 Å), UDP-N-acetylglucosamine enolpyruvyl transferase (3.38 Å), and topoisomerase IV (4.35 Å). 4,4-Dimethylcyclohex-2-en-1-ol exhibited superior overall ADMET properties, oral bioavailability, drug-likeness, and medicinal chemistry.

Conclusion: Conclusively, Stigmasterol and 4,4-Dimethylcyclohex-2-en-1-ol might be responsible for the antibacterial effect of XA. Although the latter showed better interaction with the target proteins, the former showed better ADMET properties, oral bioavailability, drug-likeness, and medicinal properties. However, improvement in these properties might enhance their antibacterial activity.

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JOP

Introduction

Bacterial infections are regarded as a major culprit leading to mortality worldwide which is further complicated by the emergence of antimicrobial resistance (AR) (Uddin et al., 2021). Antibiotic abuse in clinical practice contributes to the enhanced evolution of microbes *via* mutation into more infectious and antibiotic-resistant strains, thus creating a major problem in treatment and threatening global health (Kapoor et al., 2017). In bacteria, AR emerges as a result of antibiotic inactivation, metabolic pathways bypass, modified target, and permeability (Kapoor et al., 2017). This mechanism forms a basis for survival and enhances their endurance to antibiotic effects previously lethal to their survival (Zaman et al., 2017). Additionally, biofilm formation has also facilitated AR, further complicating treatment (Uddin et al., 2021). Although the use of antibiotics is acceptable worldwide, bacteria evolution into antibiotic resistance strains is a global concern due to the re-emergence of drug-resistant infections (Gajdács & Albericio, 2019). Antibiotics target DNA replication proteins, cell walls, and macromolecule synthesis, which are processes needed for bacterial survival (Kapoor et al., 2017). However, the instinct for survival and adaptation to adverse conditions associated with bacteria drives evolutionary mechanisms including genetic modifications that lead to AR (MacGowan & Macnaughton, 2017).

Although multiple drugs or combined therapies are applied in the treatment of bacterial infections, the problem of multidrug resistance further emerges (Uddin et al., 2021), thus the prospects for alternatives such as plant-based sources. Plant-based drugs include phytochemicals produced in plants against predators and climate change to ensure their survival (Doughari, 2012). Some of these phytochemicals are lethal to bacteria targeting multiple processes and pathways simultaneously and synergistically (Dahiru et al., 2023b). Thus, might be regarded as an alternative to conventional drugs in combating AR. The use of plants in the management of bacterial ailments garnered attention due to their affordability, safety, and acceptability. Thus, various plants of varying efficacy are used for antibiotic purposes to achieve therapeutic goals (Chassagne et al., 2021). Moreover, previous studies reported the antibacterial activity of different plant extracts revealing the bactericidal and inhibitory effects of the plant extracts (Dahiru et al., 2023a; Dahiru et al., 2023b; Ebbo et al., 2019). *X. americana* has been associated with different antibacterial effects (Agustina & Nugroho, 2021; Kiessoun et al., 2018; Maikai et al., 2009) including inhibiting biofilm formation (Bakrim et al., 2022), and antibiotic synergistic effect (de Menezes et al., 2019). In traditional medicine, the plant is used for urinary tract infections, diarrhea, anti-parasitic, leprotic ulcers, antiseptic, and skin infections (Monte et al., 2012).

Different computational techniques are employed in drug discovery and design to predict the possible mechanism of action of compounds including molecular docking, molecular dynamics, and the ADMET (absorption, distribution, metabolism, excretion, and toxicity) approach (Clegg & Mac Gabhann, 2015; Lin et al., 2020; Sliwoski et al., 2014). In the molecular docking approach, ligands (compounds) and targets (proteins) are downloaded from various databases and prepared by energy minimization, water molecules, and heteroatom removal for docking (Raval & Ganatra, 2022). The ligand is docked into the binding pocket of the target to calculate the binding affinity or energy and the interactions (Raval & Ganatra, 2022). This can be further subjected to molecular dynamics simulation to predict the possible residue motion with the structure of the target (Hollingsworth & Dror, 2018). Previous studies revealed many compounds with various antimicrobial properties present in X. *americana* (Dahiru et al., 2022). However, the exact mechanisms of action of the compounds are yet to be identified. In this study, the antibacterial activity of the compounds previously identified in *X. americana* was investigated *in silico via* molecular docking and molecular dynamics to determine its major antibacterial compounds followed by ADMET predictions of the compounds for drug- and lead-likeness potential.

Materials and methods

Materials

Hardware Specification

A personal computer was used for the present study with 8 GB RAM with an AMD 2.1 GHz to 2.9 GHz Elite Quad-core A10-5745M accelerated processer and AMD Radeon HD 8610G graphics with up to 3053 MB total graphics memory.

Ligands and Targets

The ligands (compounds) previously identified in our study (Dahiru et al., 2022) and targets used in our study were downloaded from the PubChem (https://pubchem.ncbi.nlm.nih.gov) and RSCB (https://www.rcsb.org) databases in SDF and PDB formats respectively. The ligands were energy-minimized using the PyRx – Python Prescription software (version 0.8) while water molecules, heteroatoms, attached ligands, and extra chains were removed from the targets using AutoDockTools (version 1.5.7) (Sanner, 1999) before docking. The list of ligands is presented in Table 1 including their PubChem ID.

Table 1: List of Ligands

Name	PubChem ID
Catechol	289
Phloroglucinol	359
Hydroquinone	785
Palmitic Acid	985
Pyrogallol	1057
Methyl palmitate	8181
5-Methyl-1H-pyrazole-3-carboxylic acid	9822
Hydroxyquinol	10787
Tridecane	12388
Pentadecanoic acid	13849
2-Isopropoxyphenol	20949
2-dodecoxyethanol	24750
5-Acetoxymethyl-2-furaldehyde	66349
6-Methylpyridazin-3(2H)-one	83346
3,4-dimethylcyclohexanol	97960
2,6-Heptanedione	100532
5-Butylnonane	300476
Oleic Acid	445639
3,8-Dimethyldecane	519396
Tetradec-13-enal	522841
Stigmasterol	5280794
1-(1-Butenyl) pyrrolidine	5357122
7,11-Hexadecadien-1-ol, acetate, (7Z,11Z)-	5363265
9-Tetradecenal, (Z)-	5364471
4,4-Dimethylcyclohex-2-en-1-ol	19771306

The docking targets are presented in Table 2 depicting their RSCB PDB ID and the docking grid box center.

Table 2: List of Targets

Name	PBD ID	Grid box center		
		X	Y	Z
Enoyl-acyl-carrier-protein Reductase (FabI)	1LX6	-2.28	22.61	134.79
Penicillin-binding protein 2X (PBP2X)	5OJ0	33.51	-16.54	54.36
Dihydrofolate reductase (DHFR)	1RG7	-1.21	21.34	21.20
Dihydropteroate synthase (DHPS)	5V79	-18.37	7.09	103.33
UDP-N-acetylglucosamine enolpyruvyl transferase (MurA)	2RL2	28.79	-53.59	45.05
Topoisomerase IV (TopoIV)	3FV5	12.93	-0.80	2.39

Methods

Molecular Docking (MD) and Molecular Dynamics Simulation (MDS)

The docking pockets were initially identified using the Prankweb online server (https://prankweb.cz) (Jendele et al., 2019) to determine the docking coordinates. The docking protocol was carried out using the PyRx software *via* the Vina wizard to determine the binding affinity (BA) with exhaustiveness set to 16. The inhibition constant (Ki) was evaluated from the BA using the equation Ki = exp ΔG/RT where T=298.15 K (temperature) and R=1.985 x 10^{-3} $kcal^{-1}$ mol^{-1} k^{-1} (the universal gas constant) and ΔG = binding affinity (Ortiz et al., 2019). Furthermore, the ligand-target docked complexes were saved in PDB and visualized in 2D and 3D using Biovia Discovery Studio Visualizer (version 16.1.0). Additionally, only the top three compounds with the least BA and Ki were selected and presented. Lastly, the top docked complex with the least BA and Ki was subjected to MDS using the iMODs server (https://imods.iqfr.csic.es) (iMODS) (López-Blanco et al., 2014) and CABs-flex v2.0 (http://biocomp.chem.uw.edu.pl/CABSflex2/index) (Kurcinski et al., 2019) online servers to identify cluster and residue displacements denoted by the root-mean-square fluctuation (RMSF) respectively.

ADMET Prediction

The ADMET of the compound with the least BA and Ki interaction was further predicted using the pkCSM online server (https://biosig.lab.uq.edu.au/pkcsm) (Pires et al., 2015) and the SwissADME server (http://www.swissadme.ch) (Daina et al., 2017) for its drug-likeness and medicinal chemistry.

Results

The BA and Ki of the compounds docked with FabI and PBP2X are displayed in Table 3. Stigmasterol exhibited superior binding interaction with FabI demonstrating the least BA (-7.1 kcal/mol) and Ki (6.16 µM) followed by 4,4-Dimethylcyclohex-2-en-1-ol with -5.5 kcal/mol and 92 µM next to 7,11-Hexadecadien-1-ol, acetate, (7Z,11Z) with -5.0 kcal/mol and 214.24 µM. The docking interactions with PBP2X revealed stigmasterol as the most favorable among the compounds with BA and Ki of -8.8 kcal/mol and 0.35 µM respectively next to 4,4-Dimethylcyclohex-2-en-1-ol with -7 kcal/mol and 7.30 µM, while the least favorable was 7,11-Hexadecadien-1-ol, acetate, (7Z,11Z) with -5.3 kcal/mol and 129.05 µM, a similar pattern observed in that of FabI.

The binding interactions of FabI with stigmasterol are presented in Figure 1 showing the 2D and 3D dock pose and depicting the hydrogen bonds and alkyl interactions. Single conventional hydrogen was observed with LYS163 while six alkyl interactions were also observed.

Table 3: Binding affinity and Ki of the interaction of FabI and PBP2X with the compounds

Target	Ligand	Binding Affinity (kcal/mol)	Ki (µM)
FabI	Stigmasterol	-7.1	6.16
	4,4-Dimethylcyclohex-2-en-1-ol	-5.5	92.04
	7,11-Hexadecadien-1-ol, acetate, (7Z,11Z)	-5.0	214.24
PBP2X	Stigmasterol	-8.8	0.35
	4,4-Dimethylcyclohex-2-en-1-ol	-7.0	7.30
	7,11-Hexadecadien-1-ol, acetate, (7Z,11Z)	-5.3	129.05

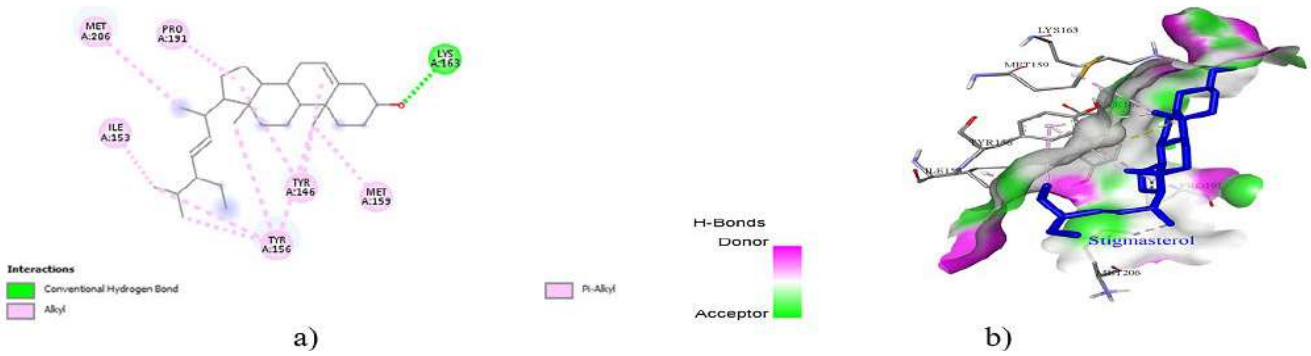

Figure 1: Binding interactions and docked pose of stigmasterol with FabI; a) 2D and b) 3D

The residue fluctuation and cluster displacement of FabI and stigmasterol-FabI docked complex are shown in Figure 2. Increased residue fluctuations were observed at GLY2 (11.33 Å), SER16 (2.12 Å), ASN41 (2.50 Å), CYS210 (1.14 Å), and ASN257 (3.52 Å) though there was a decrease at ASP103 (2.51 Å) compared to the apoenzyme. Although the highest cluster movement was observed at the N-terminal end of the peptide, a lesser displacement was also observed close to the midchain.

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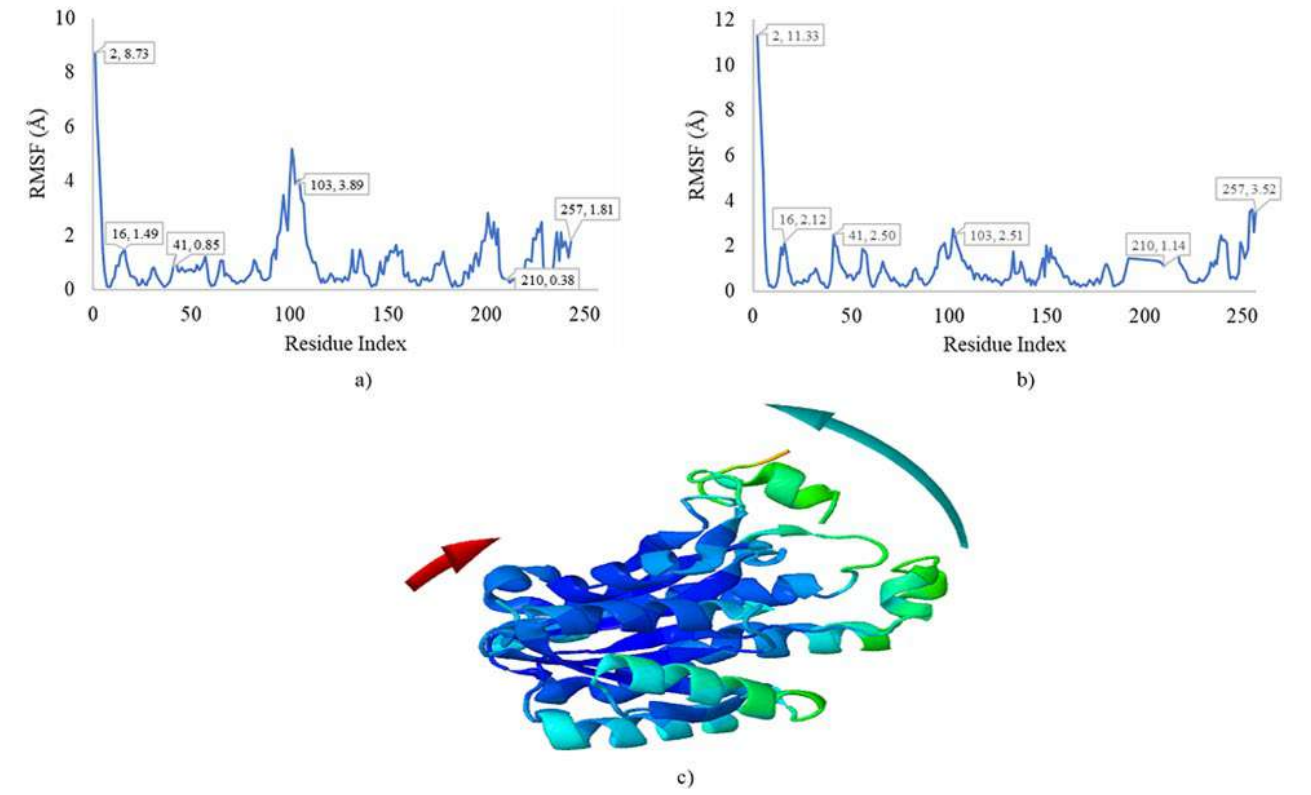


Figure 2: MDS result depicting residue fluctuation of; a) FabI, b) Stigmasterol-FabI docked complex, and c) Cluster displacement of the docked complex (blue and red arrows indicate higher and lower displacements respectively)

The binding interactions of PBP2X with the stigmasterol are presented in Figure 3 highlighting the residue involved in 2D and 2D dock pose. A π-alkyl interaction with PHE450 was observed in addition to a π-sigma with TRP374.

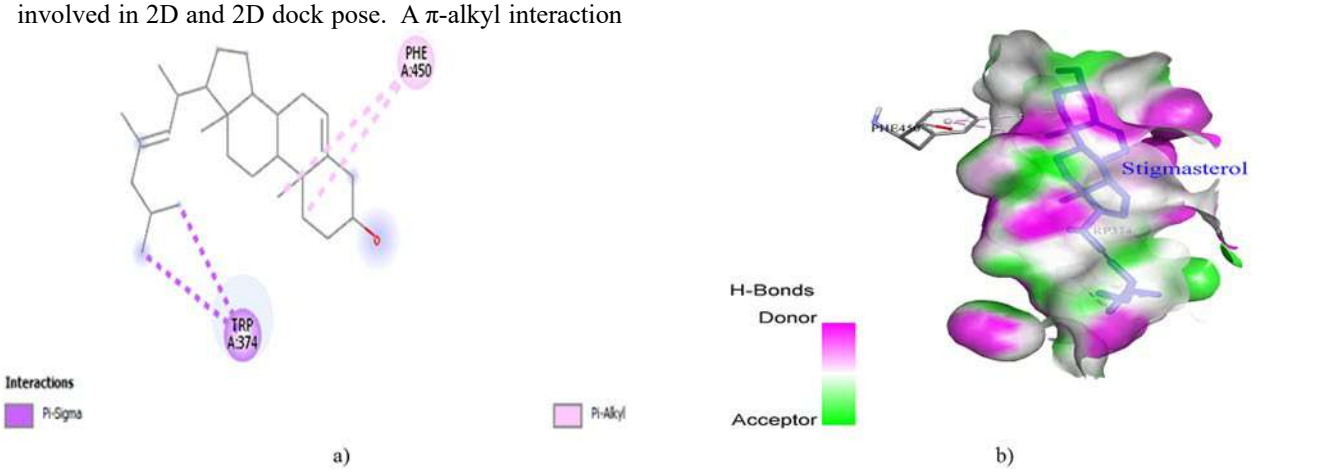


Figure 3: Binding interactions and docked pose of stigmasterol with PBP2X; a) 2D and b) 3D

Figure 4 presents the MDS results for PBP2X and stigmasterol-PBP2X docked complex showing the fluctuating residues and cluster movement. Several increased residue displacements were observed with the highest by ILE318 (4.67 Å) while others include PRO144 (3.31 Å), GLN629 (4.54 Å), GLY677 (3.13 Å), and ASP750 (4.24 Å). However, a notable decrease in ASP555 (2.07 Å) and ASN580 (3.09 Å) was also observed. Additionally, cluster displacements were seen at both terminal clusters of the peptide and within its structure though the highest was at the N-terminal.

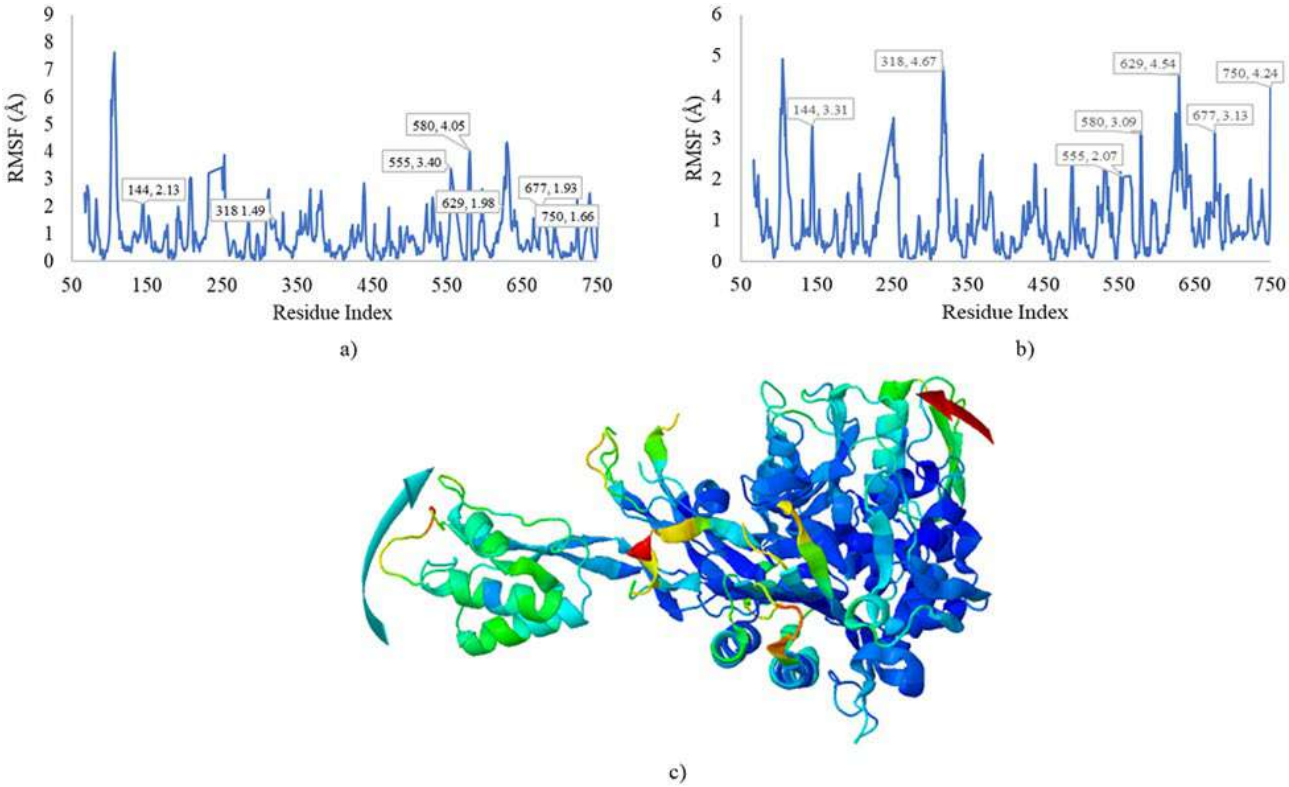


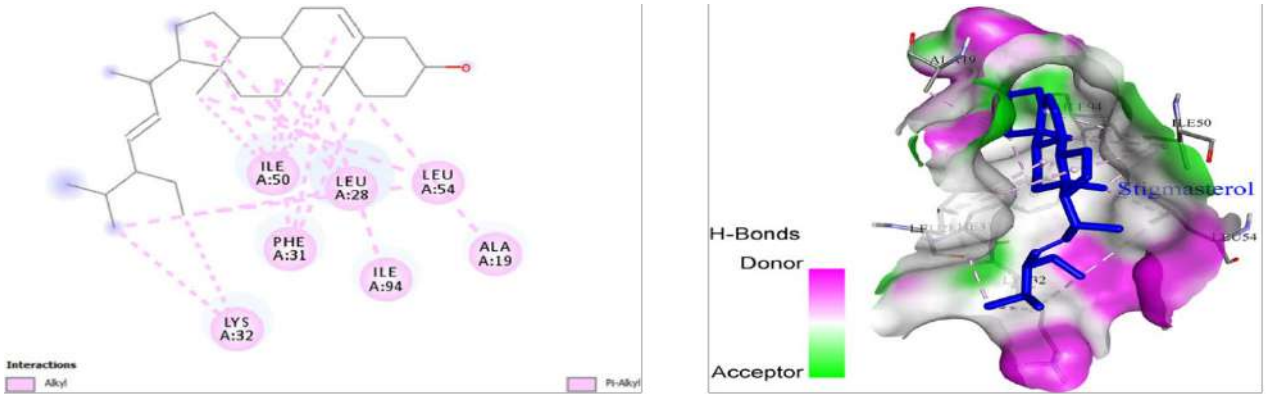
Figure 4: MDS result depicting residue fluctuation of; a) PBP2X, b) Stigmasterol-PBP2X docked complex, and c) Cluster displacement of the docked complex (blue and red arrows indicate higher and lower displacements respectively)

Table 4 displays the BA and Ki of the binding affinity and Ki of DHFR and DHPS with the compounds. Stigmasterol exhibited a superior and more favorable interaction with DHFR with BA and Ki of -9.6 kcal/mol and 0.09 µM respectively followed by 4,4-dimethylcyclohex-2-en-1-ol with -7.7 kcal/mol and 2.24 µM. Oleic acid exhibited the highest BA (-6.5 kcal/mol) and Ki (17 µM) when docked with DHFR. Furthermore, stigmasterol showed the BA (-7.8 kcal/mol) and Ki (1.89 µM) when docked with DHPS. These values are more favorable interactions compared to the other compounds. Specifically, 4,4-dimethylcyclohex-2-en-1-ol showed a BA of -5.7 kcal/mol and Ki of 65.65 µM, while 5-acetoxymethyl-2-furaldehyde demonstrated the least favorable values with -5.3 kcal/mol and 129.05 µM, respectively.

The 2D and 3D dock poses depicting the binding interactions of the stigmasterol-DHFR docked complex are presented in Figure 5. A total of seven alkyl interactions were observed with ILE50, LEU28, LEU54, PHE31, ILE94, ALA19, and LYS32.

Table 4: Binding affinity and Ki of the interaction of DHFR and DHPS with the compounds

Target	Ligand	Binding Affinity (kcal/mol)	Ki (µm)
DHFR	Stigmasterol	-9.6	0.09
	4,4-Dimethylcyclohex-2-en-1-ol	-7.7	2.24
	Oleic Acid	-6.5	16.99
DHPS	Stigmasterol	-7.8	1.89
	4,4-Dimethylcyclohex-2-en-1-ol	-5.7	65.65
	5-Acetoxymethyl-2-furaldehyde	-5.3	129.05



a) b)

Figure 5: Dock poses and interactions of stigmasterol with DHFR; a) 2D and b) 3D

Figure 6 displays the MDS result of DHFR and stigmasterol-DHFR complex revealing the residue fluctuation and cluster movement. All the residues were displaced with the highest (3.61 Å) displacement observed around the midchain (GLY67) of the peptide with cluster movement at both terminals. Additionally, notably increased displacements by PRO21 (2.70 Å) and a decrease by GLY56 (1.91 Å) were observed. Cluster displacements were observed within the peptide chain with the highest around the N-terminal to midchain of the peptide.

The binding interactions of stigmasterol with DHPS are shown in Figure 7 revealing the docked pose in 2D and 3D. Exactly five alkyl interactions were observed with ARG63, HIS257, ILE20, PHE190, and LYS221 stabilizing the docked complex.

Figure 8 depicts the cluster displacement and residue fluctuation of DHPS and stigmasterol-DHPS docked complex MDS. The highest (4.97 Å) residue displacement was seen at the midchain (THR147) of the peptide though there were increased displacements by MET1 (2.19 Å), HIS14 (2.48 Å), and ASN35 (3.33 Å) with decrease by PRO80 (0.22 Å), ALA111 (0.56 Å), LEU134 (0.38 Å), ALA170 (0.38 Å), and LYS221 (1.78 Å). The C-terminal demonstrated the highest cluster displacement though both terminals were displaced including the lower displacement at the midchain of the peptide.

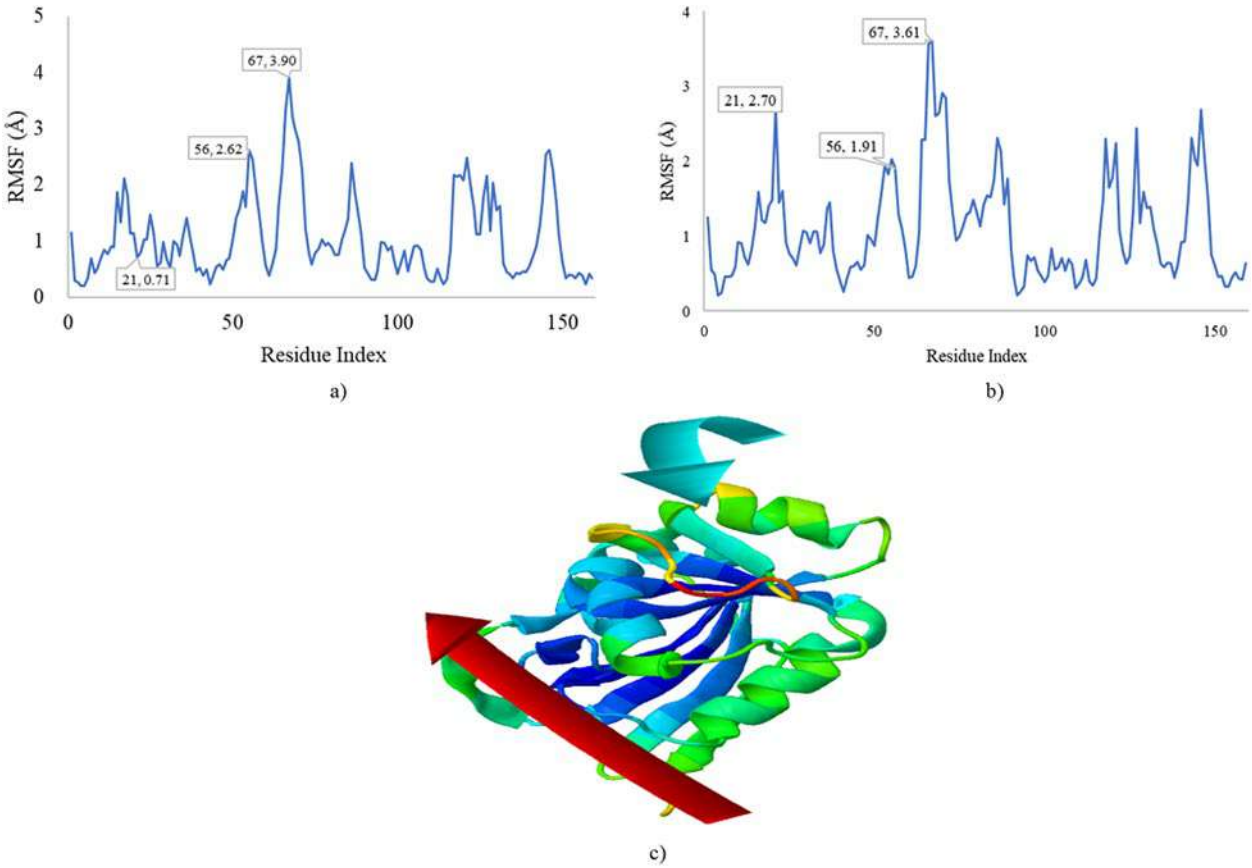


Figure 6: MDS result depicting residue fluctuation of; a) DHFR, b) Stigmasterol-DHFR docked complex, and c) Cluster displacement of the docked complex (blue and red arrows indicate higher and lower displacements respectively)

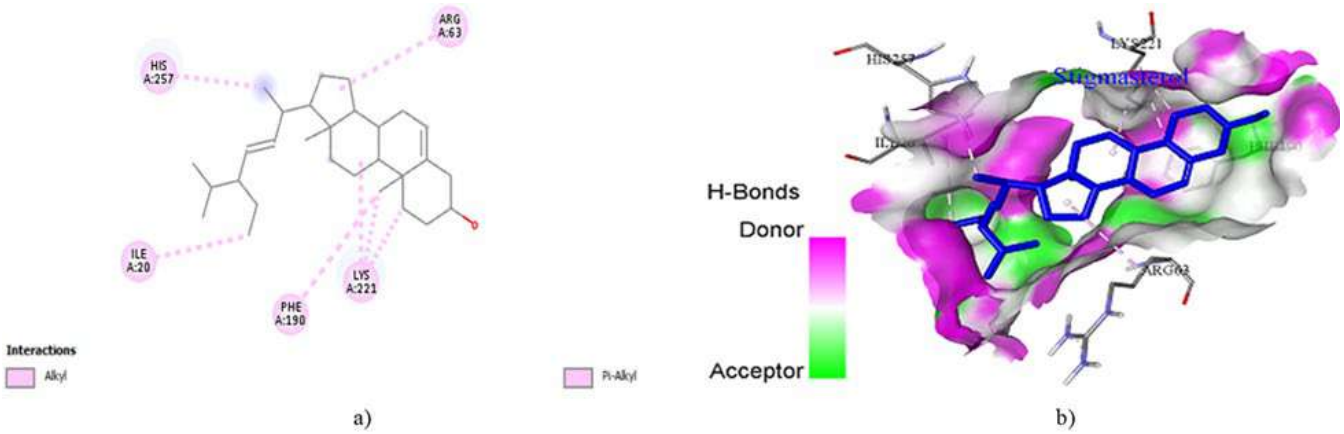


Figure 7: Dock poses and interactions of stigmasterol with DHPS; a) 2D and b) 3D

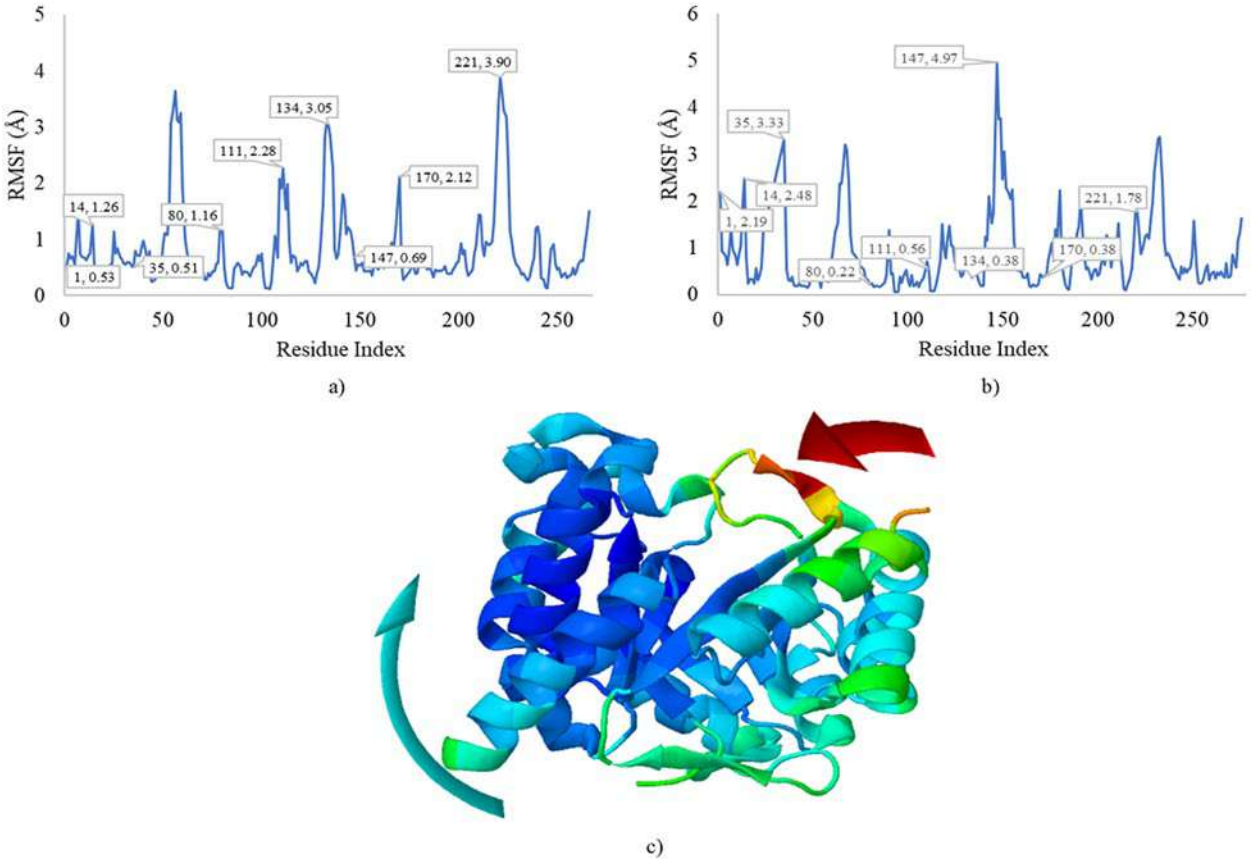


Figure 8: MDS result depicting residue fluctuation of; a) DHPS, b) Stigmasterol-DHPS docked complex, and c) Cluster displacement of the docked complex (blue and red arrows indicate higher and lower displacements respectively)

The docking interaction of MurA and TopoIV is presented in Table 5 revealing the BA and Ki. The most favorable docked pose with MurA was exhibited by stigmasterol demonstrating the least BA (-7.1 kcal/mol) and Ki (10.23 µM) followed by 4,4-dimethylcyclohex-2-en-1-ol with -6.8 kcal/mol and 10.23 µM respectively. Moreover, 5-acetoxymethyl-2-furaldehyde showed the highest BA (-5.1 kcal/mol) and Ki (180.94 µM). Stigmasterol also exhibited the least BA (-7.8 kcal/mol) and Ki (1.89 µM) interacting with TopoIV next to 4,4-dimethylcyclohex-2-en-1-ol with -5.7 kcal/mol and 65.65 µM respectively while 5-acetoxymethyl-2-furaldehyde had the highest -5.3 kcal/mol and 129.05 µM respectively.

The docking interaction of the stigmasterol-MurA docked complex is presented in a 2D and 3D docked pose in Figure 9. A single conventional hydrogen bond was observed with Lys90, while the alkyl bonds were observed with PHE330, ILE119, CYS117, and ARG93, all contributing to the stability of the complex.

Table 5: Binding affinity and Ki of the interaction of MurA and TopoIV with the compounds

Target	Ligand	Binding Affinity (kcal/mol)	Ki (µM)
MurA	Stigmasterol	-7.1	6.16
	4,4-Dimethylcyclohex-2-en-1-ol	-6.8	10.23
	5-Acetoxymethyl-2-furaldehyde	-5.1	180.94
TopoIV	Stigmasterol	-7.8	1.89
	4,4-Dimethylcyclohex-2-en-1-ol	-5.7	65.65
	5-Acetoxymethyl-2-furaldehyde	-5.3	129.05

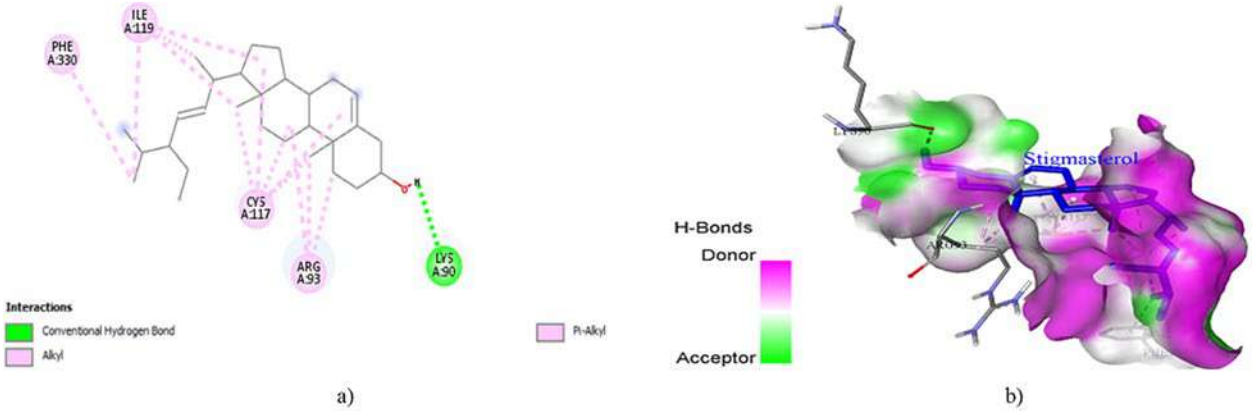


Figure 9: Dock poses and interactions of stigmasterol with MurA; a) 2D and b) 3D

The cluster displacement and residue fluctuation of MurA and stigmasterol-MurA docked complex observed from MDS are presented in Figure 10. All the residues fluctuated with the highest (3.38 Å) by THR69 though both terminals also fluctuated. Additionally, increased fluctuations were exhibited by GLU87 (1.84 Å) and LEU113 (2.51 Å) while CYS117 (1.90 Å), SER212 (1.93 Å), and HIS301 (1.41 Å) were decreased. Moreover, cluster displacement showed higher movement around the N-terminal than the C-terminal with the clusters moving in opposite directions.

Figure 11 depicts the 2D and 3D dock pose and binding interactions of the stigmasterol-TopoIV docked complex. A conventional hydrogen bond was formed between the ligand and ASP69, with additional alkyl interactions with residues MET74, ILE90, ALA86, LEU86, ARG93, and PRO75.

The cluster displacement and residue fluctuation of MDS of TopoIV and stigmasterol-TopoIV docked complex are presented in Figure 12. The highest (4.35 Å) fluctuation was exhibited by ALA61 (2.76 Å), though PRO196 (2.00 Å), ASN207 (2.21 Å), and TY215 (1.91 Å) exhibited increased displacements while GLY15 (2.85 Å), GLY73 (2.12 Å), and ASN207 (2.21 Å) were decreased. Moreover, a higher cluster displacement was observed from the N-terminal to the midchain with lower displacement at the C-terminal.

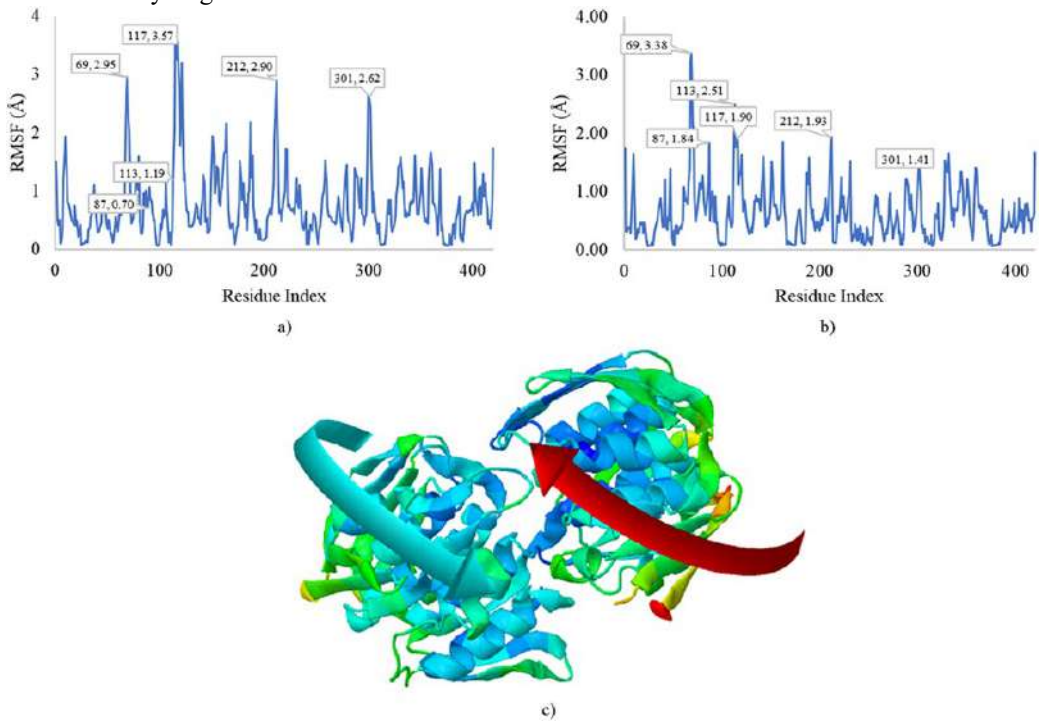


Figure 10: MDS result depicting residue fluctuation of; a) MurA, b) Stigmasterol-MurA docked complex, and c) Cluster displacement of the docked complex (blue and red arrows indicate higher and lower displacements respectively)

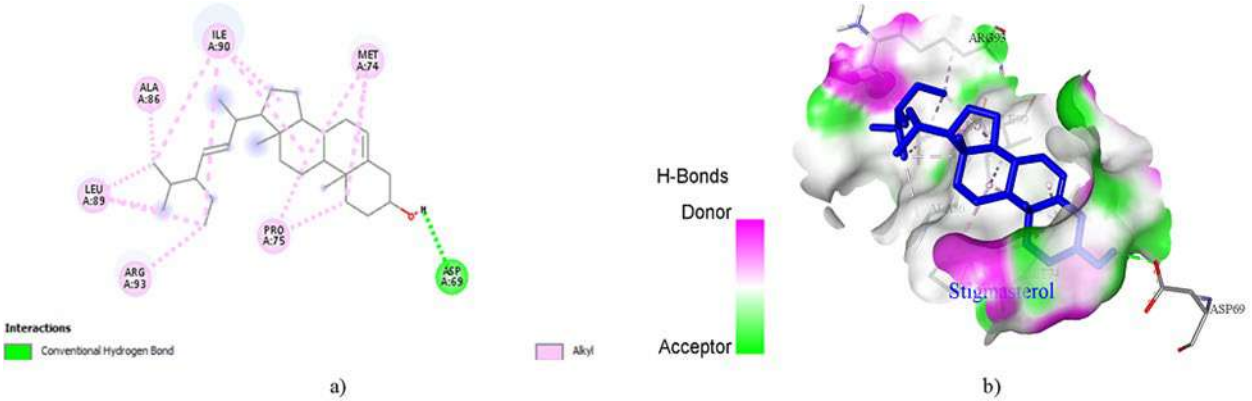


Figure 11: Dock poses and interactions of stigmasterol with TopoIV; a) 2D and b) 3D

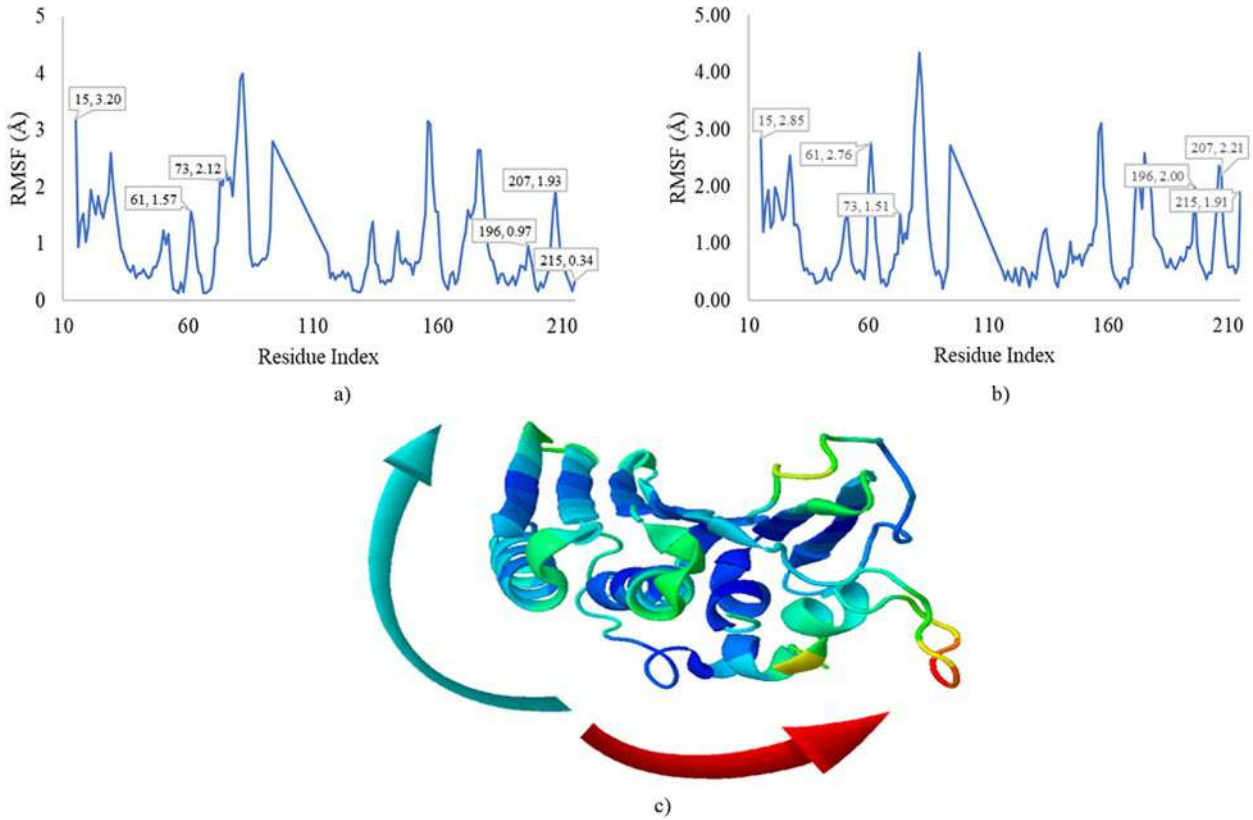


Figure 12: MDS result depicting residue fluctuation of; a) TopoIV, b) Stigmasterol-TopoIV docked complex, and c) Cluster displacement of the docked complex (blue and red arrows indicate higher and lower displacements respectively)

The ADMET prediction results of stigmasterol and 4,4-dimethylcyclohex-2-en-1-ol are shown in Table 6. Stigmasterol was predicted to be poorly soluble with low gastrointestinal absorption in addition to being a non-substrate but a P-glycoprotein inhibitor. Moreover, it has lipophilicity, water solubility, and skin permeation values of 6.98 Log $P_{o/w}$, -1.82 Log *S*, and -2.74 cm/s respectively. Predictively, stigmasterol have no blood-brain barrier permeability (BBB), fraction unbound (Fu) and volume of distribution (VD) and central nervous system (CNS) permeability of 0.176 log L/kg and -1.691 log PS respectively. Additionally, it's a CYP3A4 substrate and CYP2C9 inhibitor with a renal clearance of 0.618 log ml/min/kg and a non-renal organic cation transporter 2 (OCT2) substrate. Furthermore, stigmasterol is neither hepatotoxic, carcinogenic, mutagenic, nor, cytotoxic with 2.35 mol/kg LD50 and low toxicity (class 4).

For 4,4-dimethylcyclohex-2-en-1-ol, it is a moderately soluble compound with high gastrointestinal absorption and neither substrate nor inhibitor of the glycoproteins though it has lipophilicity, water solubility, and skin sensation of 3.90 Log $P_{o/w}$, -4.47 Log *S*, and -4.81 cm/s respectively. Furthermore, it is BBB permeant with VD, Fu, and CNS permeability of 0.553 L/kg, 0.177, and -2.23 log PS, respectively. However, it is a CYP2D6, CYP3A4, and renal OCT2 substrate and CYP2D6 inhibitor with a total clearance of 0.061 log ml/min/kg. Moreover, it is neither hepatotoxic, carcinogenic, mutagenic, nor cytotoxic but skin sensible with LD50 of 2.11 mol/kg.

Table 6: ADMET predictions of stigmasterol and 4,4-Dimethylcyclohex-2-en-1-ol

Parameters		Stigmasterol	4,4-Dimethylcyclohex-2-en-1-ol
Absorption	Lipophilicity (consensus Log $P_{o/w}$)	6.98	3.90
	Water solubility (consensus Log *S*)	-1.82	-4.47
	Solubility class	Poorly soluble	Moderately soluble
	GI absorption	Low	High
	Skin permeation [Log K_p (cm/s)]	-2.74	-4.81
	P-glycoprotein substrate	No	No
	P-glycoprotein I inhibitor	Yes	No
	P-glycoprotein II inhibitor	Yes	No
Distribution	The volume of distribution (log L/kg)	0.176	0.553
	Fraction unbound	0	0.177
	BBB permeability	No	Yes
	CNS permeability (log PS)	-1.691	-2.23
Metabolism	CYP1A2 inhibitor	No	No
	CYP2D6 substrate	No	Yes
	CYP3A4 substrate	Yes	Yes
	CYP2C19 inhibitor	No	No
	CYP2C9 inhibitor	Yes	No
	CYP2D6 inhibitor	No	Yes
	CYP3A4 inhibitor	No	No
Excretion	Total clearance (log ml/min/kg)	0.618	0.061
	Renal OCT2 substrate	No	No
Toxicity	LD50 (mol/kg)	2.35	2.11
	Toxicity Class	4	4
	Hepatotoxicity	No	No
	Skin Sensitisation	No	Yes
	Carcinogenicity	No	No
	Mutagenicity	No	No
	Cytotoxicity	No	No

The oral bioavailability radar of stigmasterol and 4,4-dimethylcyclohex-2-en-1-ol is presented in Figure 13 depicting its drug-like properties. Although the size, flexibility (FLEX), insaturation (INSATU), and polarity (POLAR) are within the accepted range, lipophilicity (LIPO) and insolubility (INSOLU) were out of range. However, the oral bioavailability radar of 4,4-dimethylcyclohex-2-en-1-ol falls within the acceptable pink range as shown in Figure 13.

The drug-likeness and medicinal chemistry of stigmasterol and 4,4-Dimethylcyclohex-2-en-1-ol are presented in Table 7. A bioavailability score of 0.55 was predicted for stigmasterol with single violations of Lipinski's and Verber rules though without any Egan violation. Although no PAINS alert was predicted, there were two lead-likeness violations with a synthetic accessibility of 6.21. For 4,4-dimethylcyclohex-2-en-1-ol, it has the same bioavailability score (0.55) as stigmasterol with neither violation of Lipinski's rule, Egan rule, nor Veber's rule, and PAINS alert, however, one lead likeness violation and 2.93 synthetic accessibility were observed.

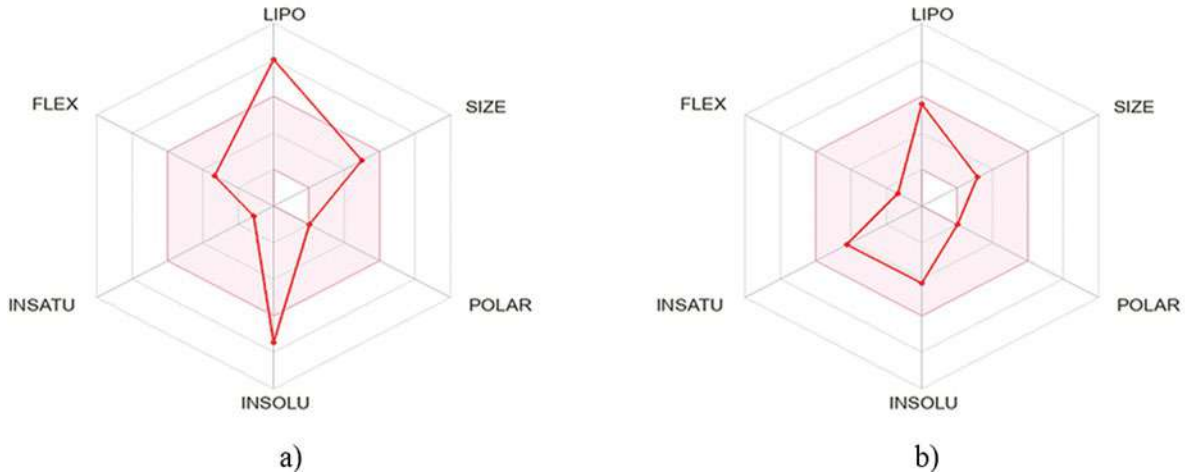


Figure 13: Oral bioavailability radars showing the LIPO (lipophilicity), flexibility (FLEX), insaturation (INSATU), and insolubility (INSOLU) of; A) Stigmasterol and B) 4,4-Dimethylcyclohex-2-en-1-ol

Table 7. Drug-likeness and medicinal chemistry of compounds stigmasterol and 4,4-Dimethylcyclohex-2-en-1-ol

Parameters		Stigmasterol	4,4-Dimethylcyclohex-2-en-1-ol
Druglikeness	Lipinski	Yes; 1 violation: MLOGP>4.15	Yes; 0 violation
	Egan	Yes	Yes
	Veber	No; 1 violation: WLOGP>5.88	Yes
	Bioavailability score	0.55	0.55
Medicinal chemistry	PAINS	0 alert	0 alert
	Lead likeness	No; 2 violations: MW>350, XLOGP3>3.5	No; 1 violation: XLOGP3>3.5
	Synthetic accessibility	6.21	2.93

Discussion

In the present study, the binding energy and interaction of identified compounds in XA with antimicrobial targets were investigated to determine the inhibitory potential of the compounds. The targets were selected due to their roles in the survival of bacterial cells *via* replication and metabolism of important metabolites. The FabI enzyme catalyzes the elongation step during fatty acid synthesis requiring NADH and NADPH as cofactors and, thus, a target for antibacterial therapeutics (Hopf et al., 2022). Stigmasterol interaction with FabI with low BA and Ki in the present study might demonstrate inhibition of the enzyme due to high affinity for the ligand, further revealed by the MDS with the several residue displacements within the protein structure. The last stage of bacterial cell wall synthesis (peptidoglycan) is catalyzed by PBP2X vital for the maintenance of cellular integrity, growth, and survival (Schweizer et al., 2014). PBP2X is a target of the antibacterial class of drugs β-lactams though resistance to drugs is possible by the lactamase enzymes produced by bacteria, which inactivate the enzymes (Peters et al., 2021). In our study, stigmasterol showed the most favorable docking with PBP2X with least BA and Ki, furthermore, the MDS showed several residue fluctuations, a possible inhibition of enzyme activity by stigmasterol.

DHFR is crucial to bacterial replication and survival due to its role in folate synthesis (Cao et al., 2018). It catalyzes the synthesis of (6s)-5,6,7,8-tetrahydrofolate (essential enzyme for DNA synthesis) from 7,8-dihydrofolate by reduction requiring NADH for the hydride transfer (Askari & Krajinovic, 2010; Cao et al., 2018). This enzyme is thus, a target of antibacterial drugs due to its absence in humans. Stigmasterol showed a favorable docked pose within the binding pocket of the enzyme with a low BA and Ki, possibly disrupting its activity due to its high affinity for the ligand. This is further supported by the MDS result which revealed fluctuations of the residues and cluster displacement depicting the flexibility of the formed complex. DHPS is another crucial enzyme required for the folate required for DNA synthesis, thus, an antibacterial drug target for the sulfonamides (Griffith et al., 2018). Specifically, it catalyzes the synthesis of 7,8-dihydropteroate (the substrate for DHFR) from 6-hydroxymethyl-7,8-dihydropteridine pyrophosphate and para-aminobenzoic acid (PABA) *via* condensation (Satuluri et al., 2020). In our study, stigmasterol also exhibited inhibitory potential against DHFR revealed by the docking interactions and low BA and Ki demonstrated by the docked complex. Furthermore, the MDS of the docked complex showed flexibility depicted by the hinge regions due to residue displacements.

MurA is a vital enzyme of peptidoglycan synthesis catalyzing the first committed step, the enolpyruvate group transfer from phosphoenolpyruvate to UDP-N-acetylglucosamine yielding UDP-N-acetylglucosamine enolpyruvate (de Oliveira et al., 2022; Hrast et al., 2014). Thus, an antibacterial target by its inhibitors like fosfomycin, covalently binding and inactivating the enzyme (de Oliveira et al., 2022). Stigmasterol also exhibited stable docking interaction with this enzyme with low BA and Ki possibly inhibiting its activity attributed to the high affinity for the ligand. Additionally, the MDS result showed several hinges regions depicting flexibility within the enzyme structure and cluster displacement at both peptide terminals, a possible disruption of its tertiary structure and activity. TopoIV is another vital bacterial enzyme involved in DNA synthesis during template separation and subsequent segregation of the daughter chromosomes (Helgesen et al., 2021). Therefore, it is a target for the antibacterial drugs fluoroquinolones, which decrease the rate of DNA synthesis and replication, generating double breaks leading to cell death (Hooper & Jacoby, 2016). Stigmasterol also showed promising antibacterial potential *via* inhibition of this enzyme due to the higher affinity of the enzyme for the ligand evidenced by the low BA and Ki interaction. Moreover, the MDS result also supports its potential due to the cluster movement and residue fluctuations.

We further investigated the pharmacological properties of stigmasterol and 4,4-dimethylcyclohex-2-en-1-ol for their ADMET drug-likeness and medicinal chemistry. 4,4-dimethylcyclohex-2-en-1-ol exhibited superior absorption properties with higher solubility and gastrointestinal absorption than stigmasterol, though both are not P-glycoprotein substrates. The P-glycoprotein acts as a barrier detoxifying the cell by extruding toxins and foreign compounds from the cell (Pires et al., 2015). Moreover, both compounds are skin permeable as a compound with skin permeability >-2.5 cm/h is considered permeable (Pires et al., 2015). Moreover, a steady-state volume of distribution (VDss) <-0.15 and >0.45 are regarded as low and high respectively (Pires et al., 2015). The compound with CNS >-2 is regarded as CNS penetrant. Stigmasterol has a moderate VDss while 4,4-Dimethylcyclohex-2-en-1-ol has higher VDss with the former being BBB permeable while the former has higher CNS permeability (Pires et al., 2015). Although both compounds are of the same toxicity class, 4,4-dimethylcyclohex-2-en-1-ol has skin sensation with lower LD50. Furthermore, the oral bioavailability showed 4,4-Dimethylcyclohex-2-en-1-ol demonstrate better drug-

likeness than stigmasterol without violations of the Lipinski's, Verber's, or Egan rule though both compounds are without PAINS alerts and lead-likeness. Summarily, 4,4-dimethylcyclohex-2-en-1-ol exhibited better pharmacological properties than stigmasterol.

Conclusion

In the present study, the antibacterial potential of the compounds identified in XA from our previous study was explored *via* molecular docking and molecular dynamics study. Furthermore, we explored the possibility of applying the top-docked compound as an antibacterial drug *via* its ADMET, lead-likeness, and medicinal properties. Stigmasterol exhibited the most favorable interaction with all the tested targets demonstrating the least BA and Ki among the compounds. In terms of drug-likeness, 4,4-dimethylcyclohex-2-en-1-ol showed good antibacterial potential with better drug and lead likeness than stigmasterol. Although stigmasterol has inferior drug and lead likeness properties, it exhibited better binding properties with all the enzymes, thus, structural modification might improve its weakness. Conclusively, stigmasterol and 4,4-dimethylcyclohex-2-en-1-ol might be responsible for the antibacterial effect of XA.

Authors Contributions

Conceptualization, D.M.M., A.M.A., and I.Y.; methodology, D.M.M.; investigation, D.M.M.; resources, A.M.A., and I.Y.X; writing-original draft preparation, D.M.M.; writing-review and editing, D.M.M. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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ORIGINAL ARTICLE

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Variation of bromine concentration as an essential trace element in human milk over lactation stages

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ABSTRACT

Introduction: Bromine has been newly discovered in human milk but its importance in the growth and development of infants is unclear. Only a few studies have reported the concentration of bromine in human milk and considered it as an essential element, whereas others highlighted its toxicity of bromism in humans. This study aimed to determine the concentration of bromine as an essential trace element in human milk using a validated acid digestion method and discuss its variation over lactation stages.

Method: Human milk samples were collected from three postpartum mothers and analysed using inductively coupled plasma mass-spectrometry (ICP-MS). The concentration of bromine was determined over a certain postpartum period, analysed using Microsoft Excel 2016, and reported descriptively.

Results: Method validation parameters for bromine showed good linearity ($R^2 > 0.999$), limit of detection (0.003 µg/L), limit of quantification (0.01 µg/L), accuracy (96%), inter-day (3.76%RSD) and intra-day (3.35%RSD) repeatability. The median concentration of bromine in human milk decreased over six months of lactation, in µg/L: 1210, 674, 722, 671, 511 and 538. At later lactation months which were 12th, 13th, 14th, 15th and 21st, the median bromine concentration was in µg/L: 780, 815, 645, 846, 910, respectively.

Conclusion: The acid digestion method by ICP-MS was robust and accurate in determining bromine concentration in human milk. The consistent variation of bromine in human milk over lactation stages may indicate its importance in supporting infant development in the first two years of age. Future research should explore the role of bromine in infants' development, its chronobiological importance, and the risk of deficiency or toxicity.

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Introduction

Human milk contains essential trace elements that can vary in concentration over the postpartum period to meet an infant's nutritional needs. These include zinc (Zn), copper (Cu), selenium (Se), iodine (I), molybdenum (Mo), manganese (Mn), iron (Fe), and bromine (Br) (Mohd-Taufek et al., 2016a). Unlike other well-reported elements, very few studies have discussed about bromine in human milk. It was the first time reported in 2016 that median bromine concentration (1066 µg/L) was relatively higher than other elements such as selenium, iron, and iodine, but comparable to those of zinc (1639 µg/L) in an Australian population (Mohd-Taufek et al., 2016b). Bromine has been discovered as an essential trace element in human for its role as a key cofactor in tissue development in the type IV collagen scaffold presented in basement membranes, which support epithelial cells (McCall et al., 2014). However, data and information about bromine concentration and its role in humans remain scarce and require further research.

The essentiality of trace element bromine could have been overlooked due to its prominent toxicity profile that has been widely highlighted in the literature (Frances et al., 2003; Lugassy & Nelson, 2009; Rho & White, 2018). Although rare, the occurrence of bromide intoxication or bromism in adults characterised with neuropsychiatric symptoms have been reported from medicinal exposure (James et al., 1997; Frances et al., 2003). Moreover, a case of a 22-day-old girl with bromism who presented with excessive sleepiness and decreased oral intake due to ingestion of elixir containing potassium bromide was also reported (Lugassy & Nelson, 2009). Despite being the first drug as anti-epilepsy, potassium bromide has a narrow therapeutic index, of which toxicity characteristics include severe skin reactions, lethargy, cachexia, delirium/psychosis, and exacerbation of seizure activity (Rho & White, 2018). The neuropsychiatric symptoms were reported with a serum bromide concentration of 1717 mg/L (Frances et al., 2003) whereas severe bromism was found in a patient with serum bromide of 3180 mg/L (Horowitz, 1997). Therefore, its clinical use has declined for decades but bromide can still be found in prescription and over-the-counter (OTC) preparations owing to its sedative effect (Lugassy & Nelson, 2009). For example, a case of chronic bromide toxicity has been reported from the recurrent abuse of OTC dextromethorphan hydrobromide (Monks, Yen & Myers, 2020). Additionally, severe bromism could also happen from consumption of cola containing brominated vegetable oil (Horowitz, 1997).

In recent years, bromine has been recognised as part of more than 20 elements that are essential for life. It is unclear about the role of bromine in infants other than tissue development. Nevertheless, infants are generally at high risk of trace element deficiency due to higher metabolism and need for their rapid growth and development (Angelova et al., 2014). Unlike bromine toxicity, its deficiency has been rarely reported, and thus reference ranges in human biological samples have not been established. A review has summarised that decreased bromine concentration was found in insomnia and long-term dialysis patients and suggested that evaluation of human bromine status might be useful in correcting bromine-dependent health issues (Canavese et al., 2006). It was reported that bromine was significantly lower in the scalp hair samples of dialysis patients compared to healthy subjects (Ochi et al., 2011). The analysis of dried blood spot also has been reported for bromine and iodine (He et al., 2020). However, different biological matrices contain different concentrations of trace elements, which require specific reference range.

The presence of bromine in human milk at a relatively similar concentration to zinc poses questions about whether infant's need of bromine is sufficiently sourced from breast milk. In infants, high metabolism may deplete bromine reserve in the body and signs and symptoms of its deficiency have not been identified. For example, zinc deficiency in exclusively breastfed infants have been frequently misdiagnosed as eczema or impetigo before the correct diagnosis of nutritional zinc deficiency attributable to the decreased zinc content in human milk at the later stage of lactation (Kienast et al., 2007). The composition of human milk nutrients has been reported to vary over lactation stages with some trace elements such as zinc and copper had been observed to decrease throughout the postpartum period (Terrin et al., 2015). Some nutrients may decrease or maintain their concentration due to various intrinsic and external factors. However, there are limited data on the variation of bromine concentration in human milk. It is important to document more data about bromine in human milk to evaluate the longitudinal changes relative to the development of infants. This study aimed to determine bromine concentration in human milk of postpartum mothers using a validated method and discuss its essentiality in infants.

Methodology

This study received ethics approval from the International Islamic University Malaysia (IIUM) Research Ethics Committee (IREC) (ID No.: IREC 2021-053).

Participant recruitment, data collection, sample collection, and analyses have been conducted following the protocol as described in the previously published article (Mohd Taufek et al., 2023). The developed and validated method has been used to analyse bromine concentration in human milk. The certified reference material for bromine in human milk is currently unavailable, thus spiked samples were used to measure accuracy using 1000 mg/L of Br single-element standard solution (Merck Certipur).

The 0.1 ml of Br single-element standard solution 1000 mg/L was diluted with 1% (v/v) nitric acid up to 50 ml for the purpose of standard stock solution. Then, 1 mL of each of 5 samples of milk was spiked with 0.005 g/mL bromine and made up to 10 mL by adding 1% (v/v) nitric acid. The 1% (v/v) nitric acid was made by diluting 15.4 mL of 65% (v/v) nitric acid (Merck Suprapur) with 1 L of water. A total of 105 milk samples were analysed using the method. All the results were analysed for descriptive analysis using Microsoft Excel version 2016.

Results

A total of three participants consented to the study and were included as case studies. Participant X was a 30-year-old mother who did not take any supplements and donated her milk at her time convenience in the first six months postpartum. Participant Y was a 27-year-old mother who took supplements for selenium, manganese, zinc and copper. Participant Z was a 27-year-old mother who took supplement for zinc and iron. Both participants Y and Z donated their milk at the later stage of lactation which were 12^{th} month until 21^{st} month at their convenience. All participants were Malay, had normal body mass index at the time of milk collection, and delivered male infants of the same birth weight (3 kg) at full term (37≤ weeks of gestation). Only participant Z had a history of gestational diabetes and asthma. Other details regarding the participants have been published in a previous study (Mohd Taufek et al., 2023)

The calibration data for bromine is presented in Table 1, with $R^2 > 0.999$ is considered good linearity. The inter-day and intra-day repeatability of spiked bromine samples are shown in Table 2 which indicate good percentage recovery (80-120%) and %RSD (Mohd Taufek et al., 2023).

Table 3 showed that median bromine concentration was the highest in the first month postpartum in comparison to the later months for Participant X. The minimum and maximum range provided more information on the variation of bromine across six months of the lactation period. Although the median bromine concentration dropped to half in the second month, it seemed to remain stable until the sixth month.

Table 4 showed that bromine concentration observed in two different postpartum mothers at the 12^{th}, 13^{th}, 14^{th}, 15^{th} and 21^{st} month postpartum were stable with little fluctuations.

Table 1: LOD and LOQ, slope and correlation coefficient.

Trace element	LOD (µg/L)	LOQ (µg/L)	Slope	Correlation coefficient (R^2)
Br	0.003	0.01	y= 420x + 539	0.9994

Table 2: Percentage recovery, inter-day (n=3), and intra-day repeatability

Trace element	Percentage recovery (%)	Inter-day [%RSD]	Intra-day [%RSD]
Br	96	3.76	3.35

Table 3: Concentration of bromine of participant X over six months postpartum.

Trace element	Mean/Median	Month postpartum					
		1	2	3	4	5	6
		n = 15	n = 8	n = 10	n = 15	n = 6	n = 10
Br (µg/l)	Mean ± SD	1340 ± 503	678 ± 101	718 ± 123	739 ± 259	540 ± 95	574 ± 163
	Median (range)	1210 (886-3010)	674 (517-847)	722 (528-902)	671 (494-1500)	511 (431-678)	538 (431-1000)

n: number of milk samples available

Table 4: Concentration of bromine of participants Y (12^{th} – 15^{th}) and Z (21^{st}) month postpartum.

Trace element	Mean/Median	Y				Z
		Month postpartum				
		12	13	14	15	21
		n = 16	n = 4	n = 6	n = 4	n = 11
Br (µg/l)	Mean ± SD	788±144	863±252	641±79	879±210	888±224
	Median (range)	780 (573-1190)	815 (614-1210)	645 (545-746)	846 (696-1130)	910 (354-1180)

n: number of milk samples available

Discussion

We report a validated acid digestion method to determine bromine concentration in human milk that obtained good values and acceptable validation parameters. These are comparable to a previous study that used an alkaline dissolution method (Mohd-Taufek et al., 2016a). Our method is applicable to monitor bromine concentration in human milk in the future to establish the reference range and assist in the identification of bromine deficiencies or toxicities. For the current study, the method successfully measured bromine in the human milk of three postpartum mothers.

Despite different individuals providing milk samples at different periods of postpartum at their convenience, our findings provided important information about bromine concentration in human milk up to 21 months postpartum. We propose that the consistently high bromine concentration in different individuals at different postpartum periods may indicate that infants require abundant of bromine for growth and development. The nutritional aspect of bromine across two years of infants age needs to be explored, and its concentration should be maintained within a certain reference value to ensure sufficient intake. Exclusively breastfed infants aged six months and above are generally recommended to be fed with complementary food to prevent nutritional deficiencies based on developmental readiness (Pérez-Escamilla et al., 2019). Considering that bromine concentration in human milk was comparable to zinc which presented at a higher concentration than other elements (Mohd-Taufek et al., 2016b; Mohd Taufek et al., 2023), we speculate that deficiency in infants is possible, and its identification requires further investigation. For example, inadequate levels of zinc were reported to increase the risk of infections, impairment of growth, neurological function, and several complications particularly affecting rapidly growing preterm infants such as necrotising enterocolitis (Terrin et al., 2015). Additionally, higher risk of pneumonia and stunting were seen in children with zinc deficiency (Hamed et al., 2019; Khairun et al., 2019). Since bromine has not been routinely monitored in human milk as well as in the blood of mother-infant dyads, its deficiency or toxicity are currently unknown. Future studies evaluating this aspect are recommended.

In participant X, the median bromine concentration dropped to half in the second month (674 µg/L) compared to the first month (1210 µg/L) then were relatively stable throughout the six months postpartum (Table 3). Limited data are available addressing bromine concentration in human milk. A study in Australia has reported that bromine concentration was not significantly different before and after pasteurisation, ranging from 834 to 1443 µg/L in 16 different mothers who donated their milk (Mohd-Taufek et al., 2016b). These values were similar to our findings, when referring to the concentration range. The difference between the minimum and maximum values of bromine concentration in each month suggests that chronobiological changes in bromine contribute to huge variation longitudinally. This aspect can be explored like other nutrients in human milk, since dysregulated circadian biology may affect the concentration of bromine and thus infant health outcomes (Hahn-Holbrook et al., 2019; Italianer et al., 2020). On the other hand, organobromine pollutants have been reported to contaminate the breast milk of Swedish population due to environmental contamination (Norén & Meironyté, 2000). Bromine exposure from the environmental pollutants have been readily reported in the literature from agricultural products (Shtangeeva, 2022) and important highlight was the genotoxicity risk owing to increased bromide body burden (Nusair et al., 2019). More data are needed to clarify the essentiality and toxicity of bromine in human and specifically in human milk.

Table 4 showed the bromine concentration of different participants who donated their breast milk after 12 months postpartum. Interestingly, the concentration of bromine was relatively consistent with median level of

between 600-900 µg/l between 12 to 21 months postpartum. This study observed that bromine concentration in human milk varied over the postpartum period. The factors for this variation could be similar to those of the other nutrients including lactation stages, dietary intakes or supplements (Samuel et al., 2020). We were not able to confirm any significant changes in the pattern of bromine variation due to insufficient study population size. Future research is needed to confirm the changing pattern of bromine concentration in human milk at the population level, and its association with infants' health outcomes.

This study has the limitation of the small number of study participants. However, this study validated a simple and robust method that is applicable to monitor bromine concentration in human milk. Studies with large sample sizes are required to determine any association between maternal factors and bromine concentration. However, the findings from this study open more opportunities to explore bromine from nutritional aspects of the growth and development of infants.

Conclusion

The relatively high concentration of bromine in human milk indicates its essential role in infant growth and development. Bromine exhibited longitudinal variation in concentration in human milk like other essential trace elements such as zinc. Future studies may determine the reference range, and explore potential deficiency and toxicity.

Author Contribution

NHMT, ASMS & JB designed the study, collected samples and data. NBA, USMJ and ARFN analysed the samples and data. All authors wrote and reviewed the manuscript.

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Ethical Approval Statement

This study received ethics approval from the International Islamic University Malaysia (IIUM) Research Ethics Committee (IREC) (ID No.: IREC 2021-053).

Informed Consent Statement

Informed consent was obtained from all individual participants included in the study.

Conflict of Interest

All the authors declare that there is no conflict of interest.

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ORIGINAL ARTICLE

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Characterisation of *Maclura cochinchinensis* (Lour.) Corner Trunk Heartwood Extract and its Toxicity Evaluation

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ABSTRACT

Introduction: *Maclura cochinchinensis* is widely used as a natural dye for clothing in Indonesia. Besides, there are some researches about its activities as an antioxidant, antimicrobial, and antidiabetic. However, there is a lack of comprehensive information regarding the standard characteristics and safety of use of its heartwood extract. Therefore, this study aimed to characterize *M. cochinchinensis* heartwood extract and to evaluate its toxicity.

Method: To obtain the extract, coarse powder of *M. cochinchinensis* heartwood was macerated using 70% ethanol and evaporated by vacuum rotavapor. Subsequently, phytochemical screening and thin-layer chromatography profiling were carried out, while the toxicity evaluation was conducted using brine shrimp lethality test.

Results: The phytochemical screening showed that this extract contained flavonoids, saponins, tannins, steroids, triterpenoids, coumarins, and essential oils. The extract exhibited a dark brown colour, distinct odour, flavourlessness, the value of water-soluble content, ethanol-soluble extract content, loss on drying, moisture content, moisture content, total ash content, acid-insoluble ash content, and water-soluble ash content of 31.44%±1.31, 50.44%±8.48, 9.51%±0.32, 1.62%±0.48, 4.93%±0.27, 1.10%±0.11, 3.81%±0.19, respectively. The residual solvent and heavy metal contamination were undetectable. Microbial contamination was minimal and dominated by a semipolar compound, and brine shrimp assay indicated low toxicity with an LC_{50} value of 174.40 mg/L.

Conclusion: *M. cochinchinensis* heartwood extract has good quality and tends to be safe for the environment. This research obtained data that could be used as a supporting evidence for more specific utilization of *M. cochinchinensis* heartwood and its isolated compounds.

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JOP

Introduction

Indonesia has the second-largest biodiversity after Brazil, and among its diverse array of species is *Maclura cochinchinensis*, also referred to as tegeran (synonym, *Cudrania javanensis* Trécul). This plant is commonly used as a natural dye for clothing due to the presence of a flavonoid compound called morin, which is also the major compound of *M. cochinchinensis*. Natural dye from plants, like *M. cochinchinensis*, is used as an alternative to reduce environmental pollution caused by synthetic dye. However, there has been limited attention to the activities of this plant (Darsih et al., 2020). To date, some studies indicated *M. cochinchinensis* heartwood exhibits some biological activities such as antioxidant, antidiabetic, antiinflammation, and anti-hyperuricemia (Chewchinda et al., 2021; Darsih et al., 2020; Sato et al., 2020).

To determine the safety of using *M. cochinchinensis* and the effectiveness of remediation methods for hazardous substances, comprehensive testing is required. Various methods, including the use of brine shrimp (*Artemia salina*), can be used for testing. Brine shrimp (*A. salina*) are widely used in analysis of pesticide residues, anaesthesia, and toxins in the marine environment. The principle behind this testing is to compare the number of shrimp larvae that die when exposed to a solution of the sample compound until the LC_{50} value is obtained (Marzuki et al., 2019; Meyer et al., 1982). Therefore, this study aims to analyse toxicity of *M. cochinchinensis* heartwood extract using Brine Shrimp Lethality Test (BSLT) method in order to provide a comprehensive understanding of the safety of *M. cochinchinensis* wood extract in terms of its potential impact on human health and the environment.

Methodology

Plant Collecting and Determination

M. cochinchinensis plant and the heartwood of the trunk pieces were obtained from Cirebon, Indonesia. The plant was taken near the neighbourhood, since it was not cultivated. Then, it subsequently identified in the Biota Collection Room at Universitas Indonesia on 20 June 2023 with specimen code JI23-P-077 by the Botanical and Zoological Specimen Curator, Alexander Tianara. Then, the *M. cochinchinensis* heartwood was dried in room temperature for 48 hours.

Extraction

The heartwood of the trunk from *M. cochinchinensis* were sorted and ground into a coarse powder. A total of 500 grams of coarse powder were macerated with 5 L of 70% ethanol. The mixture was stirred continuously at 290 rpm during a soaking period for 6 hours, followed by 18 hours of undisturbed soaking at room temperature. The resulting macerate was filtered using a Whatman No. 1 filter paper. The obtained filtrate was then evaporated into a thick extract using a vacuum rotavapor (Heidolph, Germany) at 40°C (Directorate General of Pharmaceutical and Medical Devices, 2017).

Phytochemical Screening

Phytochemical screening of the coarse powder and thick extract of *M. cochinchinensis* heartwood was performed using the Farnsworth method. This screening included testing for the presence of secondary metabolites such as alkaloids, flavonoids, saponins, quinones, tannins, steroids, triterpenoids, coumarins, and essential oils (Farnsworth, 1966).

Extract Quality Characterisation

Characterisation of extracts quality included evaluating various parameters, such as organoleptic properties, water-soluble content, ethanol-soluble content, loss on drying, moisture content, total ash content, acid-insoluble ash content, water-soluble ash content, residual solvent, total plate count (TPC), yeast and mould number (YMN), Pb and Cd metal contamination. The procedures were conducted based on general standard parameters for medicinal plant extract (Directorate General of Pharmaceutical and Medical Devices, 2000).

Thin Layer Chromatography (TLC) Profiling

Silica gel 60 GF254 (Merck, Germany) was used as the stationary phase, cut into 10 cm lengths and 2 cm in width, and then was activated by heating in an oven at 120 °C for 30 minutes. Each chromatographic chamber was saturated with the respective mobile phases of toluene/ethyl formate/formic acid (50/40/10), methanol/chloroform/n-hexane (7/2/1), ethyl acetate/formic acid/water (8/1/1), and chloroform/n-hexane/methanol (5/4/2) using filter paper. The sample solution was prepared by diluting 1 mg of *M. cochinchinensis* extract into 10 mL of methanol, and 5 µl of the sample solution was spotted using a capillary pipette on the TLC plate. After undergoing the elution process in each saturated chamber, the TLC plates were subsequently allowed to aerate at room temperature, and their spots were observed under UV light (CAMAG, Swiss) at a wavelength of 254 and 366 nm (Gwatidzo et al., 2018; Maleš et al., 2004; Poole et al., 2000).

BSLT

Hatching Process of A. salina

The container used for brine shrimp hatching consisted of two sections, the dark side (hatching compartment) and the light side (illuminated compartment), both filled with synthetic seawater. The eggs of *A. salina* were placed

within the hatching compartment, while the opposite area was illuminated using an 18-watt TL lamp. After 24 hours, the newly hatched Artemia nauplii, which had migrated to the illuminated compartment, were isolated into a separate container and left undisturbed for the next 24 hours. Therefore, the tested nauplii were 48 hours old (Meyer et al., 1982).

Preparation of Tested Sample Solution

The stock solution of extract was prepared by dissolving 20 mg of *M. cochinchinensis* thick extract in 2 mL of 96% ethanol, resulting in a concentration of 10000 mg/L. This stock solution was used to prepare vials containing concentrations of 1000, 100, and 10 mg/L, each with three replications. In cases of insolubility, 1% DMSO was added to the vials. Control vials were prepared by adding 96% ethanol without extract. Then all of the vials were put in a water bath to dry the solvent (Marzuki et al., 2019; Meyer et al., 1982).

Data Analysis

Each vial, free of solvent was filled with 10 nauplii and 5 mL of synthetic seawater. The surviving nauplii were counted after 24 hours. The lethal concentration for 50% mortality after 24 hours of exposure (LC_{50}) was established through probit analysis, serving as an indicator of extract toxicity. Then toxicity of extract was classified based on the LC_{50} value obtained (Marzuki et al., 2019; Meyer et al., 1982).

Results

Extraction

Extraction of 500 g coarse powder with a solvent ratio of 1/10 produced 122.1 grams of thick extract, with 24.42% yield and 4.0950 for the DER-native.

Phytochemical Screening

The coarse powder and the thick extract of *M. cochinchinensis* heartwood qualitatively contained flavonoids, saponins, tannins, steroids, triterpenoids, coumarins, and essential oils, as shown in Table 1.

Extract Quality Characterisation

M. cochinchinensis heartwood extract obtained a thick, dark brown colour, distinct odour, and no taste. Table 2 shows the results for *M. cochinchinensis* extract quality characterisation, which had good quality by fulfilling the requirements value of The Indonesian Food and Drug Authority Regulation No. 32 of 2019.

Table 1: Phytochemical screening result.

Phytochemical Screening	Results	
	Coarse Powder	Thick Extract
Alkaloids	-	-
Flavonoids	+	+
Saponins	+	+
Quinones	-	-
Tannins	+	+
Steroids	+	+
Triterpenoids	+	+
Coumarins	+	+
Essential oils	+	+

TLC Profiling

The obtained spots indicated the presence of compounds as shown in Figure 1.

BSLT Analysis

The result from the 24-hour observation of *A. salina* Leach larvae towards *M. cohinchinensis* heartwood extract obtained the LC_{50} value of 174.40 mg/L, which was in the range of 100–1000 mg/L based on Meyer index showed low toxicity (Marzuki et al., 2019). The LC_{50} value were obtained by an antiLog of the x value from the linear regression equation below as shown in Figure 2.

Discussion

Using natural sources in the form of extract must meet criteria related to quality, safety, and effectiveness. Maintaining quality of extract is crucial throughout the entire process, starting from the collection of plant materials. This includes accurate plant identification and classification to prevent any inadvertent mixing with other plant species, and this stringent method should continue until the final extract is obtained. The proper identification for medicine plant is also critical for maximizing its efficacy and minimizing potential toxicity adulteration (Klau & Hesturini, 2021; Upton et al., 2020). In this study, extract yield was lower than in the previous investigation, where a yield of 30.2% was achieved using the same method and solvent (Sato et al., 2020). This may be caused by the amount of remaceration carried out, which the more remaceration was performed, the more extract yielded (Siddiq et al., 2022). However, this maceration with a solvent of 70% ethanol was preferable to the other conventional method to extract *M. cochinchinensis*, because it yields more extract than other solvent and 70%

Table 2: Extract quality characterisation results.

Formulation	Smell	Taste
Specific characteristics		
Water-soluble extract content	31.44 ± 1.31	-
Ethanol-soluble extract content	50.44 ± 8.48	-
Non-specific characteristics		
Loss on drying (%)	9.51 ± 0.32[a]	≤10
Moisture content (%)	1.62 ± 0.48[a]	≤10
Total ash content (%)	4.93 ± 0.27[b]	-
Acid-insoluble ash content (%)	1.10 ± 0.11[a]	-
Water-soluble ash content (%)	3.81 ± 0.19[a]	-
Total plate count (CFU/g)	Too few to count	$\leq 1\times10^4$
Yeast and mould number (CFU/g)	Too few to count	$\leq 1\times10^3$
Pb metal contamination (mg/L)	Undetectable	10
Cd metal contamination (mg/L)	Undetectable	0.3
Residual solvent (%)	Undetectable	≤1

[a] The data were presented in mean ± SD, n = 3
[b] The data were presented in mean ± SD, n = 6

ethanol extract produces the lowest colour degradation on fabric, which is beneficial for dye as the common use of *M. cochinchinensis* extract in Indonesia (Atika, 2017; Sato et al., 2020).

M. cochinchinensis produces colour as a natural dye for clothing due to its secondary metabolites (Darsih et al., 2020). To determine the phytochemical compound contained in the plant, phytochemical screening was conducted. The result of the phytochemical screening of *M. cochinchinensis* showed both the coarse powder and extract did not contain alkaloids, while previous investigation indicated alkaloids in the methanol extract of *M. cochinchinensis* (Swargiary & Ronghang, 2013). The difference in phytochemical screening results can occur according to several factors, such as geographic location and climate, various types of soil, or extraction methods (Farida et al., 2023).

Extraction method is also possibly affecting the safety and stability during storage for a long period of extract obtained. Therefore, extract's quality should be characterized to ensure it is safe and feasible to use. Quality characteristics of extract can be divided into specific and nonspecific categories. The specific characterisation provides information about the plant itself, whereas the non-specific characterisation mostly shows the safety and stability parameters that should be fulfilled (Directorate General of Pharmaceutical and Medical Devices, 2000).

The specific characterisation parameters consist of organoleptic properties to identify extract using human senses and dissolved compounds in certain solvents that provide an initial description of the number of compounds contained. The value of ethanol-soluble content in *M. cochinchinensis* heartwood extract was higher, indicating the plant contained more secondary metabolites dissolved in ethanol solvent. This can happen because ethanol is a solvent that has universal properties, which can attract compounds that are polar, semipolar, and nonpolar. Subsequently, compounds that tend to be extracted in ethanol are saponins, flavonoids, steroids, and triterpenoids, while in water, the presence of saponins and flavonoids (Farida et al., 2023).

The nonspecific characteristics consist of loss on drying, moisture content, total ash content, acid-insoluble ash content, water-soluble ash content, residual solvent, TPC, YMN, Pb, and Cd metal contamination. All the results fulfilled the requirements from Indonesian Food and Drug Authority Regulation No. 32 of 2019 (Indonesian Food and Drug Authority, 2019).

The loss on drying analysis was conducted to establish an upper threshold for the loss of compounds during the drying process. This consists of volatile compounds such as essential oils, thermolabile compounds, and water content within extract. This parameter is measured based on the remaining substance after drying at 105°C until a constant weight is expressed as a percent value. An ideal loss on drying value should be below 10%, as this figure also accounts for the evaporated water content within extract. The moisture content shows the amount of water in extract, which should be controlled since it affects the stability of extract

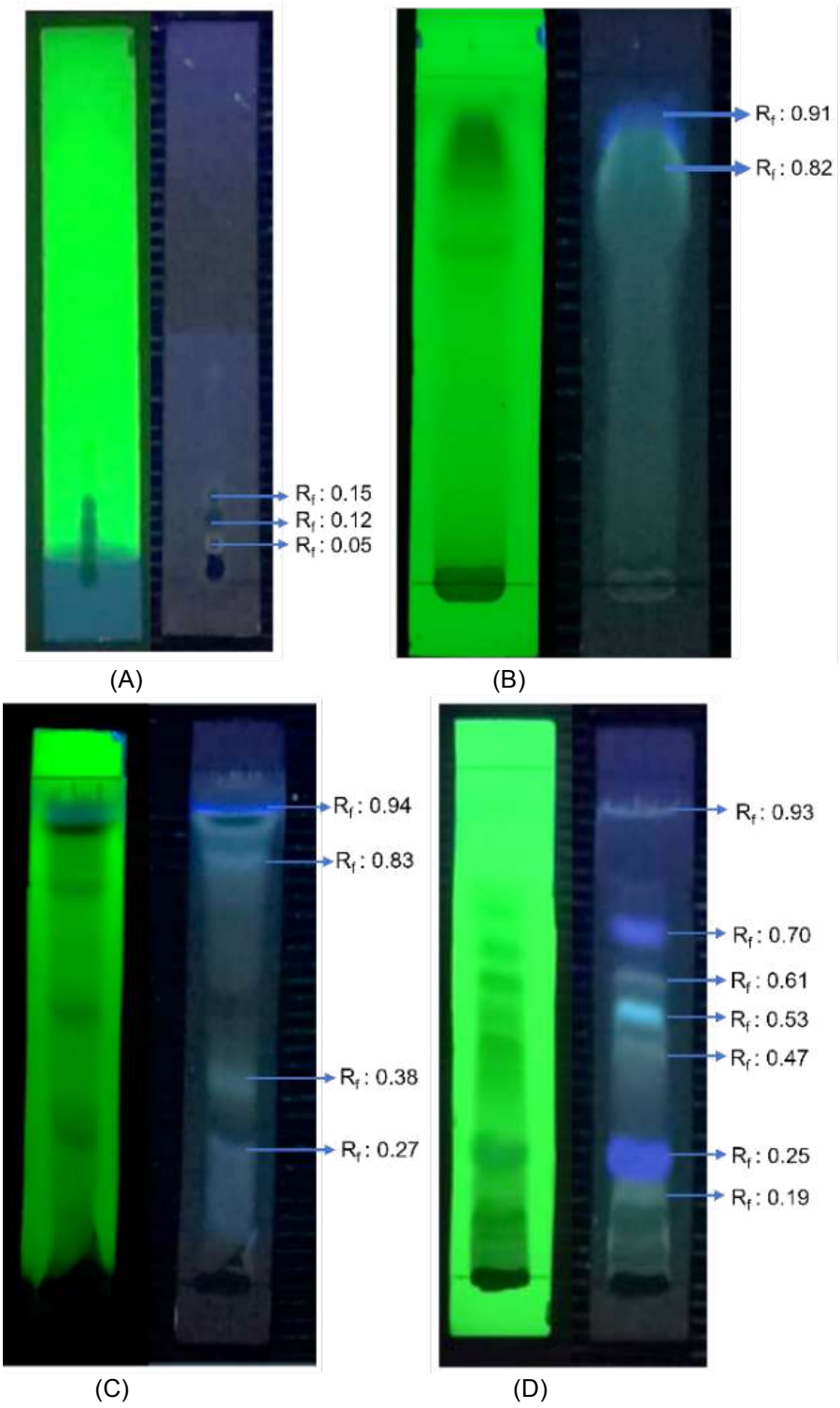

Figure 1: TLC result of *M. cochinchinensis* heartwood extract with mobile phases of (A) toluene/ethyl formate/formic acid (50/40/10) (B) methanol/chloroform/n-hexane (7/2/1) (C) ethyl acetate/formic acid/water (8/1/1) (D) chloroform/n-hexane/methanol (5/4/2)

against microbe contamination. The ash content represents an overview of the internal and external mineral composition from the initial process to extract yield. Additionally, the ash content is determined by the

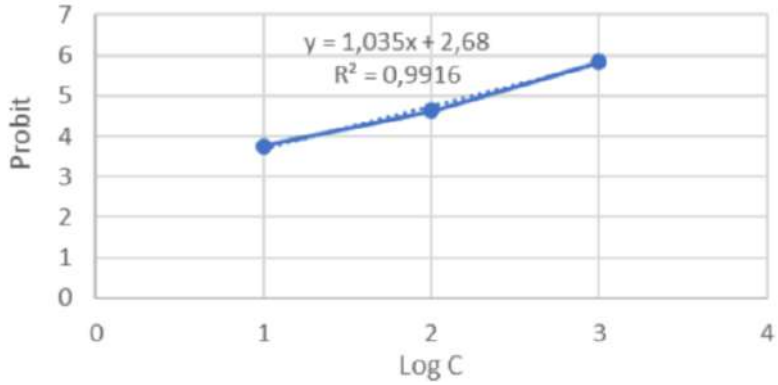

Figure 2: Correlation between log concentration and probit to determine LC_{50}

gravimetric method, which has the principle of heating the material to a temperature where the organic compounds and their derivatives are destroyed and evaporated leaving behind only mineral and inorganic elements. The total ash content indicates the amount of physiological and non-physiological ash, while the acid-insoluble ash content indicates the presence of silica and heavy metals such as Pb, Hg, and Cd. No regulation specifies the requirement value for *M. cochinchinensis* heartwood extract, however, it can be assessed by comparing it to similar plants with wood or trunk components. The result of *M. cochinchinensis* heartwood total ash content was a little higher than the average total ash content value of wood extract that are listed in the Indonesian Herbal Medicine Pharmacopoeia Second Edition, namely bidara laut, sanrego, secang, and kuning wood. The microbial contamination test, including YMN and TPC, was carried out to confirm that extract did not contain pathogenic or non-pathogenic microbes that exceeded the established limit because they could affect the stability of extract and be toxic to health. The result showed that *M. cochinchinensis* heartwood extract is safe to be used in pharmaceutical preparations and can be stored for a long time. Subsequently, heavy metal contamination testing was conducted to ensure that extract did not exceed the permissible levels of heavy metals, as these metals pose health risks. Extract that fulfils the requirement for heavy metal contamination can be used as a preparation material as it is considered safe for use. The determination of residual solvent was conducted to ensure that no solvent residues were left during the process, as they should ideally be absent. Extract should be less than 1.0% to be declared safe from residual solvent remaining from the maceration process (Directorate General of Pharmaceutical and Medical Devices, 2000, 2017; Farida et al., 2023).

Chromatogram profiling, an integral part of extract quality characterisation, offers a detailed view of the potential chemical composition (Directorate General of Pharmaceutical and Medical Devices, 2000). Among the four chromatograms produced, only chromatogram (D) has the best quality. Subsequently, the chromatogram is considered of high quality when spots are within the Rf value range of 0.2-0.8, exhibit no tailing, and have symmetrical shapes. This phenomenon can occur because eluents with lower polarity often enhance compound visibility, resulting in favourable Rf (retention factor) values (Wulandari, 2011). The solubility of compounds from each flavonoid group in specific solvents is a crucial factor in selecting eluents to achieve well-separated chromatogram profiles. A diverse group of compounds is indicated in *M. cochinchinensis*, with flavonoids being the most prominent class. Flavonoids are subdivided into various groups based on their polarity, where compound solubility is influenced by hydroxyl and sugar groups binding together. For example, isoflavones, flavanones, and flavonols are relatively less polar, making them more soluble in ether or chloroform. These unknown compound spots can be further identified to determine the specific compounds present in the chromatogram (Medic-Saric et al., 2008). Subsequently, prior to these results, *M. cochinchinensis* extract characterisation lacked scientific investigation, making this study a potential starting point for establishing uncharted parameter requirements.

Toxicology tests were performed to ascertain the degree of toxicity of a chemical. In this study, the BSLT method was used because it is mostly used, cheap, fast, easy, and reliable (Hamidi et al., 2014). The result showed low toxicity, suggesting the safe use of *M. cochinchinensis* heartwood extract in the environment. When used as a dye, its waste is less likely to result in water contamination, thereby reducing potential risks to aquatic ecosystems. This assertion is supported by the study's results, which indicate that *A. salina*, a sensitive environmental stress indicator, exhibited minimal mortality rates. Therefore, the presence of *M. cochinchinensis* heartwood extract is unlikely to have a significant adverse effect on aquatic ecosystems and

humans (Hamidi et al., 2014; Meyer et al., 1982). This result is in line with previous investigations that showed low toxicity of chloroform, ethyl acetate, and methanol extract of *M. cochinchinensis* against *A. salina* with the same range of LC_{50} (Sato et al., 2020).

Conclusion

In conclusion, 70% ethanol extract of *M. cochinchinensis* heartwood showed high quality, primarily containing a dominant compound. Its TLC (Thin-Layer Chromatography) profile indicated semi-polar characteristics. Furthermore, it exhibited low toxicity, as evidenced by an LC_{50} value of 174.40 mg/L, suggesting its relative safety for the environment. As the research on the characteristics and toxicity of 70% ethanol extract of *M. cochinchinensis* heartwood for human utilization with its environmental impact is limited, this study provides valuable data that can serve as supporting evidence for more targeted utilization of *M. cochinchinensis* heartwood and its isolated compounds. Moreover, it is recommended that further research be conducted to explore the secondary metabolite for the targeted use of *M. cochinchinensis*.

Author Contribution

Conceptualization, E.M. and D.Y.K.; methodology, E.M., D.Y.K., R.D. and D.K.P.; formal analysis, D.Y.K.; resources, E.M., D.Y.K., R.D. and D.K.P.; writing—review and editing, E.M., D.Y.K., R.D. and D.K.P.; supervision, E.M., R.D. and D.K.P; project administration, D.K.P.; funding acquisition, R.D.

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Conflict of Interest

The authors declare that we do not have any conflicts of interest related to this work.

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ORIGINAL ARTICLE

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Evaluating the taste-masking ability and sensory attributes of alginate-encapsulated black seed oil

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ABSTRACT

Introduction: This study aimed to evaluate the sensory attributes of alginate-encapsulated black seed oil in 12 healthy volunteers. Black seed oil, derived from *Nigella sativa* seeds, is known for its therapeutic properties but is characterized by a pungent taste. Alginate, a hydrophilic polysaccharide polymer derived from brown seaweeds, forms water-insoluble gels in the presence of divalent metal ions such as calcium ions. Alginate finds applications in various fields, including food, pharmaceuticals, and biotechnology engineering.

Method: The ionic gelation method was employed to encapsulate black seed oil within alginate beads for taste-masking and to enhance its sensory characteristics. Sensory analysis was conducted to assess the smell, taste, taste masking, aftertaste, and texture acceptability of different formulations, including blank beads (negative control), black seed oil (positive control), black seed oil-alginate beads with and without added flavours (vanilla, chocolate, orange, and sugar), in both dried and wet forms, and a commercial black seed oil soft-gelatine capsule.

Results: The results showed that encapsulating black seed oil within alginate beads significantly improved its taste and aftertaste, and the addition of flavours further enhanced the smell. Vanilla and orange flavours were found to be the most effective in improving palatability and taste masking ability of the beads in both wet and dried forms, surpassing sugar and chocolate flavours. However, sugar is preferred in the dried form alone.

Conclusion: Overall, the study demonstrated the potential of alginate encapsulation to mask the taste of black seed oil and improve its sensory attributes, offering broad applications for enhancing the palatability of other unpleasant bio-compounds

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Introduction

A broad spectrum of medications possesses low organoleptic properties and, unless formulated meticulously, may be highly unpalatable. Therefore, there is significant interest in taste masking for active pharmaceutical ingredients with unpleasant tastes, as well as certain food supplements. This aspect has gained importance in the formulation of medications for paediatrics and other sensitive patient populations. Palatability, encompassing the acceptability of orally administered medications, is closely linked to various organoleptic properties such as mouthfeel (the texture of medications) and aroma (Andrews et al., 2020). While medications effectively treat illnesses, their unappealing taste remains a primary obstacle, hindering a considerable number of patients from consuming them easily. The application of taste masking to these medications enhances patient compliance, thereby improving their overall quality of life (Al-Kasmi et al., 2018).

However, numerous techniques have been employed to mask the bitter taste of drugs in pharmaceutical applications. These methods include introducing flavours and sweeteners (Abay & Ugurlu, 2015), utilising lipophilic vehicles (Abay & Ugurlu, 2015), forming solid dispersions (Zheng et al., 2018), employing salting-out layers (Abay & Ugurlu, 2015), employing complexation with ion exchangers and cyclodextrins (Al-kasmi et al., 2017), encapsulation into microspheres and microcapsules (Zheng et al., 2018), and applying physical barriers or coatings (Zheng et al., 2018).

In a recent study, sucrose octa-acetate served as a model for bitter-tasting drugs encapsulated in lipid microspheres. These microspheres were then integrated into rapidly dissolving edible films containing both masking and flavouring agents (Smutzer et al., 2020). The formulation was employed in a study focusing on suppressing bitter taste, with taste-masking efficacy evaluated in 24 healthy volunteers. Results demonstrated a significant reduction in the bitter taste of sucrose octa-acetate with this formulation (Smutzer et al., 2020). Another study utilised three techniques to assess taste-masking effectiveness: *in vivo* testing with healthy volunteers, *in vitro* drug dissolution, and electronic tongue analysis (Wasilewska et al., 2020). This investigation aimed to develop orodispersible minitablets containing rupatadine fumarate, formulated in taste-masked ethylcellulose-based microparticles produced through spray drying. Rupatadine fumarate, a recent antihistamine, served as a model bitter drug. The study concluded that the three aforementioned methods were valuable for evaluating taste-masking effectiveness, confirming that the fabrication of rupatadine fumarate in ethylcellulose microparticles and subsequent preparation in orodispersible minitablets yielded a satisfactory taste-masking outcome (Wasilewska et al., 2020). Furthermore, *in vitro* assessment and palatability evaluation were conducted to gauge the taste-masking efficacy of microencapsulated paracetamol in chitosan-coated alginate beads (Almurisi et al., 2020). Palatability was assessed with 12 human participants. Results indicated that the ability of dried beads to mask the bitterness of paracetamol surpassed that of wet beads. Even though wet beads were evaluated similarly to the marketed paracetamol suspension, the aftertaste evaluation was superior (Almurisi et al., 2020).

Utilising alginate beads for the microencapsulation of unpleasant drugs is a promising and straightforward approach applicable for a wide range of active compounds, offering scalability for large-scale preparation. Alginate, a hydrophilic polysaccharide polymer derived from brown seaweeds, has witnessed increasing utilisation across diverse fields, including food, pharmaceuticals, and biotechnology engineering. Its widespread application is attributed to its remarkable qualities of non-toxicity, biocompatibility, biodegradability, and the ability to form water-insoluble gels in the presence of divalent metal ions such as Ca^{2+}. Alginate is composed of (1–4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues, arranged in chain homo sequences of MMMMM and GGGGG, interspersed with MGMGMG hetero sequences (Jain & Bar-Shalom, 2014; Leong et al., 2016). The carboxylic acid groups of G units can crosslink with Ca^{2+}, and this gelation process follows the "egg-box" mechanism, where one divalent cation interacts with four –COOH groups (Abasalizadeh et al., 2020). Alginate encapsulation acts as a physical barrier, effectively shielding drugs or supplements from interacting with taste buds and, consequently, masking unpleasant tastes (Chirag J et al., 2013). This encapsulation process relies on the capacity of alginate to undergo water-insoluble gelation in the presence of divalent metal ions like calcium, leading to the formation of cross-linked beads (Chirag J et al., 2013; Liu et al., 2017).

Black seed oil (BSO), extracted from the seeds of *Nigella sativa*, commonly known as black cumin or black seeds, has garnered significant attention in recent studies (Majeed et al., 2020). Several investigations have documented the therapeutic effects of BSO, highlighting its anti-hypertensive, anti-microbial, anti-cancer, anti-inflammatory, and anti-diabetic properties (Majeed et al., 2020; Mazaheri et al., 2019; Mukhtar et al., 2019). Traditionally, BSO has served as a natural remedy for various ailments, such as relieving pain in osteoarthritis, treating influenza, asthma, headaches, rheumatism, and bronchitis (Begum & Mannan, 2020; Majeed et al., 2020; Mazaheri et al., 2019; Mukhtar et al., 2019). Moreover, a separate study suggested that genetically incorporating

BSO into a gene delivery carrier could potentially enhance therapeutic benefits for Alzheimer's disease treatment (Doolaanea et al., 2016).

Nevertheless, BSO is characterised by a bitter and pungent taste and, similar to other oils, is susceptible to oxidation and degradation during storage. This necessitates the use of high-quality packaging that can delay the process of oxidation and degradation over time (Chakraborty et al., 2017; Martins et al., 2017). Employing the technique of ionic gelation to encapsulate BSO within alginate beads could serve as an excellent strategy to mask its taste and enhance its sensory characteristics. Therefore, the aim of the present study was to assess the effectiveness of the ionic gelation method in masking the taste of BSO and evaluating the sensory attributes of the developed beads.

Methodology

Materials

Black seed (*Nigella sativa*) oil (BSO) was purchased from Blessed Seed Sdn. Bhd. (Kuantan, Malaysia). High stiffness gelation type sodium alginate IL-6G (KIMICA Corporation, Tokyo, Japan). Calcium chloride dihydrate was purchased from CFL-Chemische Fabrik Lehrte GmbH & Co. KG (Köthenwaldstraße, Germany). Polysorbate 80 (Tween 80) was purchased from Guangdong Runhua Chemistry Co., Ltd (Yingde, China). BSO soft-gelatine capsules (Baraka, 450 mg) were purchased from a local pharmaceutical outlet in Malaysia. Sugar and flavouring agents of vanilla, chocolate, and orange were purchased from a local market in Malaysia.

Formulation of BSO-alginate beads following ionic gelation

The emulsification of BSO was achieved by incorporating BSO into an alginate solution using Tween 80 as a stabiliser. The BSO-alginate beads were then fabricated using the ionic gelation method (Alkhatib et al., 2022). The concentrations of the ingredients used in the formulation of BSO-alginate beads are presented in Table 1. To create the beads, the BSO-alginate emulsion was slowly dripped through a 22-gauge stainless-steel needle using a syringe into a gelation bath containing a 1% w/v calcium chloride solution, as depicted in Figure 1. Following the gelation process, the beads were allowed to cure in the bath for 20 minutes. Subsequently, the beads were collected using a metal mesh, rinsed with distilled water, and filtered in preparation for their use in the sensory analysis study.

Table 1: Ingredients of BSO-alginate emulsion formulation.

No	Ingredients	Percentage (w/w %)	Quantity (g)
1	BSO	10	1
2	Alginate	2	0.2
3	Tween 80	3	0.3
4	Sugar / Flavouring agents	1	0.1
5	Distilled water	84	8.4
	Total	100 %	10.00

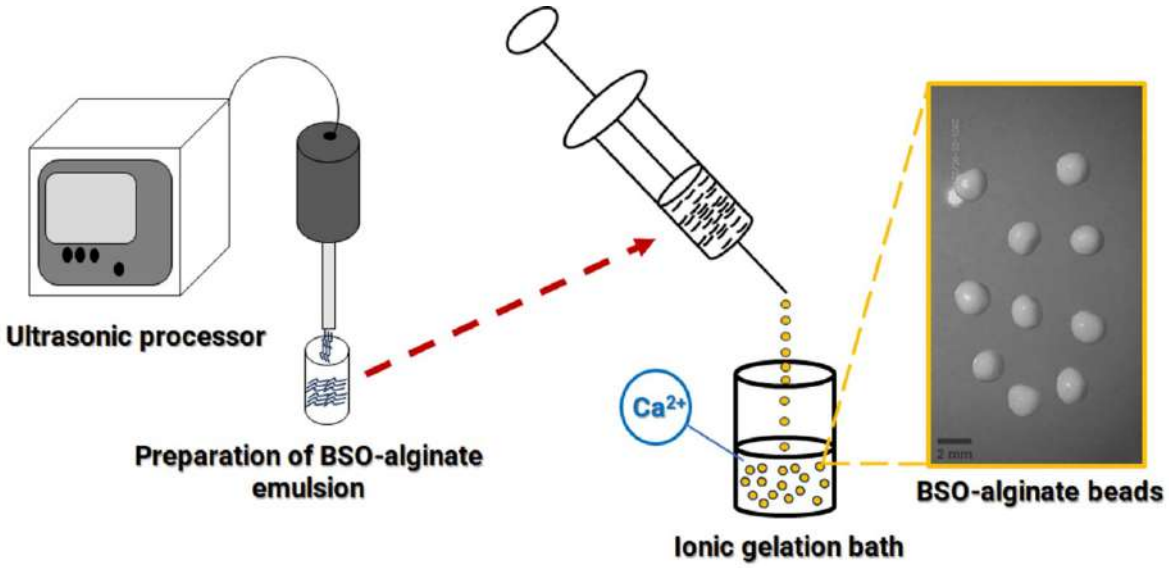

Figure 1: Formulation of BSO-alginate beads by the ionic gelation.

Sensory analysis of BSO in alginate beads

A blinded palatability study involving 12 healthy volunteers was carried out, and their informed consent was obtained in accordance with the university's procedure (IIUM Research Ethics Committee (IREC) reference no: IIUM/504/14/11/2/IREC 2019-156). The study protocol followed the methodology described by Alkhatib et al. (2020). The eligibility criteria included healthy individuals aged 18 to 35 years, while exclusion criteria encompassed smokers, individuals with cough and flu symptoms, use of prescribed medication, pregnant women, and individuals with known allergies to any component present in the formulations.

Six males and six females evaluated the smell, taste, taste masking, aftertaste, and texture acceptability of the following formulations: 100 mg of blank beads (alginate beads without BSO were used as a negative control), 100 mg of BSO (positive control), wet BSO-alginate beads, vanilla wet BSO-alginate beads, chocolate wet BSO-alginate beads, orange wet BSO-alginate beads, sugar wet BSO-alginate beads, dried BSO-alginate beads, vanilla dried BSO-alginate beads, chocolate dried BSO-alginate beads, orange dried BSO-alginate beads, sugar dried BSO-alginate beads, and BSO soft-gelatine capsule (used as a commercial product for comparison). The content of BSO in all beads formulations was 100 mg. The dried beads were made by overnight drying in an oven at 30□C. All the additive flavours and sugar were added to the BSO-alginate beads formulations at the concentration of 1 % w/w.

The participants were instructed to refrain from consuming any food or beverages for one hour prior to the test. A disposable plastic teaspoon was used to administer the tested material to the participants. Each participant tested each sample once. They were asked to place the material in the centre of their tongue and hold it there for 20 seconds before spreading it across their tongue for an additional 20 seconds, after which they spat it out. Feedback from the participants was collected five minutes after spitting out the product. To neutralise the taste of the oil, the participants consumed unsalted crackers and thoroughly rinsed their mouth with plenty of water. A 10-minute interval was provided between the introductions of each product. Palatability and taste-masking attributes were evaluated using a Likert scale, ranging from 1 (very unpleasant) to 5 (very pleasant) (Han et al., 2019).

Alongside the Likert scale, the participants were also requested to provide a taste score ranging from 0 to 100, indicating the perceived intensity of the BSO taste (Albertini et al., 2004). This scoring system assigned a value of 100 to BSO liquid and 0 to blank beads.

Statistical analysis

The statistical analysis in the present study was conducted using Minitab software, version 17.1.0. For the sensory analysis data, a non-parametric Mann-Whitney test was utilised, and the results were reported as medians (n = 12). The taste score results were analysed using One-Way ANOVA, followed by a Tukey's post-hoc test, and are presented as mean ± SD (n = 12).

Results and Discussion

Sensory analysis of BSO in alginate beads

An *in vivo* palatability and taste-masking efficacy evaluation study were conducted to optimise additive flavours for the BSO-alginate beads formulation, namely vanilla, chocolate, orange, and sugar. Pure BSO served as a positive control, compared with all BSO-alginate beads formulations, while blank alginate beads were used as a negative control to neutralise the alginate bead taste and for comparing the textures of BSO-alginate beads formulations, i.e. the gritty feeling in the mouth (Table 2 and Table 3). A Likert scale, ranging from 1 (very unpleasant) to 5 (very pleasant), was utilised for the evaluation, with 3 indicating a neutral feeling.

The lowest scores for smell, taste, and aftertaste were given to pure BSO, receiving ratings of 2, 1, and 1, respectively, as shown in Table 2 and Table 3. The unpleasant smell and very unpleasant taste and aftertaste of BSO present a genuine challenge when attempting to consume this oil in its raw form. The disagreeable taste of medications/supplements is widely acknowledged as a primary reason for non-compliance with treatments, particularly among children who tend to be more sensitive to the unpleasant and unfamiliar tastes of medications than adults (Moreira & Sarraguça, 2020).

Sensory analysis of the wet BSO-alginate beads formulations

The encapsulation of BSO in alginate beads in wet form, with no flavours added, did not improve the smell of BSO, as the wet BSO-alginate beads formulation still exhibited an unpleasant smell when compared with pure BSO (p = 0.972), as shown in Table 2. The addition of flavours significantly enhanced the smell in the wet form, particularly when vanilla and orange flavours were utilised (p = 0.013 and p = 0.029, respectively), as indicated in Table 2. Although the smell of chocolate wet BSO-alginate beads and sugar wet BSO-alginate beads was rated higher than the smell of pure BSO, no significant difference was observed (p = 0.283 and p = 0.137, respectively). This lack of significance was attributed to the low scores given by volunteers when evaluating the smell of these two formulations. Moreover, the ability of vanilla and orange flavours to improve the smell of BSO in wet bead forms surpassed the ability of the commercial product of BSO

soft-gelatine capsules, which did not show a significant difference in smell compared to the smell of pure BSO (p = 0.05) (Table 2). Additionally, the evaluation results indicated a significant improvement in the taste and aftertaste feeling of BSO in the mouth cavity, as well as in taste-masking ability, after encapsulation in the matrix of wet BSO-alginate beads (p = 0.002, p = 0.007, and p = 0, respectively). Despite the statistical significance of these results, they suggest a limited ability of the wet BSO-alginate beads formulation to enhance the taste and aftertaste sensation of BSO, or to exert a substantial taste-masking impact, as shown in Table 2.

BSO droplets were initially dispersed in the aqueous phase to formulate a BSO-alginate emulsion. Subsequently, during the gelation process, these BSO droplets became entrapped within the alginate matrix. The homogeneous distribution of BSO droplets within the alginate bead matrix resulted in the presence of oil droplets among the bead walls. The encapsulation's objective is to establish a barrier between pure BSO and taste receptors, aiming to minimise the inherent taste of BSO. However, the existence of BSO droplets throughout the entire alginate bead matrix, including the bead walls, allowed some of the pure BSO taste and aftertaste sensation to persist, along with a limited taste-masking effect. In contrast, the wall of the BSO soft-gelatine capsule completely prevented the BSO material from reaching taste receptors, resulting in the highest evaluation scores for taste, aftertaste feeling, and taste-masking ability (p = 0, p = 0, and p = 0, respectively), as detailed in Table 2 and Table 3. Al-kasmi et al. (2017) reported that microencapsulation, as a taste-masking method, is employed to render medications completely insoluble in saliva. This observation is based on the understanding that the bitter taste of medications becomes apparent only when the drug is dissolved in saliva and comes into contact with the tongue's taste buds. Encapsulation in alginate beads does not alter the taste or smell of BSO, particularly when carried out without the use of additives such as flavours. In this process, encapsulation minimises the original unpleasant palatability of pure BSO, which received the lowest scores in evaluations by volunteers. However, the addition of vanilla or orange flavour significantly enhances the evaluation scores of taste, aftertaste feeling, and taste-masking in their wet alginate beads formulations (p = 0), as shown in Table 2. Conversely, using chocolate or sugar as additive flavours in the wet formulations did not contribute to the improvement of taste, aftertaste feeling, and taste-masking ability during the encapsulation process, as the evaluation results closely resembled those of wet BSO-alginate beads (Table 2).

Sensory analysis of the dried BSO-alginate beads formulations

Drying the beads generally improved the smell, except in the case of dried BSO-alginate beads with no flavours added. There was no significant difference between the smell of dried BSO-alginate beads and the smell of pure BSO (p = 0.081), even though the smell was evaluated to be higher than that of pure BSO (Table 3). Notably, the smell significantly improved in the formulation of sugar

Table 2: Scores of palatability and taste-masking evaluation of wet BSO-alginate beads formulations. The texture scores were compared with blank beads. Data were analysed using non-parametric Mann-Whitney test and are presented as medians (n = 12).

Formulation	Smell	Taste	Aftertaste	Taste-masking	Texture
Blank beads	-	-	-	-	3
Pure BSO	2	1	1	1	-
Wet BSO-alginate beads	2 p = 0.972	2* p = 0.002	2.5* p = 0.007	2* p = 0.000	3 p = 0.899
Vanilla wet BSO-alginate beads	4* p = 0.013	3* p = 0.001	3* p = 0.003	3* p = 0.000	3 p = 0.948
Chocolate wet BSO-alginate beads	2.5 p = 0.283	2* p = 0.002	3* p = 0.002	2* p = 0.000	3.5 p = 0.975
Orange wet BSO-alginate beads	4* p = 0.029	3* p = 0.000	3* p = 0.002	3* p = 0.000	3 p = 0.400
Sugar wet BSO-alginate beads	3 p = 0.137	2* p = 0.004	2* p = 0.015	2* p = 0.000	3 p = 0.547
BSO soft-gelatine capsule	3 p = 0.050	4* p = 0.000	5* p = 0.000	5* p = 0.000	-

*A statistically significant difference was indicated at $p < 0.05$.

dried BSO-alginate beads, reaching the levels of vanilla dried BSO-alginate beads and orange dried BSO-alginate beads (p = 0.002, p = 0.006, and p = 0.004, respectively). However, this improvement in smell was not observed in sugar wet BSO-alginate beads (Table 2 and Table 3). On the other hand, there was an improvement in the smell of chocolate dried BSO-alginate beads compared to the smell of pure BSO, but it did not reach the levels achieved by using vanilla and orange flavours (p = 0.029), as shown in Table 3.

The general observation from these results is that the drying process was more effective in improving the smell of BSO compared to the wet beads. In fact, pure BSO, or crude BSO, is divided into two major fractions: a fixed oil fraction (triglyceride) accompanied by a minor fraction of volatile oil (E. Edris, 2021). It is expected that the volatile oil is responsible for the strong smell of BSO. The evaporation of water content from the formulations during the drying process might combine with some of the volatile content of BSO, thereby minimising the strong smell of BSO. Moreover, two major factors control the rate of aroma release from products, namely the volatility of the aroma compounds in the product base (thermodynamic factor) and the resistance to mass transfer from the product to air (kinetic factor) (de Roos, 2003). This observation aligns with the obtained results in the current study, where the smell transfer of pure BSO was limited after encapsulation in alginate beads, preventing the BSO smell from reaching the surrounding air in comparison with the smell of pure BSO. Furthermore, the high-water content in the wet formulations (≈ 80%) made the smell transfer to the surrounding air easier in wet beads compared to the dried beads, as this water content could act as an intermediate phase between the beads (solid phase) and the surrounding air (gas phase).

Additionally, the concentration of the additive flavours increased in the beads after the drying process due to water loss, enhancing the effect of the additive flavours. This was particularly evident in sugar-dried BSO-alginate beads, where this formulation obtained the highest evaluation scores with significant improvements in taste, aftertaste feeling, and taste-masking ability among the dried beads (p = 0). Moreover, chocolate dried BSO-alginate beads also achieved a level comparable to vanilla and orange dried BSO-alginate beads in terms of the significant ability to enhance the palatability aspects of taste and aftertaste feeling, in addition to the taste-masking efficacy (p = 0), as shown in Table 3.

Evaluation of the texture of BSO-alginate beads

The texture of blank beads received favourable evaluation scores, suggesting that the use of alginate beads is preferred as an oral formulation. Furthermore, the incorporation of BSO into alginate beads, with or without the addition of flavourings, did not result in a significant decrease in mouthfeel texture for both wet and dried bead forms (p > 0.05), as shown in Tables 2 and 3.

Table 3: Scores of palatability and taste-masking evaluation of dried BSO-alginate beads formulations. The texture scores were compared with blank beads. Data were analysed using non-parametric Mann-Whitney test and are presented as medians (n = 12).

Formulation	Smell	Taste	Aftertaste	Taste-masking	Texture
Blank beads	-	-	-	-	3
Pure BSO	2	1	1	1	-
Dried BSO-alginate beads	3 p = 0.081	2.5* p = 0.000	3* p = 0.001	2* p = 0.000	3 p = 0.318
Vanilla dried BSO-alginate beads	4* p = 0.006	3* p = 0.000	3* p = 0.001	3* p = 0.000	3 p = 0.182
Chocolate dried BSO-alginate beads	3* p= 0.029	3* p = 0.000	3* p = 0.000	3* p = 0.000	3 p = 0.367
Orange dried BSO-alginate beads	4* p =0.004	3* p = 0.000	3* p = 0.001	3* p = 0.000	2.5 p = 0.058
Sugar dried BSO-alginate beads	4* p = 0.002	4* p = 0.000	4* p = 0.000	4* p = 0.000	3 p = 0.190
BSO soft-gelatine capsule	3 p = 0.050	4* p = 0.000	5* p = 0.000	5* p = 0.000	-

*A statistically significant difference was indicated at p < 0.05.

Evaluation of the taste feeling of BSO

The results of BSO taste feeling in the fabricated alginate beads formulations were consistent with the findings obtained from the palatability evaluation, as shown in Figure 2. The taste feeling of BSO significantly decreased following encapsulation in alginate beads, measuring 67.17 ± 21.62% in wet BSO-alginate beads and 45.5 ± 22.43% in dried BSO-alginate beads ($p < 0.05$). The additive flavours generally enhanced the efficacy of alginate beads in reducing the taste feeling of BSO. Sugar dried BSO-alginate beads were the only formulation that reached the level of the commercial product of BSO soft-gelatine capsule with no significant difference ($p > 0.05$), as shown in Figure 2. The taste feeling of BSO in both sugar dried BSO-alginate beads and BSO soft-gelatine capsule was significantly reduced to 21.08 ± 16.31% and 2.67 ± 4.92%, respectively ($p < 0.05$). However, the efficacy of adding sugar or chocolate flavour to reduce the taste feeling of BSO in wet BSO-alginate beads formulations was less than the efficacy of vanilla and orange flavours (Figure 2).

The overall observation from the palatability, taste-masking, and BSO taste feeling evaluation studies showed that both vanilla and orange flavours surpassed sugar and chocolate flavours in improving the acceptability of BSO in wet beads formulations, and their efficacy remained stable in the dried beads formulations. In a study conducted by Lopalco et al. (2019), orange flavour was found to be the preferred taste corrector for the bitter-salty taste of sodium dichloroacetate. The palatability assessment in their study covered a range of flavours, including orange, tropical fruits, berries, and vanilla. Orange flavour emerged as a preferred flavouring agent and yielded satisfactory results in the current study for enhancing the palatability of BSO, masking its unpleasant taste, and reducing its taste feeling in the alginate beads formulation. Therefore, it is recommended to be selected as a flavouring agent in the formulation of BSO-alginate beads.

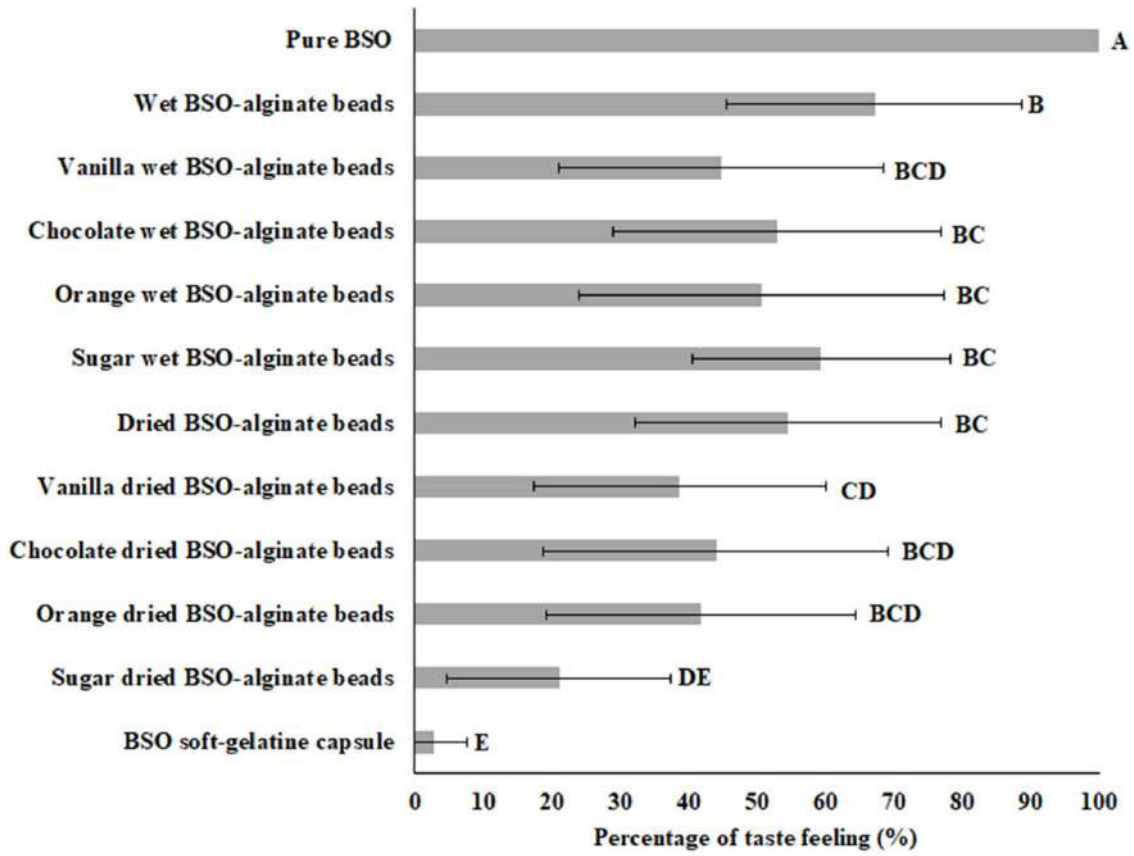

Figure 2: Percentage of BSO taste feeling score. Different letters indicate significant differences among means after Tukey Post-Hoc test ($p < 0.05$). Error bars represent the standard deviation of the mean values (n = 12).

Conclusion

In conclusion, this study demonstrated that alginate-encapsulated BSO successfully enhanced its sensory attributes and masked its unpleasant taste. Wet BSO-alginate beads, particularly those formulated with vanilla and orange flavours, exhibited significant improvements in smell, taste, and aftertaste compared to pure BSO. The alginate encapsulation method effectively shielded the taste buds from interacting with BSO, thereby enhancing its acceptability. Drying the beads further improved the texture and mouthfeel, making the dried formulations more pleasant for consumption. Overall, this approach holds promising implications for enhancing the palatability and patient compliance of BSO and similar compounds, thereby improving the quality of healthcare delivery.

Author Contribution

H. A.: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Validation; Visualisation; Writing - original draft; Writing - review & editing. A. S.: Project administration; Supervision; Writing - review & editing. A. R. S.: Writing - original draft; Writing - review & editing; Formal analysis. M. A.: Data curation; Methodology; Formal analysis. F. M.: Resources; Supervision; Writing - review & editing. A. D.: Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Visualisation; Writing - review & editing.

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Ethical Approval Statement

Ethical approval for this study was obtained in accordance with the university's procedure. The study protocol was reviewed and approved by the IIUM Research Ethics Committee (IREC) under reference no: IIUM/504/14/11/2/IREC 2019-156.

Informed Consent Statement

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

Conflict of Interest

The authors declare no conflict of interest.

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Physicians' Perception on Prescribing Potentially Inappropriate Medications for Older Patients: A Qualitative Study from Malaysia

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ABSTRACT

Introduction: Many quantitative studies reported that potentially inappropriate medication (PIM) is quite prevalent among older adult patients. However, the issue is less explored qualitatively from the perspective of physicians. **Objective:** To qualitatively explore hospital physicians' perception regarding PIMs, associated factors and the possible interventions to control this phenomenon. **Method:** A qualitative study using individual semi-structured and in-depth interview research method was constructed on 15 physicians serving in a Malaysian hospital. The purposive sampling technique was used at the beginning followed by the snowball sampling process. **Results:** It was found that the physicians have inadequate knowledge about PIM and the published PIM criteria . Several factors were perceived as barriers of appropriate prescribing. Firstly, physicians' lack of knowledge and training in geriatric medicine as well as lack of time. Secondly, some of the physicians were skeptical about the applicability of PIM criteria in daily practice due to limited alternative medications. Lastly, complexity of the cases due to multimorbidity, polypharmacy and patient's poor knowledge about their medications. The proposed interventions to optimize prescribing for older patients were education (for patients and physicians), optimization of healthcare workforce and activation of deprescribing. **Conclusions:** Prescribing for older patients is a complex process that is affected by numerous patient-related and doctor-related factors. Improvement strategies should target the patient, physicians and the work environment activating a joined-up working between the physician and other healthcare providers.

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Introduction

Older adults' population is keep increasing worldwide including Malaysia. The percentage of older adults was expected to increase from 5% in 2010 to 14.5% by 2040 in Malaysia (Department of Statistics, 2016). Increasing age is usually associated with multimorbidity which requires the use of multiple medications (polypharmacy) to control the conditions. Multimorbidity, polypharmacy and age-related physiological and pathological changes affect the pharmacokinetics and pharmacodynamics of the medications leading to a higher incidence of adverse drug reactions (ADR), drug-drug interactions, drug-disease interactions and drug-related hospitalization (DRH) compared with younger populations (O'Connor et al., 2012). On top of that, older adults are usually excluded from clinical trials which led to the absence of solid evidence about the efficacy and safety of medications in this population. Consequently, medications approved based on efficacy studies that ruled out older patients are unlikely to suit these patients in daily practice (Beers et al., 2014). All of the above make prescribing medications for older patients quite challenging for physicians. Therefore, experts in the field of geriatric pharmacotherapy developed tools to identify medications that should be avoided in older patients which called potentially inappropriate medications (PIM). PIMs are group of medications that carry more risks than benefits when used in older people as there are safer and effective alternatives (O'Connor et al., 2012). The most commonly used generic lists are Beers criteria (Fick et al., 2019) and STOPP (Screening Tool of Older Person's Prescriptions) criteria (O'Mahony et al., 2015). So far, there is a plethora of published studies linked the use of PIM in older people with several negative health outcomes including ADR, DRH, reduced quality of life and increase healthcare expenses (Xing et al., 2019). Because of that, inappropriate prescribing in older people is getting more attention as it is highly prevalent in different healthcare settings. A recent systematic review from central and eastern Europe reported a median prevalence of 34.6% with a wide range of 6.5–95.8% based on the study setting and the used tool (Brkic et al., 2022). Another systematic review estimated the pooled prevalence of PIM to be 47% (Mekonnen et al., 2021). The prevalence in Malaysia was found also to be similar to these results. Two studies from Pahang state reported a prevalence of PIM among hospitalized patients of 55.3% using Beers criteria (Chee Teng et al., 2020) and 27% based on the STOPP criteria (Akkawi & Mohamed, 2018).

Many studies investigated the factors associated with prescribing PIMs and the barriers to medications optimization for older patients. They identified several patient-related (such as polypharmacy, multimorbidity and inadequate knowledge) and prescriber-related (such lack of knowledge and time, clinical inertia and poor communication with other healthcare providers [HCP]) factors (Xu et al., 2021). Although the majority of these studies quantitatively investigated the prevalence of PIM and associated factors, there are a few studies that assessed this issue qualitatively from the patient's, physician's and nurse's perspectives (Cullinan et al., 2014; Xu et al., 2021). However, most of the published qualitative trails focused on a specific drug class (e.g., benzodiazepines or antidepressants) or on older patients with a particular disease (e.g., dementia). Additionally, almost all of those studies were conducted in primary care settings involving general practitioners (GPs). Available studies from Malaysia are only quantitative focusing on the prevalence and pattern of PIMs with no data available about the prescribers' points of view regarding this phenomenon (Chang et al., 2021). While it is important to identify the prevalence, causes and consequences of prescribing PIM among geriatric patients in Malaysian hospitals, we believe that hearing openly from physicians about their perception of PIM is pivotal to planning for strategic interventions to reduce PIM prescribing in Malaysian hospitals. To the best of the authors' knowledge, there is no qualitative study investigated the issue of appropriate prescribing in older patients neither in Malaysia. The objective of this study is to qualitatively explore the perception of physicians serving in a Malaysian hospital regarding PIMs and the associated factors as well as the possible interventions to control this phenomenon.

Methods

Study Design and Sampling Strategy

This qualitative study was conducted among physicians serving in a teaching hospital in Pahang, the largest state of Peninsular Malaysia. The purposive sampling technique was used at the beginning followed by the snowball sampling process. The physicians were recruited from internal Medicine, cardiology as well as ear, nose and throat (ENT) departments. These three departments were chosen because the physicians in these departments deal with most of the older adult patients in the hospital. All the physicians are serving in the outpatient specialist clinics and in the general medical wards. The physicians were approached during their break time at clinics and an appointment was made if the physician was willing to participate in this study. Six physicians declined to participate in the study due to their packed work schedule. Along with verbal explanation, participant information sheet (PIS) was given to the participants detailing the objectives of the research and the methodology. The interviews were conducted at the participants' workplace in a separated calm room. Each participant received honorarium of 150 Ringgit Malaysia after the end of the interview.

Data Collection

Semi-structured individual face-to-face interviews were performed by a female pharmacist researcher (UMR). The researcher received an intense training on conducting qualitative interviews by an expert in this field. The interview protocol was developed based on previous qualitative and quantitative studies. Then, SZ and MEA (Pharmacy PhD holders) were involved in checking the feasibility of the interview protocol content. It was designed to cover three aspects related to medication prescribing for older patients, namely: physicians' knowledge and perception about prescribing for older patients; factors associated with PIM; the proposed interventions to reduce PIM. The phenomenological approach was also implemented in the data collection which involves an interactive interview with a range of people to elicit a detailed personal description of a phenomenon's lived experience from a small number of individuals who have experienced it. Before the interview, the interviewer explained again about the goals of the study and the structure of the interview. The interview protocol was started with broad predefined, mainly open-ended, questions (table 1). Based on the answers given by the participants, further questions were arisen spontaneously in a free-flowing conversation allowing for in-depth interview related to the aspects of interest. That was the reason for choosing semi-structured interviews for data collection as it is a flexible method for both interviewer and interviewees (McIntosh & Morse, 2015). The interviews were conducted fully in English language with an average duration of 40-60 minutes per session. Every session was recorded using a phone recorder application and notes were taken on paper during the interviews for data transcription purposes. Data collection was started in October and completed once reaching the data saturation point in December 2022.

Data Analysis

After each interview, the audio data was labelled and pseudo-anonymized to protect the privacy of the participants and the confidentiality of the study. Then, the raw audio data was transcribed verbatim using the 'transcribe' feature on Microsoft Word® 365 software. The transcription included using different languages, slang, and pronunciation errors except for the grammar because all of the participants were note English native speakers, therefore their grammar may not be too accurate. Also, small details such as brief space out or intonation changes were excluded depending on the theme of the discussion with the respondents (Clyne et al., 2016). Besides, the re-checking process was done to ensure all of the audio data have been transcribed accurately before the data analysis process. The transcripts were given back to the participants for comments or corrections. No correction/comment was received back from them. Data were sorted by UMR and MEA into small sections based on the contents summarization or impression, and they were coded to create key themes and subthemes as well as a storyline so that it will help to understand and relate more to the research questions. A deductive coding approach was used whereby the data is tested whether it is consistent with the prior assumptions, theories and hypotheses. The theories begin from significant themes, topics, or models which emerge from raw data through repeated analyzation and comparison. After organizing the pre-defined codes into a set, NVivo software was used to assist with organizing the data for analysis. By using this software, it ensures the data can be coded efficiently and also the sources are kept together so that it will be easier to be retrieved for further analysis (Zamawe, 2015). Data collection was discontinued once reaching the data saturation points where no new information appeared. Data saturation started to appear after analysing the data obtained from interviewing the 13th participant. Two additional physicians were interviewed to make sure that no new response will be given. Consolidated criteria for Reporting Qualitative research (COREQ) checklist (Tong et al., 2007) was used to validate this qualitative research ensuring that all criteria were fulfilled. (appendix)

Results

Out of the 21 physicians approached, 15 of them (5 female and 10 male) agreed to participate in the study. There were six consultants/specialists (3 from ENT; 1 from cardiology; 1 from the medical), five medical officers (MO) (2 from cardiology, 3 from medical) and four registrars (all from medical) who are senior MO on their journey to pursue as a specialist. After coding the data, ten integrated subthemes were emerged. They were arranged under the predefined themes (Table 2).

Physicians' perception and knowledge about PIM

All of the 15 participants reported that they are not familiar with the term potentially inappropriate medication (PIM) specifically or directly. Also, they have never heard of the published special criteria for prescribing in older adults such as STOPP criteria or Beers criteria. They have never encountered any of those criteria during study or throughout their profession as a physician. However, most of them do know the general understanding of inappropriate medication and were aware of some medications that should be avoided or should be given with caution to older adult patients.

> *"Based on my understanding... potentially inappropriate medication. Some kind of medication that we give to patient that might cause more harm to the patient. Maybe in terms of the side effect, the incorrect indication." (Dr. Af., MO4)*

> *"Based on my initial impression, basically I think it is*

about certain medication that should not be prescribed to the old patient in certain aspects or certain areas or in regards to certain dosing or maybe in underlying disease in certain locations." (Dr. Mu., Registrar 3).

On the other hand, we found that some of the physicians does not differentiate between PIM and drug related problems.

"For me, in appropriate medication. Number one. We give the wrong medication to the patient. They may not be based on the correct diagnosis. Number 2, the wrong route of administration. So maybe we change from oral to IV. Next is the dose dosage right? Maybe wrong dosage right? So, it should be also inappropriate and then the timing and frequency. Maybe some medication is required at certain particular time and then we give different time or supposedly, four times we give two times so. That would be also inappropriate." (Prof M., Consultant 2)

With further discussion, some of the physicians were able to give example of medications that should be used with cautions in older adults.

"Not enough knowledge. For example. Prescribing excessive sedation or usually we like to use alprazolam but in geriatric patients, they tend to be very sensitive to that. So, you know, they will have more sedative effect compared to other population group" (Dr. Ad., MO3)

"Of course, the first in my opinion is actually the painkillers. Because I see a lot of side effects from the painkillers in my patient, they are geriatric patient, so they are fragile. They are renal impairment patients, so they will develop side effects from all those painkillers whether the peptic ulcers or even the worsening of renal functions. That's the most common… because actually if you ask the geriatric patient the main problem is actually pain. I think we need to be careful in selecting the most less side effect painkillers for the patient." (Dr. S, MO 1)

Most of the physicians did not see PIM as a big issue during their practice. This is because they follow the guidelines, they are experienced staff and they have never encountered, any serious side effects or hospitalization related to PIM prescribing.

"OK, to be honest, I think it's not. It's not common. It's not common here. First, probably because here we have, we have specialists and medical officers. We don't have houseman officers (junior doctors)" (Dr. H., R1)

"Not so sure actually but I think no (not a big issue) because we already follow all the guidelines, discussion, experiences and judgement. So inshaAllah (hopefully) so far, not really I think. (Dr. N., Specialist 2).

Causes of PIM prescribing

Patient-related factors: Complexity of the cases

Most of the participants see older patients as complicated cases due to their comorbidities and being treated by multiple doctors in different healthcare settings. This will make it difficult to trace patient's current diseases and medications especially that most of the older adults having inadequate knowledge about their medical history.

"Right now, we are tracing (medication) based on the patient appointment book. If patient bring the book or the medication itself, then we will know the medications of the patient but if the patient didn't bring the medications or book, it will be difficult for us as well. So, when the patient didn't bring the book, we might give the same medication twice or double the dose maybe." (Dr. Af, MO 4).

"Polypharmacy can lead to PIM. Because sometimes they took from 2 centers then might overlap and sometimes double the doses. Or maybe different types of drugs for the same indication. So that can cause harm to the patient now." (Dr. Ab, Registrar 2).

Physician-related factors

Lack of knowledge and training in geriatric medicine

Participants referred to the lack of knowledge about diseases, medications and special precautions for older adults as a key factor for prescribing PIM. This was confirmed by other participants who admitted of having inadequate knowledge about geriatric medicine/pharmacotherapy and that they treat elderly patients as any other adult patients, i.e., they standardize the treatment for all patients as they do not have any contraindication to the prescribed medication.

"For us, we don't really have any specific training for geriatrics like what kind of special attention that they need? Because we tend to treat everyone as the same standard. So, we're not really giving specific care about the geriatrics. Really, we treat everyone standardly." (Dr. Ad, MO 3)

"One thing for me, one thing is actually the education itself, (because) not all of us are exposed to many geriatric patients. It's more to experience and practice, I think." (Dr. S, MO 1)

"OK. Number one maybe lack of knowledge of the disease of the elderly. Number 2 maybe some of them cannot differentiate between elderly and non-elderly. So maybe they just standardize all the prescription to

the either elderly or non-elderly. Number 3 maybe because certain condition maybe they are not really familiar of certain diagnosis. And then they mix up elderly and non-elderly management." (Prof. M, Consultant 4)

Almost all of the interviewees have limited understanding about the job scope of geriatrician despite knowing the existence of geriatric medicine in Malaysia. On top of that, some of them were not aware whether there is a geriatrician in the hospital or not. Additionally, the participants do admit that geriatric training is still lacking among physicians because of the lack of exposure to this field in Malaysia.

"If we talk about geriatric population, I don't think we have a lot of exposure here because we don't have geriatric consultant yet. We will receive one maybe next year. So, we are not properly guided to manage geriatric patients. We just managed them as in general population." (Dr. F, MO 2)

Following the previous question, medical officers were asked about their interest of being specialist in geriatric medicine. They were reluctant because they considered it as a relatively new subspecialty in Malaysia and also because dealing with older patients require extra patience and passion.

"To deal with geriatric, you need to love the geriatric population. Not all can handle the geriatric population where we need someone who able to understand them, love them and happy to take care of them. I think because all the physicians have the same knowledge about managing but geriatric specialist physician have deeper understanding and deeper knowledge and passion towards managing the population, yeah." (Dr. F, MO 2)

Lack of time

Several physicians raised an issue of staff shortage - especially in the wards- which leads to lack of time allocated for each patient.

"Factors can include lack of time because of the business of the ward round and now here (outpatient clinic), we already have lot of patients. So yeah, perhaps that's the part of the contributing factors to cause.." (Dr. H, Registrar 1)

The applicability of the geriatric specific criteria

Some of the interviewees were skeptical about the applicability of these criteria in practice. According to them, this is mainly because of the limited number of drugs available as alternative to the medications to be avoided. Therefore, they sometimes see that prescribing PIM is unavoidable.

"Yeah, so what's the point? You ban everything but you don't get alternative in which patient need that type of group of medications. And the alternatives must be the same price or cheaper. Because the geriatric populations is increasing in numbers. So, if the price is expensive, it will cause more burden to us actually so cost is something that we have to bear in mind because we are under subsidised system." (Dr. AZ Consultant 2)

"Sometimes it's difficult to find alternative. The medication is not available in the hospital. Patients have to buy outside. Sometimes the alternative is quite expensive" (Dr. Ad MO3)

Interventions to enhance appropriate prescribing

Participants were asked about the possible interventions that could be taken in order to reduce prescribing PIM in general and specifically in this hospital. The answers can be summarized in three subthemes: Education, optimization of healthcare workforce and activation deprescribing.

Education and effective communication

As patient's ignorance was reported as one of the factors for PIM prescribing, the interviewees emphasized on educating the patients about their diseases/medications and the effective communication with the physicians and other healthcare professionals.

"Normally educated patient they also want to know their medication. So, they know this medication is for what? What are the side effect right? How to take them right? For the elderly, some are also really educated, so no problem. The one that I think we need to give counselling or education is those who are not really educated. Or maybe they not really concern about the medication." (Prof. M, Consultant 4)

"I think communication with the patient is the key. Emphasizing enough to the patient and relatives regarding their diseases, why do they need to take the medication. The importance of compliance and stuff like that to ensure that if the patient polypharmacy they know how to take correctly because some medication have specific way to take it." (Dr. Ab, Registrar 2)

Education issue is also extended to the physicians who believed that they did not get enough education and training about geriatric pharmacotherapy.

"Yeah, it's important for us physician to at least get the list of high-risk medications, so that we are more careful to prevent the side effects." (Dr. H, Registrar 1)

Optimization of healthcare workforce

This includes increasing the number of physicians and

having a joined-up working approach. All of the participants agreed that discussion the patient's case with other specialists, pharmacists and healthcare providers is a top-tier approach to result in the best health outcomes for the older adult patients. Despite understanding the significance of the joined-up working, most of them commented that discussions with pharmacists are done only for certain cases which might include dose adjustment for renal patients.

> *"So, we did consult with the pharmacist who joined the round, but here (outpatient clinic) we don't have yet a clinical pharmacist, but sometimes if we are not sure, we will consult the pharmacist, but most of the cases the pharmacy department call us if they have any concern about the prescription…So I think that's very important for discussing with the other teams, especially the pharmacist or consultant." (Dr. H)*

Activation of Deprescribing

The interviewees believe that deprescribing unnecessary drugs does contribute to a more appropriate prescribing for older patients.

> *"Yeah, I mean sometimes maybe the patient is indicated for that medication, but then because of the side effects, we need to stop. Whatever causing harm to patient and risk outweigh benefit, we need to stop. I think that's the way we go about it. It's a bit difficult. That's why we always have to weigh benefit and the risk of all of the medication. If you think it's more risk, then that's it." (Dr. S, MO 1)*

However, the process is quite challenging because most of older adult patients come with comorbidities and being treated in different medical centres (fragmentation of care). In addition, the cost and limited options of medications in certain facilities stave off the process, therefore, some patients might still be prescribed despite the unsuitability of the drugs.

> *"If we actually detect that early then yes, definitely there's benefit. But again, like you said, if patient need that medication, do you have any alternatives or not? what are the alternative that has better or at least similar effect? We shouldn't go downgrade right? By right (they should have) the same or better effect. But again, we have to choose between risk and benefit. Again, is there any alternative that can be given or not? You can ban everything, but if you are not giving alternative then how we can treat patients." (Prof. A, Consultant 2).*

Another obstacle for activation of deprescribing is the absence of local guidelines for deprescribing which makes the decision of deprescribing solely based on the physician's knowledge and judgment.

Discussion

The interviews showed a lack of understanding of PIM concept among the physicians and unawareness of existence of special criteria for prescribing in older adults. This finding was not unexpected because previous study on 82 physicians from other two hospitals in Pahang portrayed that 60% of them never heard of those criteria and only 7.3% had ever used such criteria in practice (Akkawi & Nik Mohamed, 2018). Other qualitative studies from other countries reported similar findings about the limited knowledge of physicians on PIM and published PIM criteria (Anderson et al., 2014; Voigt et al., 2016). This result is directly correlated to the fact that there is a lack of education and training related to geriatric medicine for undergraduate and postgraduate physicians. This issue is perceived by our participants as one of the factors contributing to prescribing PIMs. Data from 2018 depicted that out of 34 registered institutions under the Ministry of High Education Malaysia, only five of them included geriatric medicine as part of the undergraduate curriculum which resulted in a very low number of geriatricians around Malaysia (Tan et al., 2018). Another qualitative study has also found that lack of specific education and training of the physicians was a barrier to appropriate prescribing for older patients (Cullinan et al., 2015). All participants were open for any recommendations from the pharmacists about prescribing for older adults. Additionally, they were keen to know the list of medications to be avoided because they believe that this would be useful in their daily practice. This finding contradicts what has been reported in another qualitative study where the general practitioners (GPs) were skeptical about the usefulness of the PIM criteria in the real world as they are designed based on a controlled research environment. Most of the participants in that study think that altering a medication is worse than continuing a PIM which led physicians to find that those criteria may not be useful in the daily practice (Clyne et al., 2016). Another doctor-related barrier to appropriate prescribing was the lack of time. The physicians complained about the increasing number of patients -including older patients- in both inpatient and outpatient settings with no change in the number of the involved physicians. This issue increases the workload and shortens the time allocated for each patient, especially as there are no houseman officers (junior doctors) available in this hospital. Voigt et al in their study also showed that lack of time during consultation at practices was mentioned by most of family physicians as a barrier of appropriate prescribing (Voigt et al., 2016). Furthermore, a systematic review also reported low number of staff, heavy work burden and lack of time as reasons of inappropriate treatment of older adults (Lundby et al., 2019).

Another perceived cause of prescribing PIM by our participants was impracticality of the PIM criteria in some cases due to limited available options for the treatment.

They mentioned that the recommendations and proposed alternatives are based on studies conducted mainly in the western world which might not be applicable in Malaysia. The physicians did admit that the medication may not be perfectly suitable for the patient but the limitation of medications choices in certain facilities -especially in rural areas- hinder the chance of providing a better choice to them. This finding is supported by a German study where family practitioners revealed that prescribing PIM is sometimes unavoidable because of having no alternative especially in patients with multiple comorbidities (Voigt et al., 2016).

Complexity of the geriatric cases was reported by all participants as a substantial contributor towards inappropriate prescribing. Polypharmacy, multiple comorbidities and patient's ignorance were perceived as factors contributing to the complexity of the cases. All of these factors were identified as barriers of appropriate prescribing in other qualitative studies (Anderson et al., 2014; Clyne et al., 2016). The number of prescribers involved in the treatment was reported in the literature as a predictor of PIM prescribing (Holmes et al., 2013). In tandem with that, our participants refer to inability to do medication reconciliation as a main barrier to appropriate prescribing. This problem was attributed to having multiple prescribers in different centers (fragmentation of care) and patient's ignorance about their medical history. Patients are referred from secondary and tertiary care facilities to the hospital due to certain conditions. The problem is that most of the healthcare facilities in Malaysia including Ministry of Health (MOH) settings have not implemented any electronic system to record their patients' medical histories. All of the participants mentioned that they usually try to trace back as much as they can, especially if the medication can put the patients at a dangerous risk such as warfarin. However, if the medications still cannot be retrieved, this will cause errors and PIM which can be due to double dose or drug-drug interaction.

To reduce inappropriate prescribing in older adults, the interviewed physicians agreed on several interventions. First and foremost was enhancing physicians' knowledge and offering training related to geriatric pharmacotherapy. Some of the participants suggested that familiarization of medications and treatment, continuous self-practice and revision are paramount to a better prescribing outcome for older adult patients. A previous study concluded that awareness-raising strategies helped to improve the poor insight of prescribers regarding prescribing PIM. It was also suggested that greater attention from healthcare professionals is needed whereby familiarization themselves with evidence of PIM criteria and thorough medication review of PIM can prevent unnecessary harm associated with polypharmacy (Abdulah et al., 2018). Likewise, educational interventions for healthcare professionals regarding appropriate prescribing were effective in reducing PIM prescribing (Rodrigues et al., 2022). Similar promising results were reported from Malaysia as well (Akkawi et al., 2020). In order to cover the shortage in the field of geriatric medicine in Malaysia, the MOH Malaysia continuously fund fellowships for geriatric training every year which has led to an increasing number of geriatricians from only 14 in 2014 to 39 in 2018. However, the numbers are still insufficient compared to the current aging population now (Tan et al., 2018).

The second proposed intervention was optimization of the workforce by providing more staff and activating the joined-up working environment. Although all the participants agreed that sharing decisions with pharmacists would reduce PIM, the majority of them revealed that their discussion with the pharmacists take place only for certain conditions like dose adjustment in patients with renal impairment. Therefore, they believe that having a clinical pharmacist in each unit may help them optimizing prescriptions for older patients. The positive impact of pharmacist in reducing PIM in older patients in hospital settings is well established in the literature (Alosaimy et al., 2019; Ammerman et al., 2019). Nevertheless, the implementation of this strategy in the wards is laborious (Alosaimy et al., 2019).

Enhancing the physicians' knowledge and exposure to geriatric medicine and involving a pharmacist in the treatment decisions would pave the road for a seamless deprescribing process. Deprescribing of unnecessary medications was referred by the participants as an essential approach to reduce PIM and enhance patients' clinical outcomes. It was found that providing deprescribing guidelines can help physicians to identify the appropriate medications and thus preventing ADEs and in older patients (Lundby et al., 2019; Schuling et al., 2012).

Last but not least, patient's education and effective communication with the prescribers was repeatedly mentioned by the interviewees as a key factor for appropriate prescribing. Engaging older patients in making decision regarding their treatment is limited -in top of other factors- by the poor knowledge of this group about their diseases and medications (Clyne et al., 2016). Based on that, enhancing patient's medical knowledge and communication would contribute to appropriate prescribing. For example, discussing the negative effects of long-term use of benzodiazepines for older patients led to a significant reduction in the use of this class of medication. (Tannenbaum et al., 2014). Effective communication with the patients was also considered as an essential factor for appropriate prescribing. Paternalistic doctor-patient relationship was reported in several qualitative studies as a barrier to appropriate prescribing (Clyne et al., 2016; Spinewine et al., 2005). Whitin this relationship, the patient just follows -without any discussion- what the physician prescribes who is viewed as an authority figure (a parent)

that makes decisions for the best patient's interest.

Recently, a group of Malaysian researchers developed the Malaysian potentially inappropriate prescribing screening tool (MALPIP) to be used while prescribing for older adults in the Malaysian healthcare settings (Chang et al., 2023). The findings of the current study could help policy makers on the implementation of that tool in daily practice. For instance, as lack of time was reported as one of the barriers towards appropriate prescribing, MALPIP can be converted to a clinical decision support software (CDSS) and connected to the hospital prescribing system. The CDSS will alert the prescriber when detecting any PIM based on the patient's information and proposed prescription. CDSS was reported to be effective in improving medication appropriateness and in reducing the incidence of nontrivial ADRs in the intervention group compared with the control group (O'Sullivan et al., 2016).

The current study has some limitations. First, the snowball technique of sampling may lead to a biased sample composition bias as participants invite colleagues who usually share common thoughts and background. Second, the study was conducted in one hospital only and did not use random sampling which may limit the generalizability of its findings. Having said that, the sample involved in this study was diverse in terms of specialty and level of experience. Additionally, data collection was halted after reaching saturation point.

Conclusions

The in-depth interview with the hospital physicians found that they have inadequate knowledge about PIM and the related published criteria. Several factors were perceived as barriers of appropriate prescribing. Doctor-related factors included lack of knowledge and training in geriatric medicine/pharmacotherapy as well as lack of time due to low number of staff and compared with the high number of patients encountered every day. Some of the physicians were skeptical about the applicability of PIM criteria in daily practice due to limited options of the available medications. Patient-related factors were summarized by the theme of complexity of the cases which is attributed to the presence of multimorbidity, having polypharmacy and patient's poor knowledge about their medications. The proposed interventions to optimize prescribing for older patients were patient's education as well as encouraging effective communications with the physicians, optimization of healthcare workforce and activation of deprescribing. This study not only reveals the existing challenges but also provides a roadmap for targeted interventions that can lead to improved prescribing practices for older patients in Malaysia. The significance of these findings impacts public health outcomes and contributing to the ongoing discourse on geriatric pharmacotherapy in the region. As we move forward, it is imperative to consider these insights in the development of policies and practices that prioritize the well-being of older adults in healthcare settings.

Ethical Considerations

Ethical approval was obtained from the IIUM Research Committee (IREC 2022-130) and from the research unit of the hospital (IIR 22-37) before conducting the study. A consent form was collected from each participant before proceeding with the interview.

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Disclosure of interest

The authors declare no conflict of interest related to this work. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions

Conceptualization: M.E.A., S.Z. Methodology: M.E.A., S.Z., U.M.R. Formal analysis: U.M.R., M.E.A. Writing original draft: U.M.R. Writing review & editing: M.E.A., S.Z., U.M.R.

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Table 1: Predefined broad questions of the interview protocol.

1. What do you know about potentially inappropriate prescribing? Are you familiar with the term PIM/PIM?
2. Are you familiar with START or Beers criteria? Have you ever used them?
3. What are the causes that may lead physicians to prescribe potentially inappropriate medication for older inpatients?
4. What are the factors contributing to PIM? Is lack of knowledge/training/time one of them?
5. What intervention can be applied in this hospital to optimize prescribing for older patients?
6. Do you think any discussion with other colleagues (e.g., within physicians or with pharmacists) are important prior any prescribing activity towards geriatric patients? Do you practice that?
7. What do you think about deprescribing process? How could it be applied?

Table 2: Themes and subthemes emerged after data analysis.

Theme 1	Physicians' knowledge and perception about PIM
	Understanding of the PIM term
	Familiarization with the available PIM lists
	Perception on the importance of PIM issue
Theme 2	Factors associated with prescribing PIM
	Complexity of the cases
	Lack of knowledge and training in geriatric medicine
	Lack of time
	Perception on the applicability of the PIM lists
Theme 3	Interventions to reduce prescribing of PIM
	Education and effective communications
	Optimization of the workforce
	Activation of deprescribing

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ORIGINAL ARTICLE Open Access

Green-synthesized silver nanoparticles from *Anisophyllea corneri* leaf extract and its antimicrobial and cytotoxic activities

Ika Rizky Fadhillah[1], Muhammad Taher[2,3*], Mokhamad Nur[1] & Deny Susanti[4]

ABSTRACT

Introduction: The escalating global threat of multidrug-resistant pathogens necessitates innovative approaches to combat drug resistance. Silver nanoparticles (AgNPs) have emerged as promising candidates due to their potent antimicrobial and anti-cancer properties. Green synthesis of AgNPs using plant extracts offers an eco-friendly and cost-effective method. This study focuses on the green synthesis of silver nanoparticles (AC-AgNPs) using *Anisophyllea corneri* leaf extracts and evaluates their antimicrobial and cytotoxic activities.

Materials and methods: An eco-friendly synthesis approach was employed, utilizing *A. corneri* leaf extracts as reducing agents. Liquid Chromatography-Mass Spectrometry (LC-MS) was utilized for phytochemical profiling. The synthesis process was optimized at various temperatures (60ºC, 70ºC, 80ºC) and pH levels (4, 9) to achieve optimal AgNPs outcomes. Characterization of AC-AgNPs included UV-Vis spectrophotometry, FTIR, SEM, Zeta potential, and Particle Size Analyzer (PSA). Antimicrobial evaluation was conducted against four bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*) using paper disc diffusion. Cytotoxicity was assessed through the MTT assay on MCF-7 (breast cancer cell line).

Results: *A. corneri* leaf extract exhibited abundant active compounds facilitating the reduction of silver ions. Optimization revealed that 70ºC at pH 9 produced AC-AgNPs with a minimal particle size of 135.5 nm and a stable zeta potential (-45.1±11.7 mV). AC-AgNPs displayed a spherical morphology. Antimicrobial trials demonstrated moderate efficacy against the tested bacteria, with inhibition zones ranging from 8 to 10 mm. Additionally, AC-AgNPs exhibited cytotoxic potential with a moderate IC_{50} of 74.9 µg/mL.

Conclusion: The green synthesis, characterisation and biological activities of AgNPs from *A. corneri* leaf extracts have been established. It is recommended to optimise the synthesis process and validate the biological activities.

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Introduction

The rise of multidrug-resistant pathogens has significantly contributed to the surge in infectious diseases, becoming a leading cause of global mortality (WHO, 2023; Tanwar et al., 2014). The extensive use of antibiotics has been a primary driver of bacterial resistance, leading to severe consequences such as increased mortality, prolonged hospitalization, and substantial economic losses (O'Bryan et al., 2018; Patel et al., 2008). To address this critical issue, innovative strategies to combat drug resistance effectively are required.

Nanotechnology has emerged as a promising solution to tackle drug resistance, with silver nanoparticles (AgNPs) gaining considerable attention for their potent antimicrobial properties. Various studies have highlighted the efficacy of AgNPs against drug-resistant bacteria, including *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, and *Salmonella enteritidis*, showcasing their potential to inhibit microorganisms (Loo et al., 2018). Additionally, AgNPs have demonstrated high cytotoxicity against cancer cells such as MCF-7 and Caco-2 (Shelembe et al., 2022; Böhmert et al., 2012).

Despite their therapeutic potential, conventional methods for AgNP synthesis involve the use of hazardous chemicals, posing risks to human health and the environment. In response to these challenges, researchers have shifted towards green synthesis approaches, utilizing natural extracts to produce AgNPs. Green synthesis offers environmental friendliness by eliminating the need for harmful chemicals (Zhang et al., 2016). In this context, *A. corneri*, a plant rich in active compounds and known for its antimicrobial potential, presents an opportunity for green AgNP synthesis (Bari et al., 2021; Onivogui et al., 2016).

This study aims to employ *A. corneri* leaf extract for the green synthesis of AgNPs and investigate their antimicrobial and cytotoxic activities. Antimicrobial efficacy will be assessed against four bacterial strains (*Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*) using the disc diffusion method. Cytotoxicity will be evaluated on the breast cancer cell line (MCF-7) through the MTT assay. Additionally, the active compounds present will be analysed using Liquid Chromatography-Mass Spectrometry (LC-MS).

Materials and methods

Plant Sample Collection and Extraction

Fresh leaves of *A. corneri* (voucher specimen PIIUM0317) were collected and deposited at the Herbarium Kulliyyah of Pharmacy, International Islamic University Malaysia Kuantan Campus, Kuantan, Malaysia. The leaves underwent air-drying at 40°C, followed by mechanical grinding into a fine powder. A total of 50 grams of the powdered leaves were mixed with 500 mL of ethanol-water (80:20 v/v, Ethanol 95%, GENE Chemical) in a 500 mL Erlenmeyer flask. Ultrasonic-assisted extraction (UAE) was conducted at 48°C for 40 minutes using a Qsonica Ultrasonic Sonicator Converter Model CL-334. The resulting leaf extract was filtered using NICE Qualitative 102 filter papers and stored at 4°C (Eze et al., 2019).

Liquid Chromatography Mass Spectrometry Quadrupole Time-of-Flight (LC/MS-QTOF) Analysis

The *A. corneri* leaf extract was dried with a rotary evaporator at 130 rpm and 50°C. The dried extract was reconstituted in methanol to a final concentration of 10 mg/ml, then further diluted to 1 mg/ml with methanol. Prior to LC/MS-QTOF analysis (Agilent Technologies, SA, USA), the extract was filtered through a 0.22 µm pore size syringe filter. The LC/MS analysis employed an Agilent ZORBAX Eclipse Plus C18 Rapid Resolution HT (2.1 x 100 mm) 1.8 µm column with a gradient elution program. Mass spectrometry was operated in positive electrospray ionization (ESI) mode. Data analysis was performed using Agilent Mass Hunter Qualitative Analysis B.05.00 software (Agilent Technologies, Santa Clara, CA, USA).

Preparation of Silver Nitrate ($AgNO_3$) Solution

1 mM $AgNO_3$ solution was prepared by dissolving 0.0459 grams of $AgNO_3$ powder (EMSURE, Macedonia) in 270 mL of deionized water.

Green Synthesis of AC-AgNPs

In the green synthesis process, 30 mL of *A. corneri* leaf extract was gradually added to a 270 mL $AgNO_3$ 1 mM solution under continuous stirring (100 rpm) using a magnetic stirrer. The mixture was divided into three equal portions of 100 mL each and stirred and heated at temperatures of 60, 70, and 80°C for 90 minutes. After 90 minutes, each mixture was further divided into two equal portions of 50 mL each. In one portion of each temperature treatment, 1 M NaOH was added drop by drop until reaching pH 9. The synthesized solution was then centrifuged at 11,000 rpm for 30 minutes at 4°C for purification using a Supra 22K centrifuge (Korea). The resulting pellet of AC-AgNPs was resuspended in deionized water and dried at 40°C, stored at room temperature for future use.

Characterization of AC-AgNPs

UV-Vis Spectrophotometer Analysis: AC-AgNPs were sampled at different time points for analysis using a UV-Visible double-beam spectrophotometer (SHIMADZU UV-1800, Japan) in the scanning range of 300-800 nm.

Particle Size and Zeta Potential Analysis: An aqueous solution of AC-AgNPs was analysed using a Malvern Zetasizer instrument at 25°C.

FTIR Analysis: AC-AgNPs powder samples were analysed using a PerkinElmer Dual FT-IR Spectrometer, covering the 400–4000 cm^{-1} range.

SEM Analysis: Images of AC-AgNPs were captured using a Zeiss EVO-50X Scanning Electron Microscopy (SEM) instrument.

Antimicrobial Assay

Four bacterial strains, including two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and two Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria, were cultured in nutrient broth medium. Subsequently, they were incubated for 18 hours at 37±1°C. The antimicrobial activity of AC-AgNPs was evaluated using a disc diffusion assay. The cultivated microbes were sub-cultured on Petri dishes, and 10 µL of AC-AgNPs, *A. corneri* plant extract, $AgNO_3$ solutions, and 10% DMSO (negative control) were applied to sterile discs. Amoxicillin discs were used as the positive control. Following this, the Petri dishes were placed in a CO_2 incubator (Binder) at 37°C for 24 hours. The diameter of the inhibition zones around the discs was measured in millimeters and compared with the negative control.

Cytotoxic Activity

Human epithelial breast adenocarcinoma cells (MCF-7) were cultured at 37°C in a 5% CO_2 incubator (Thermo Scientific Heraeus BB15) in Eagle's minimal essential medium (EMEM) (ATCC 30-2003, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Brazil) and 1% (v/v) penicillin-streptomycin (Nacalai Tesque, INC., Kyoto, Japan). Once the cells reached 80% confluence, they were detached and subcultured. Subsequently, the cells were seeded into a 96-well plate at a density of 15,000 cells per well. Cell Viability Assay: The cytotoxic activity of the synthesized AgNPs was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The seeded cells were exposed to various concentrations of AC-AgNPs (90 µg/mL, 45 µg/mL, 22.5 µg/mL, 11.25 µg/mL, 5.63 µg/mL, and 2.81 µg/mL), $AgNO_3$, and an anticancer drug, Tamoxifen, as a positive control. The cells were incubated for 24 hours. Afterwards, 10 µL of MTT (0.5 mg/mL) was added, and the cells were re-incubated for 4 hours at 37°C. Formazan crystals that formed were dissolved using 100 µL of DMSO (EMPLURA, USA), and they were further incubated for an additional 30 minutes at 37°C. Using a microplate reader, absorbance at 570 nm was measured to determine differences in color intensity.

Results and Discussion

LC/MS-QTOF analysis of A. corneri leaves extract

Initial compound identification was conducted through LC/MS-QTOF analysis by comparing the m/z spectrum of each compound with the METLIN mass spectrum database (Figure 1).

Table 1 provides detailed information on the chemical composition of 55 identified compounds, including their names, molecular formulas, m/z values, masses, and classifications. These compounds belong to various categories such as flavonoids, fatty acyls, tannins, and others. Notably, it is worth noting that some compounds remain unidentified as their data is not available in the METLIN database.

Numerous studies have highlighted the role of various phytochemicals, such as phenolic acids, flavonoids, alkaloids, terpenoids, amino acids, alcoholic compounds, glutathiones, polysaccharides, antioxidants, and organic acids (including ascorbic, oxalic, malic, tartaric, and protocatechuic acid), as well as quinones. These compounds are known for their ability to serve as reducing, capping, and stabilizing agents in the synthesis of AgNPs (Mustapha et al., 2022; Suriyakala et al., 2022; Zuhrotun et al., 2023). Given the observed biological activity of *A. corneri*, it appears to be a promising candidate for the synthesis of AC-AgNPs.

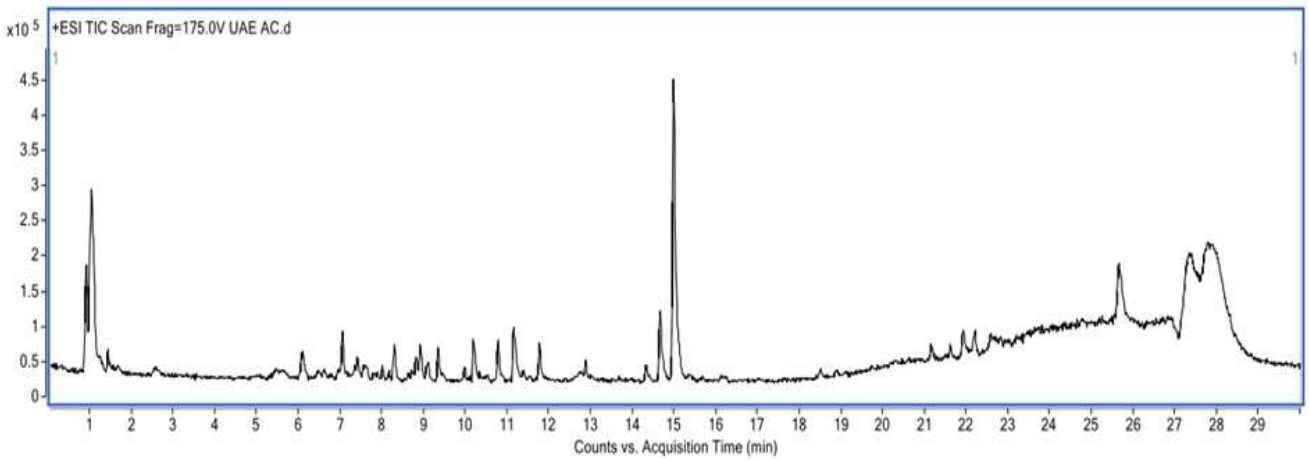

Figure 1: LC/MS Q-TOF Total Ion Chromatogram (TIC) of *A. corneri* Extract

Table 1: Compounds Predicted in *A. corneri* Leaf Extract

Retention time (min)	(M-H)- m/z	Mass	Predicted compound	Predicted molecular formula	Class
1.007	203.0535	180.0644	Paraxanthine	$C_7 H_8 N_4 O_2$	Imidazopyrimidines
1.012	282.0878	562.1613	Physalin H	$C_{28} H_{31} ClO10$	Benzene and substituted derivatives
1.02	385.0923	384.0851	5,6-Dimethoxysterigmatocystin	$C_{20} H_{16} O8$	Sterigmatocystins
1.03	381.0806	358.0916	N1-(5-Phospho-a-D-ribosyl)-5,6-dimethylbenzimidazole	$C_{14} H_{19} N_2 O_7 P$	Benzimidazole ribonucleosides and ribonucleotides
1.031	365.1058	342.117	D-(+)-Cellobiose	$C_{12} H_{22} O_{11}$	Fatty Acyls
1.045	118.0867	117.0794	Isoamyl nitrite	$C_5 H_{11} N O_2$	Organonitrogen compounds
1.047	163.0597	162.0524	3-Hydroxy-3-methyl-glutaric acid	$C_6 H_{10} O_5$	Organooxygen compounds
1.054	295.095	256.1313	2-[4-(3-Hydroxypropyl)-2-methoxyphenoxy]-1,3-propanediol	$C_{13} H_{20} O_5$	Phenol ethers
1.06	236.1498	218.1159	3-hydroxy-sebacic acid	$C_{10} H_{18} O_5$	Hydroxy acids and derivatives
1.57	132.1015	131.0943	N,N-Diethylglycine	$C_6 H_{13} N O_2$	Carboxylic acids and derivatives
2.576	166.0862	165.0788	Gentiatibetine	$C_9 H_{11} N O_2$	Pyranopyridines
5.016	205.0963	187.0625	Deethylatrazine	$C_6 H_{10} Cl N_5$	Triazines
5.453	802.1075	784.0736	Pedunculagin	$C_{34} H_{24} O_{22}$	Tannins
5.531	970.1136	952.0792	Geraniin	$C_{41} H_{28} O_{27}$	Tannins

6.079	307.081	306.0737	(+)-Gallocatechin	$C_{15}H_{14}O_7$	Flavonoids
6.123	595.1447	594.1367	Kaempferol 3-(5"-feruloylapioside)	$C_{30}H_{26}O_{13}$	Flavonoids
6.463	402.1552	401.1474	Margrapine A	$C_{21}H_{23}NO_7$	Quinolines and derivatives
6.591	652.1166	634.083	Punicacortein A	$C_{27}H_{22}O_{18}$	Tannins
6.641	344.1344	326.1011	Acetylaminodantrolene	$C_{16}H_{14}N_4O_4$	Azolidines
6.754	954.1187	936.0827	Casuarictin	$C_{41}H_{28}O_{26}$	Tannins
6.956	579.1491	578.142	Apigenin 7-(3"-p-coumaroylglucoside)	$C_{30}H_{26}O_{12}$	Flavonoids
7.042	453.104	430.1154	N-Ethylmaleimide-S-glutathione	$C_{16}H_{22}N_4O_8S$	Maleimides
7.042	321.0619	320.0547	2,3-Dihydrogossypetin	$C_{15}H_{12}O_8$	Flavonoids
7.266	386.1601	385.1524	Papaverrubine B	$C_{21}H_{23}NO_6$	Rhoeadine alkaloids
7.327	291.0865	290.0794	Oritin-4beta-ol	$C_{15}H_{14}O_6$	Flavonoids
7.35	471.0187	470.0111	Sanguisorbic acid dilactone	$C_{21}H_{10}O_{13}$	Tannins
7.403	207.1383	206.1309	2-Phenylethyl 3-methylbutanoate	$C_{13}H_{18}O_2$	Fatty Acyls
7.99	437.1077	436.1006	Homomangiferin	$C_{20}H_{20}O_{11}$	Benzopyrans
7.991	305.0664	304.0589	Dihydrorobinetin	$C_{15}H_{12}O_7$	Flavonoids
8.162	479.0828	478.0755	Isoetin 4'-glucuronide	$C_{21}H_{18}O_{13}$	Flavonoids
8.285	465.1019	464.095	5,6,7,3',4'-Pentahydroxy-8-methoxyflavone 7-apioside	$C_{21}H_{20}O_{12}$	Flavonoids
8.285	319.0445	318.0374	Rhodocladonic Acid	$C_{15}H_{10}O_8$	Flavonoids
8.612	491.2857	473.2522	LysoPE(0:0/18:4(6Z,9Z,12Z,15Z))	$C_{23}H_{40}NO_7P$	Glycerophospholipids
8.91	303.0498	302.0425	3,5,7,2',5'-Pentahydroxyflavone	$C_{15}H_{10}O_7$	Flavonoids
9.057	463.0877	462.0799	5,6,7,2'-Tetrahydroxyflavone 7-glucuronide	$C_{21}H_{18}O_{12}$	Flavonoids
9.08	523.2159	522.2092	Isolariciresinol 9-O-beta-D-glucoside	$C_{26}H_{34}O_{11}$	Lignan glycosides
10.168	604.2744	586.2394	Xylocarpus A	$C_{31}H_{38}O_{11}$	Terpenoid
10.514	501.3201	500.3132	Physalolactone B	$C_{30}H_{44}O_6$	Steroids and steroid derivatives
10.515	663.3742	662.3668	1-Acetyl-3,27-dihydroxywitha-5,24-dienolide 3-glucoside	$C_{36}H_{54}O_{11}$	Steroids and steroid derivatives
12.731	228.1958	210.1619	10-Tridecynoic acid	$C_{13}H_{22}O_2$	Fatty Acyls
14.313	460.2703	459.2626	Militarinone A	$C_{26}H_{37}NO_6$	Pyridine alkaloid
14.314	415.212	414.2049	Eplerenone	$C_{24}H_{30}O_6$	Steroids and steroid derivatives
14.653	421.3177	398.3284	Nb-Palmitoyltryptamine	$C_{26}H_{42}N_2O$	Indoles and derivatives

14.973	397.2007	396.193	(S)-(E)-2'-(3,6-Dimethyl-2-heptenyl)-3',4',7-trihydroxyflavanone	$C_{24}H_{28}O_5$	Flavonoids
14.974	281.1384	280.1312	Hymenoflorin	$C_{15}H_{20}O_5$	Prenol lipids
14.974	119.0852	118.078	alpha-Methylstyrene	C_9H_{10}	Terpenoid
14.977	516.2933	515.2872	Candoxatril	$C_{29}H_{41}NO_7$	Indanes
15.195	415.2123	414.2045	Armillarin	$C_{24}H_{30}O_6$	Organooxygen compounds
16.202	353.2697	352.2618	MG(0:0/18:3(6Z,9Z,12Z)/0:0)	$C_{21}H_{36}O_4$	Fatty Acyls
21.143	282.2792	281.2721	Dodemorph	$C_{18}H_{35}NO$	Oxazinanes
22.568	384.3465	383.3394	N-stearoyl valine	$C_{23}H_{45}NO_3$	Fatty Acyls
22.66	338.3422	337.3346	N-Cyclohexanecarbonylpentadecylamine	$C_{22}H_{43}NO$	Fatty Acyls
22.775	593.273	570.2842	Khayanthone	$C_{32}H_{42}O_9$	Prenol lipids
23.184	310.3111	309.3039	N-Hexadecanoylpyrrolidine	$C_{20}H_{39}NO$	Pyrrolidines
25.222	959.5668	936.5796	1,2-Di-(9Z,12Z,15Z-octadecatrienoyl)-3-(Galactosyl-alpha-1-6-Galactosyl-beta-1)-glycerol	$C_{51}H_{84}O_{15}$	Glycerolipids

Green Synthesis of AC-AgNPs

In this study, the optimization of AC-AgNPs synthesis is aimed at achieving the desired size, distribution, and physicochemical properties. According to Kredy (2018), temperature plays a significant role in controlling the reaction rate of nanoparticle formation. The addition of NaOH buffer, making the solution alkaline, enhances the reduction rate during silver nanoparticle formation. Notably, one of the observable indicators of silver nanoparticle formation is the color change of the solution from its initial light green to brown, as previously reported by Bakshi et al. (2015) (Figure 2).

The green synthesis of AC-AgNPs led to distinct color changes at temperatures of 60°C, 70°C, and 80°C. The solution's initial light green color gradually transformed to transparent yellow and eventually to brown as the synthesis proceeded. At each temperature, the formation of AC-AgNPs, as indicated by the brown color change, became visible at different time points. Notably, at 70°C and 80°C, the brown turbidity appeared earlier than at 60°C, showcasing the influence of temperature on the synthesis kinetics. Moreover, the introduction of NaOH not only affected the color but also raised the solution's pH from acidic to alkaline, indicating its role in the synthesis process.

After completing the nanoparticle synthesis, high-speed centrifugation was employed to precipitate the silver nanoparticles. The collected particles were then dried to obtain solid nanoparticles, with the corresponding yields recorded in Table 2. The results highlight that increasing the pH to an alkaline level and elevating the temperature generally led to higher yields. These findings align with Kredy's (2018) observations and indicate that optimizing pH and temperature can facilitate efficient redox reactions and enhance particle growth. The pH influences the stability and rate of silver nanoparticle formation, while temperature affects the reaction rate and overall yield.

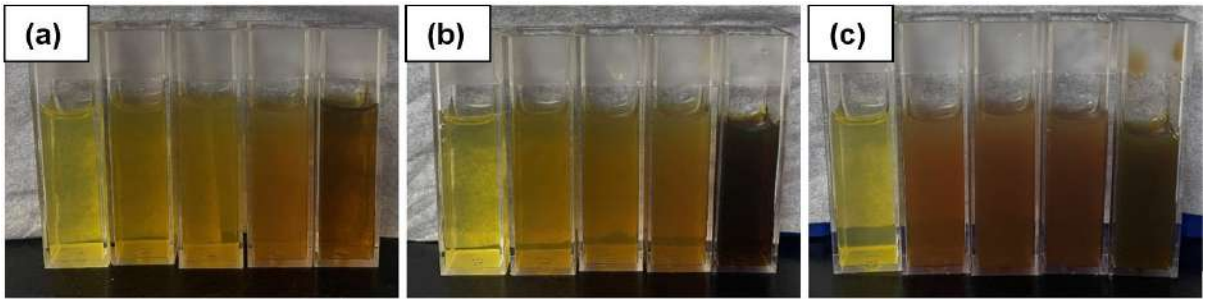

Figure 2: Results of Nanoparticle Synthesis (a) 60ºC, (b) 70ºC, and (c) 80ºC

Table 2: Results of optimization of synthesis temperature and pH on the yield of AC-AgNPs

Optimization conditions		Parameters
Temperature (ºC)	**pH**	**Dry yield (mg)**
60	4	7
	9	12
70	4	10
	9	9
80	4	6
	9	12

Characterization of AC-AgNPs

UV-Vis Spectrophotometer Analysis

The UV-Vis spectrophotometer analysis (Figure 3) reveals that absorbance increased over time at 60°C and 70°C, indicating a direct relationship between synthesis duration and the reduction rate. According to Oktaviani and Amrullah (2009), absorbance in the range of 400–450 nm is typically associated with silver nanoparticles (Ag^0), while silver ions (Ag^+) are linked with absorbance in the 370-400 nm range. The addition of NaOH at 60°C and 70°C resulted in a shift of the peak wavelength to 424 nm and 412 nm, confirming the formation of pure silver AgNPs. Lower temperatures led to larger nanoparticles with longer wavelengths, while higher temperatures yielded smaller nanoparticles. AgNPs did not form at 80°C, likely due to unsuitable conditions. This underscores the critical role of temperature and reaction time in silver nanoparticle synthesis (Ibrahim, 2015; Patra and Baek, 2014).

Particle Size and Zeta Potential Analysis

Particle size and size distribution analysis, performed with a Particle Size Analyzer (PSA) at temperatures of 60°C and 70°C and under varying pH conditions (4 and 9), revealed significant insights. The average particle size exhibited a direct relationship with pH alterations at both temperatures. At pH 4, the particle sizes were approximately 518.8 nm (60°C) and 502.6 nm (70°C). In contrast, at pH 9, the particle sizes decreased to 147.1 nm (60°C) and 135.5 nm (70°C). The smallest particles were achieved at a pH of 9 and a synthesis temperature of 70°C. This is in line with previous studies suggesting that higher pH leads to a faster reaction rate and smaller particle size (Marciniak et al., 2020). The accepted descriptive range for nanoparticle size is between 1-100 nm, as supported by numerous studies (Susanti et al., 2022). In the pharmaceutical field, nanoparticles are also defined as particles with diameters ranging from 10-1000 nm (Mazayen et al., 2022).

Zeta potential analysis, a crucial indicator of stability, revealed values of -40.4±8.79 mV (60°C) and -45.1±11.7 mV (70°C) after the addition of NaOH (Table 3). Zeta potential values exceeding +30 mV or falling below -30 mV indicate stability, with values within this range being unstable and prone to aggregation. The results demonstrate the stable conditions of AC-AgNPs, emphasizing their potential for cellular uptake and long-term structural integrity (Lin et al., 2014).

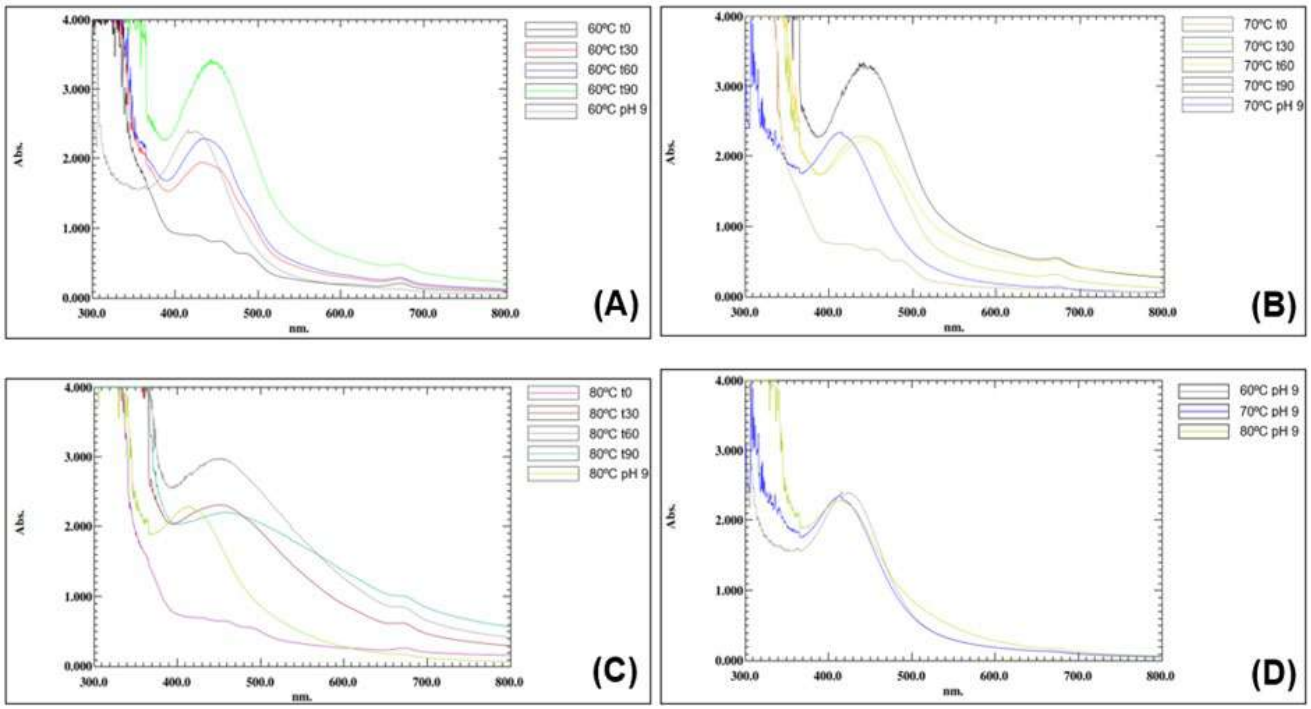

Figure 3: Comparison of solution absorbance at each temperature with time (A) 60ºC; (B) 70ºC; (C) 80ºC and (D) addition of NaOH

Table 3: Particle size and zeta potential of AC-AgNPs

Particle size analysis		
Temperature (ºC)	**pH**	**Z-Average (d.nm)**
60	4	518.8
	9	147.1
70	4	502.6
	9	135.5
Zeta potential analysis		
Temperature (ºC)	**pH**	**Zeta potential (mV)**
60	9	-40.4±8.79
70	9	Nd

Nd: not determined.

FTIR Analysis

The FTIR spectra of the synthesized silver nanoparticles (AC-AgNPs) are presented in Figure 4, revealing absorption peaks that confirm the presence of various functional groups within the plant extract involved in silver nanoparticle formation. The peak at 3363.42 cm^{-1} indicates the presence of hydrogen bonds (O-H) from alcohol compounds. Peaks at 2922.28 cm^{-1} and 2852.16 cm^{-1} signify (C-H) stretching, related to alkene groups. The absorption band at 1729.37 cm^{-1} represents carbonyl compounds like ketones, aldehydes, esters, or carboxyls, while the peak at 1607.9 cm^{-1} indicates double bonds or aromatic compounds with (C=C) stretching. The absorption bands at 1454.94 cm^{-1} and 1375.81 cm^{-1} confirm the presence of methyl compounds through bending vibrations (C-H). Furthermore, primary and secondary amine compounds with stretching (C-N) are validated by absorption bands at 1159.14 cm^{-1} and 1032.58 cm^{-1}. Previous research by Abdi et al. (2012) and Sankar and Abideen (2015) also employed FTIR to identify these functional groups, which have the ability to reduce AgNPs (Gnanadesigan et al., 2011).

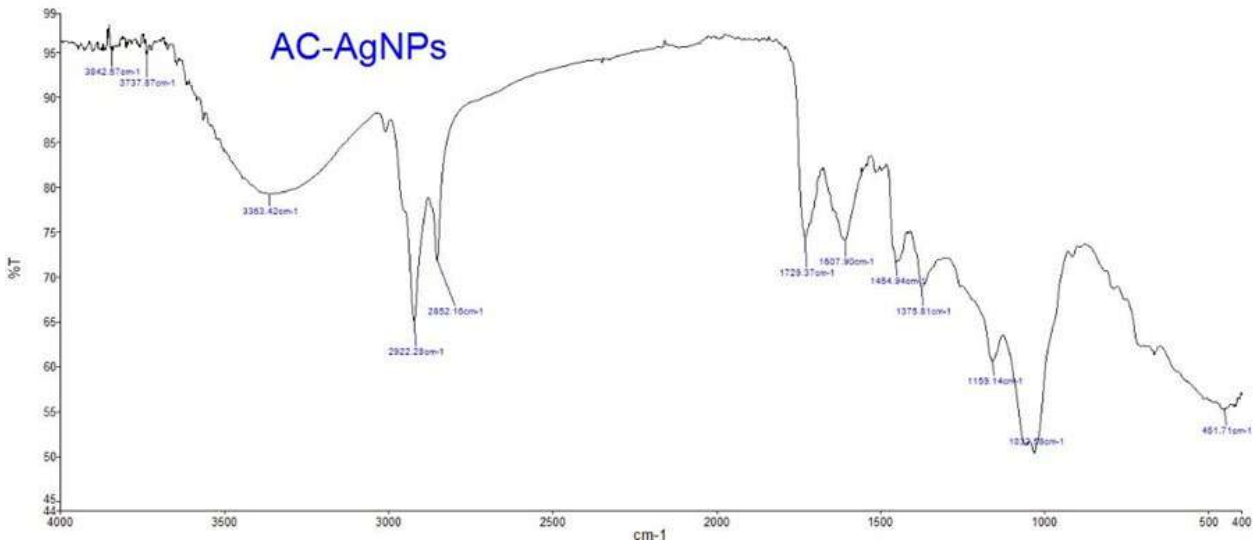

Figure 4: FTIR spectrum of AC-AgNPs

SEM Analysis

The morphology of the synthesized silver nanoparticles was characterized using Scanning Electron Microscopy (SEM) presents the observation results, indicating that silver nanoparticles produced with *A. corneri* leaf extract at 70°C and pH 9 exhibit a spherical shape. This spherical shape is typical of silver nanoparticles as they tend to form structures with the minimum surface area, which is the most thermodynamically stable configuration. The particle's growth kinetics during synthesis play a role in shaping these nanoparticles (Sau and Rogach, 2010).

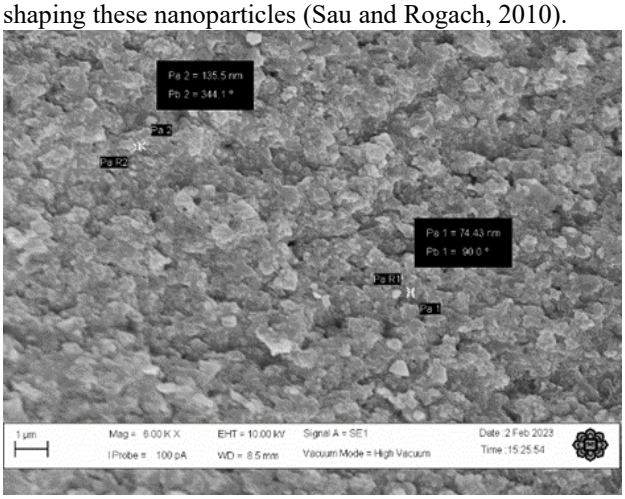

Figure 5: Morphology of AC-AgNPs Using SEM at Observation with a Magnification of 4.00 KX

Antimicrobial Activity of AC-AgNPs

The results of inhibitory zone measurements in the antimicrobial activity test are presented in Figure 6 and Table 4. The antimicrobial activity results revealed the effectiveness of the synthesized AC-AgNPs against various bacteria, emphasizing their inhibitory zones in comparison to control groups and $AgNO_3$. Testing the AC-AgNPs synthesized at 70°C with NaOH revealed inhibitory zones against all tested bacteria, with average inhibition diameters ranging from 8.33 to 10.00 mm.

Notably, these AC-AgNPs showed larger inhibition zones than the $AgNO_3$ solution, indicating the enhanced antibacterial efficacy of AC-AgNPs due to their smaller size, increased surface area, and more effective interaction with microorganisms. In contrast, *A. corneri* leaf extract and the negative control (DMSO 10%) exhibited no antimicrobial activity. The antimicrobial activity of AC-AgNPs was particularly potent against Gram-positive bacteria, with the largest inhibition observed against *S. aureus*.

This pattern aligns with previous studies, attributing the increased effectiveness against Gram-positive bacteria to differences in cell wall structure and membrane composition. While Gram-positive bacteria have peptidoglycan-rich thick cell walls that are sensitive to silver nanoparticles, Gram-negative bacteria's outer membrane structure, often fortified with lipopolysaccharides serves as a protective barrier, reducing the penetration of silver nanoparticles (Tang et al., 2013; Siddiqui et al., 2019).

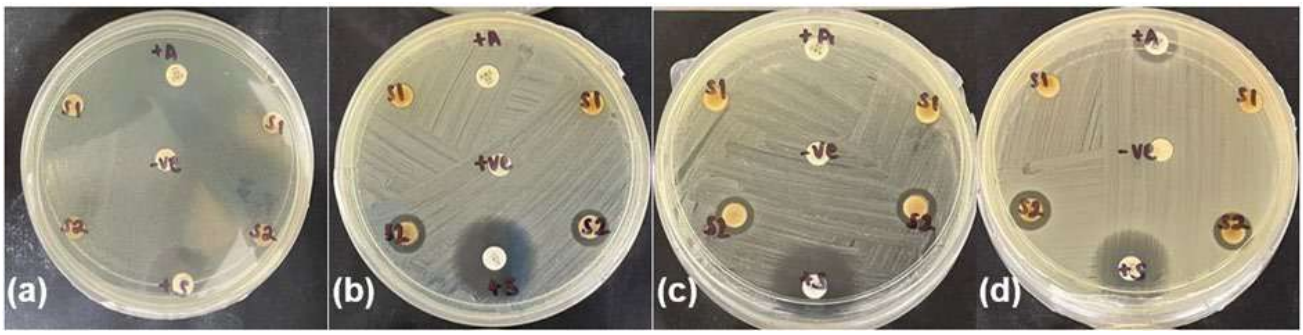

Figure 6: Antimicrobial activity of AC-AgNPs is indicated by +S on (a) *P. aeruginosa*, (b) *E. coli*, (c) *B. subtilis*, and (d) *S. aureus*

Table 4: Results of Inhibitory Zone Measurements in Antimicrobial Activity Testing

No.	Test Sample	Inhibition Zone (mm)			Average (mm)
		1	2	3	
Bacteria: *Pseudomonas aeruginosa*					
1.	Positive control (Streptomycin)	21	24	25	23.33
2.	Negative control (DMSO 10%)	0	0	0	0.00
3.	*A. corneri* leaf extract	0	0	0	0.00
4.	10 mM $AgNO_3$ solution	9	8	8	8.33
5.	AgNPs	7	9	9	8.33
Bacteria: *Escherichia coli*					
1.	Positive control (Amoxicillin)	7	0	0	2.33
2.	Positive control (Streptomycin)	24	24	15	21.00
3.	Negative control (DMSO 10%)	0	0	0	0.00
4.	*A. corneri* leaf extract	0	0	0	0.00
5.	10 mM $AgNO_3$ solution	9	9	8	8.67
6.	AgNPs	9.5	10	7	8.83
Bacteria: *Bacillus subtilis*					
1.	Positive control (Amoxicillin)	7	0	0	2.33
2.	Positive control (Streptomycin)	25	15	22	20.67
3.	Negative control (DMSO 10%)	0	0	0	0.00
4.	*A. corneri* leaf extract	0	0	0	0.00
5.	10 mM $AgNO_3$ solution	9	9	9	9.00
6.	AgNPs	10	7	11	9.33
Bacteria: *Staphylococcus aureus*					
1.	Positive control (Amoxicillin)	9	12	8	9.67
2.	Positive control (Streptomycin)	23	20	11	18.00
3.	Negative control (DMSO 10%)	0	0	0	0.00
4.	*A. corneri* leaf extract	0	0	0	0.00
5.	10 mM $AgNO_3$ solution	8	8	8	8.00
6.	AgNPs	11.5	10.5	8	10.00

Cytotoxic Activity of AC-AgNPs

The MTT assay was employed to evaluate the cytotoxicity of AC-AgNPs on MCF-7 breast cancer cells, revealing a dose-dependent reduction in cell viability. The IC_{50} value was determined to be 74.9 µg/mL, classifying AC-AgNPs' cytotoxic activity as moderate according to the US National Cancer Institute (NCI), as shown in Figure 7. This research underlines the potential of AC-AgNPs as a cytotoxic agent.

MCF-7 cells were chosen for their extensive use in cytotoxicity research and the reliability of results due to their genetic stability. Furthermore, the study draws upon previous literature supporting silver nanoparticles' effectiveness in cytotoxic assays against MCF-7 cells, showcasing their potential for cancer treatment (Ali et al., 2021; Firdhouse and Lalitha, 2015).

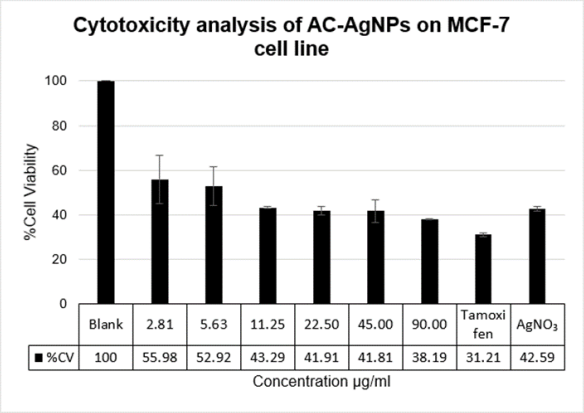

Figure 7: Cytotoxic Activity of AC-AgNPs Assessed by MTT Method

Conclusion

Green synthesis of silver nanoparticles using *A. corneri* leaf extract at a temperature of 70°C and pH 9 has successfully yielded spherical silver nanoparticles with a particle size of 135.5 nm and a zeta potential value of -45.1±11.7 mV. These nanoparticles have exhibited antimicrobial activity against both Gram-negative and Gram-positive bacteria, including *P. aeruginosa*, *E. coli* for Gram-negative bacteria, and *B. subtilis*, *S. aureus* for Gram-positive bacteria, falling within the moderate category. Furthermore, the silver nanoparticles have demonstrated cytotoxic activity against MCF-7 breast cancer cell lines, with an IC_{50} value of 74.9 µg/ml, also categorized as moderate. This research not only presents a green and eco-friendly method for synthesizing silver nanoparticles but also showcases their potential applications in combating bacterial infections and cancer treatment. The findings contribute to the advancement of nanotechnology and offer new possibilities in addressing critical health challenges.

Authors Contributions

Conceptualization, M.T.; methodology, M.T., D.S., and I.R.; conduct the research and collect the data, I.R.; writing—original draft preparation, I.R.; writing—review and editing, M.T., and M.N.; supervision, M.N., M.T., and D.S.; project administration, M.N., M.T., and D.S.; funding acquisition, M.T., and D.S. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

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

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PVA-PEG Hydrogel Incorporated with Cellulose Nanofibril of Oil Palm Empty Fruit Bunches and Antibacterial Agent Curcumin

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ABSTRACT

Introduction: The compelling characteristics of hydrogel films, resembling biological tissues, have sparked significant interest for their use in wound healing dressings.

Materials and methods: Cellulose nanofibrils (CNFs) and antibacterial agent of curcumin was incorporated into polyvinyl alcohol (PVA)-polyethylene glycol (PEG) hydrogel prepared via few cycles of freeze-thaw methods. The CNFs were extracted from oil palm empty fruit bunches (OPEFB) using alkaline-deep eutectic solvent (alkaline-DES) assisting with ultrasonication. The inclusion of CNFs and curcumin were optimized by varying their concentrations and moisture retention content (MRC) was determined as a response.

Results: The PVA-PEG/CNF-curcumin hydrogel achieved a 44.84% MRC via an optimal hydrogel composition comprising 6% (v/w) CNF and 5% (v/w) curcumin. Other physio-chemical properties of the developed hydrogel such as swelling behaviours, water vapor transmission rate (WVTR), hydrogel porosity, chemical structural, and antimicrobial resistance were determined as well to observe the effect of incorporating of CNFs and curcumin. The optimized PVA-PEG/CNF-curcumin hydrogel formulation demonstrated a swelling capacity of 26.44%, enhanced porosity of 48%, and a WVTR of 76.73 g/m²h, showed its potential as a promising dressing material with improved characteristics. The PVA-PEG/CNFs-curcumin hydrogel was observed to have high moisture retention content and demonstrated good resistance to gram-positive bacteria (*B. subtilis*) and lower resistance to gram-negative bacteria (*E. coli*).

Conclusion: In conclusion, the incorporation of CNFs and curcumin into PVA-PEG hydrogel demonstrated promising characteristics, highlighting its potential as an effective and versatile wound healing dressing with notable antimicrobial properties.

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Introduction

Over the years, polyvinyl alcohol (PVA) hydrogel had been applied widely in the in the medical field specifically for creating artificial organs, contact lenses, medication delivery systems, and wound dressings (Kamoun et al., 2021). PVA's similar physical characteristics make it compatible with human tissue. Due to its structure, which can absorb protein molecules, interact with minimum cell adhesion, and have no harmful effects, PVA membranes had been widely developed for biomedical purposes (Chen et al., 2017; Kamoun et al., 2021). However, PVA hydrogel still had some limitations that need to be improved and these limitations had restricted its usage in several medical and other industrial applications (Cui et al., 2021). Due to that, further studies are required to investigate its physiochemical properties like texture and swell ability, mechanical strength and find ways to improve it. While polyethylene glycol (PEG) is also another non-toxic polymer which is highly recognized for its high chain flexibility and superior temperature resistance, facilitating the development of hydrogels with relatively high swelling capacity (Cui et al., 2021).

Thus, in this study, the PVA-PEG hydrogel was reinforced with cellulose nanofibrils (CNFs) from empty fruit bunch (EFB) and incorporated with a natural antibacterial agent, curcumin. Previous studies reported that inclusion of nanomaterials specifically CNFs is expected to improve the mechanical properties of the PVA-PEG hydrogel (Butylina et al., 2016, 2020). A high crystallinity and aspect ratio of CNFs as well as apparently biocompatible nanomaterials make them attractive nanofillers in synthesizing the hydrogel with improved physical properties. Few studies on polymeric hydrogels incorporated with nanofillers showed that the incorporation of nanocellulose fibers can increase physical properties, thermal properties, and barrier properties (Chen et al., 2017; Cui et al., 2021; Kamoun et al., 2021). Furthermore, the incorporation of natural antimicrobial agent, curcumin may exhibit antibacterial properties which are important for polymeric hydrogel used for wound healing in preventing any wound infections (Alven et al., 2020).

Hence, the current study focuses to develop and optimize appropriate compositions of CNFs and curcumin in fabricating the PVA-PEG hydrogel via freeze-thaw process by response surface methodology (RSM). Physio-chemical properties of the optimized hydrogel was determined specifically moisture retention content (MRC), swelling behaviors, water vapor transmission rate (WVTR), hydrogel porosity, chemical structural as well as antimicrobial resistance to gram-positive bacteria *Bacillus subtilis* (*B. Subtilis*) and gram-negative bacteria *Escherichia coli* (*E. coli*).

Materials and methods

Chemicals and Biomass Raw Materials

The oil palm empty fruit bunches (OPEFB) fibers were collected from Kilang Sawit FELCRA Maran (Pahang, Malaysia). After cleaning the collected OPEFB with tap water, it was cut into small pieces, dried, and sieved to 100-micron size before kept in a sealed container at room temperature. All chemicals were purchased from Sigma-Aldrich, USA except for curcumin. All chemicals are analytical grades: 98% sodium hydroxide pellet (NaOH), 35% hydrogen peroxide (H_2O_2), oxalic acid dihydrate (OAD) (≥ 99 %), choline chloride (ChCl) (≥ 98 %), polyvinyl alcohol (PVA; M_W 89,000-98,000; 99% hydrolysed), polyethylene glycol (PEG; M_W 3350), and dimethyl sulfoxide (DMSO; ≥99.5%). Curcumin powder was used as an antibacterial agent for this research and was purchased from Merck.

Extraction of CNF from OPEFB

The extraction of CNFs from OPEFB followed the method described by Jafri et al. (2024), but with several modifications. The grounded EFB fibers were washed with hot distilled water at 80 °C for 1 h and dried in oven at 50 °C until constant weight achieved. Then, the extraction and purification of cellulose from EFB fibers comprised mainly of three steps: alkaline treatment, bleaching, and DES treatment. First, the EFB fibers were dissolved in a 4% (w/v) sodium hydroxide solution with a 1:30 (w/v) fiber-to-solution ratio. The mixture was agitated at 85 °C for 2 h. After that, the fibers were continuously rinsed in deionized water. After that, the pretreated fibers were dissolved in DES of ChCl-OAD at a fiber-to- solution ratio of 1:10 (w/v). The DES ratio of ChCl: OAD is 1:2. The suspension was then filtered, neutralized with deionized water, and dried for 24 h in an oven at 50 °C. The fibers were then bleached using 10% (w/v) hydrogen peroxide at 90 °C for 3 h with a 1:30 (w/v) fiber to solution ratio. This step was repeated three times until the fibers turned white. The obtained fibers were filtered, neutralized with deionized water, and oven-dried at 60 °C until their weight remained consistent.

Ultrasonicating the Extracted Cellulose

A specific amount of the extracted cellulose was mixed in deionized water (50 mL) to achieve concentration of 10% wt. The suspension was homogenized for 2 h ultrasonication at 80% (280 W) sonication amplitude to individualize CNFs. The ultrasonic homogenizer (FisherbrandTM Model 705 Sonic Dismembrator) fitted with a 1/8" sonication probe was used. The suspension samples were placed in an ice bath to prevent any damages to the samples as this process may generate some heat.

Preparation of PVA-PEG-CNF Hydrogel with Curcumin

The hydrogel preparation method was adopted from Altaf et al. (2021), with some modification. 15 (w/w%) of polyvinyl alcohol (PVA) was heated into 80 mL deionized water mixed with 20 (w/w%) of DMSO solution and stirred for 50 mins at 80°C. After that, 6 (w/w%) of polyethylene glycol (PEG) was added into the PVA solution, heated at 80 °C and stirred for 45 mins. Subsequently, varied amount of CNF ranging from 1 to 6 (w/w%) were added into the PVA-PEG solution, heated 80°C and stirred for 45 mins. Then, different contents of curcumin ranging from 0 to 5 (w/w%) were added in the PVA-PEG/CNF solution with the PVA crosslinking agent. Details of the optimization of PVA-PEG/CNF hydrogel with curcumin are shown in Section 2.5. The chemical crosslinking agent solution was prepared by adding 0.5 ml of glutaraldehyde (GA) and 0.05 ml of HCl in 10 mL of ethanol (Altaf et al., 2021). This solution was added to the PVA-PEG/CNF solution with constant stirring. Then 4 mL of glycerin was added with constant stirring and the solution was sonicated for 2 h. The hydrogel underwent three freeze-thaw cycles, each of which involved reducing the temperature to -20 °C for 24 hours and thawing for 4 h at room temperature. The hydrogels were then kept at 4 °C until further analysis.

Optimization of CNF and curcumin concentrations in PVA-PEG/CNF Hydrogel

The optimal formulation of the PVA-PEG hydrogel with constant concentration of PVA and PEG and varied concentration CNF (A) and curcumin (B) ranging from 1 to 6 (w/w%) and 0 to 5 (w/w%) respectively. Central composite design (CCD) using Design-Expert version 13.0.21.0 software (Stat-Ease Inc., Minneapolis, MN) was used and moisture retention content (MRC) is the response. Table 1 shows the CCD experimental design. The produced hydrogel was denoted as PVA-PEG/CNF-curcumin.

Moisture Retention Content

Prepared hydrogel membranes were cut into equal pieces of 2 cm × 2 cm with a thickness of 0.3 cm and then weighed. These samples were then placed inside an oven for 6 h at 40 °C. Later, they were removed from the oven and weighed again. Equation 1 was used to determine the moisture retention content (MRC) (Altaf et al., 2021).

$$MRC\ (\%) = (W_f/W_i) \times 100 \qquad (1)$$

Where, W_i is the initial weight and W_f is the weight of sample after 6 h of heating at 40 °C.

Swelling Behavior Test

The hydrogel was cut into equal pieces of 2 cm × 2 cm with a thickness of 0.3 cm and then weighed to measure the swelling behavior. The hydrogel samples were submerged in distilled water at room temperature for 24 h until the hydrogel reached its equilibrium swelling state. The hydrogel then be promptly removed, and any leftover solvent on the surface was swiftly blotted with absorbent paper before being weighed once more. Equation 2 used to calculate swelling behavior (Altaf et al., 2021).

$$\text{Swelling ratio } (\%) = \frac{(W_s - W_d)}{W_d} \times 100 \qquad (2)$$

Here, W_s is the weight of swelled hydrogel and W_d is the weight of dried hydrogel.

Table 1: CCD for each variable along with its corresponding ranges.

Coded	Variables	Levels		
		-1	0	+1
A	CNF concentration	1	3.5	6
B	Curcumin concentration	0	2.5	5

Antibacterial Test

The antibacterial activity was determined via disc diffusion method as described by Altaf et al. (2021), with little modifications to evaluate the effectiveness of generated hydrogels against bacteria. Several different bacterial strains were used for antibacterial studies such as *Bacillus subtilis (B. subtilis)* and *Escherichia coli (E. coli).* Inoculum was prepared by transferring a loopful of bacteria cells from the stock cultures into sterile Luria-Bertani (LB) broth placed in a 10 ml centrifuge tube which them kept at 4 °C. Then, this bacterial culture was incubated at 37 °C for 24 hr in an incubator shaker at 150 rpm. Then, 0.1 mL of overnight cell culture was inoculated on LB agar plate and were spread evenly on the surface of agar plates and left to dry for few minutes. A UV-sterilized equal-sized piece (1 cm × 1 cm) with a thickness of 0.3 cm of hydrogel sample was deposited on the bacteria-containing agar plates, and then incubated for 24 hr at 37 °C. Bacterial growth inhibition was determined by measuring the diameter of the inhibition zones around the hydrogel sample. Chlorohexidine (0.05%) and chlorohexidine gluconate (0.05%) were employed as controls.

Water Vapor Transmission Rate Measurement

To determine WVTR, 10 mL distilled water was poured into media glass bottles (small brown bottles). Measured the diameter of the mouth opening of the bottles. These bottles were covered with hydrogel membranes, wrapped through Teflon tape and then were weighed. These bottles were located at 50 °C inside an oven for 1 day. After 1 day, they were weighed again and WVTR (g/m2h) was evaluated using Equation 3 (Altaf et al., 2021).

$$WVTR = (W_i - W_t) / (A \times 24) \times 10^6 \quad (3)$$

Where, A is the area of the round opening of the bottle, W_i is the mass of bottle before heating and W_t is the weight of bottle after heating.

Hydrogel Membrane Porosity

The hydrogel samples were immersed into ethanol until they got flooded. Ethanol was used to wet the sample and immerse it. Equation 4 was used to determine the porosity (Altaf et al., 2021).

$$\varphi\ (\%) = (W_f - W_i) / (\rho V_f - \rho V_i) \times 100 \quad (4)$$

Where, W_1 and W_2 specify the weight of samples earlier and later having absorption in ethanol, respectively. V_1 is the volume of ethanol before absorption, V_2 is the volume of ethanol after absorption and ρ is density is the density of alcohol at room temperature.

Fourier Transform Infrared Spectroscopy (FTIR)

The structural changes that appeared in polymeric hydrogel membranes were evaluated by FTIR. The dried and impurity-free samples were subjected to FT-IR. The spectra were recorded by an FT-IR spectrometer equipped with an attenuated total reflection (ATR) unit in the range of 400–4000 cm^{-1}. The hydrogel samples were put directly into the FT-IR machine. All analysis was done at ambient temperature.

Results and Discussion

Statistical Design and Optimization Analysis

The impact of CNF and curcumin concentrations on the MRC of produced PVA-PEG/CNF-curcumin hydrogel were examined via RSM. Based on RSM, a total of 13 experimental runs were studied, and the experimental findings are displayed in Table 2.

Moreover, the experiment employed analysis of variance (ANOVA) to assess both the efficacy and significance of the created model. ANOVA, a statistical technique, was utilized to validate the model by gauging the significance of various factors and interactions within it (Rahmi et al., 2020; Rodrigues et al., 2019). The determination of the model's meaningfulness relied on the examination of the probability value, commonly known as the p-value. A model attains significance when its p-value is below 0.05, indicating a probable real impact on the studied system. The p-value played a crucial role in detecting and eliminating any irrelevant interactions or factors, ensuring the accurate representation of relationships within the data (Rahmi et al., 2020; Rodrigues et al., 2019).

The model attained was significance, evident in its low p-value ($p < 0.0001$) and an F-value of 33.91. Additionally, ANOVA affirmed that all terms (A-CNF concentrationand B-curcumin concentration) demonstrated p-values below 0.05, signifying their significance as model terms. This outcome establishes that variables such as CNF and curcumin concentrations significantly impact the MRC of the hydrogel. Furthermore, the evaluation of the model's fit to experimental data can be carried out using the regression coefficient R^2, adjusted R^2, and estimated R^2 (Bacha, 2022). A strong alignment between the model and the experimental data is indicated when these coefficients approach a value of 1 (Rahmi et al., 2020). Notably, the substantial R^2 of 0.8715, approaching 1, provides evidence

that the model fits the experimental data effectively. Moreover, the predicted R^2 of 0.7255 aligns well with the adjusted R^2 of 0.8458, differing by less than 0.02, affirming the model's accurate predictions (Thakur et al., 2020). The extent to which the developed model fails to predict variance is elucidated by the Lack-of-fit. The insignificance in the Lack-of-fit underscores the adequacy of the fitted model, indicating a robust correlation between process variables and output response (Shitole et al., 2019; Thakur et al., 2020).

Table 2: Experimental design.

Run	CNF concentration [A] (% v/w)	Curcumin concentration [B] (% v/w)	MRC (%)
1	3.5	2.5	41.84
2	3.5	2.5	40.39
3	3.5	0	39.21
4	1	0	33.77
5	1	5	40.56
6	1	2.5	38.38
7	3.5	5	43.29
8	6	2.5	42.59
9	6	0	41.79
10	3.5	2.5	41.67
11	3.5	2.5	42.31
12	3.5	2.5	41.56
13	6	0	44.84

The examination of the favorable statistical characteristics of the developed model can include parameters such as adequate precision, standard deviation, and the coefficient of variation (C.V.%). The model's adequate precision stands at 19.72%, indicating a sufficient signal for the response surface area (Rahmi et al., 2020; Rezvanian et al., 2017). A preferred response is indicated by an adequate precision value exceeding 4, denoting a suitable signal response to the process and its ability to move within the design space. Additionally, the model exhibits a favourable standard deviation of 1.07%, considered advantageous as it falls below 3%. A lower standard deviation signifies a close correspondence between the predicted result and the actual response (Rodrigues et al., 2019). The coefficient of variation (C.V.%) serves to assess the accuracy and validity of the investigations. The determined C.V.% level is 2.61%, less than the recommended threshold of 10%. Lower C.V.% values are preferred, as they indicate a more precise and rational experimentation approach (Altaf et al., 2021). Figure 1 illustrated the three-dimensional response surface graph of hydrogel MRC values.

According to Figure 1, the optimal formulation for the PVA-PEG/CNF-curcumin hydrogel resulted in a 44.84% MRC, achieved with 6% (v/w) of CNF and 5% (v/w) of curcumin. The enhanced MRC of the PVA-PEG/CNF-curcumin hydrogel at 44.84% can be attributed to the synergistic effects of incorporating CNF and curcumin into the PVA-PEG hydrogel matrix. The introduction of CNF, known for its high aspect ratio and hydrophilic nature, likely contributes to increased water absorption and retention within the hydrogel structure. The introduction of CNF into the PVA-PEG hydrogel structure creates a network with a large surface area, providing numerous sites for water molecules to interact and form hydrogen bonds (Rodrigues et al., 2019). The intertwined network of CNF within the polymer matrix may create capillary forces that enhanced water retention, preventing its easy evaporation and promoting sustained moisture content. This increased interaction facilitated water absorption and retention within the hydrogel, contributing significantly to the observed rise in MRC (Ahmed et al., 2018).

Furthermore, the inclusion of curcumin, a natural compound found in tumeric has antioxidant and anti-inflammatory properties thus were able to enhance its features in retaining moisture properties (Alven et al., 2020). Curcumin's hydrophilic characteristics may facilitate water absorption, while its interaction with the polymer chains could potentially enhance the hydrogel's overall water retention capacity (Alven et al., 2020). The combined effects of CNF and curcumin thus create a hydrogel formulation with improved moisture retention, offering potential applications in areas where maintaining high levels of hydration is crucial, such as wound healing (Alven et al., 2020; Miah et al., 2017).

Polyethylene glycol (PEG) further enhances the hydrogel's performance in moisture retention. PEG is a hydrophilic polymer that is well-known for its water-absorbing capabilities (Ahmed et al., 2018). By incorporating PEG into the hydrogel matrix, it provides additional sites for water molecules to interact and be retained within the structure. PEG's ability to form hydrogen bonds with water molecules and its compatibility with the other components in the hydrogel contribute to the overall enhancement of the hydrogel's water retention capacity (Cui et al., 2021). Therefore, the combined effects of CNF, curcumin, and PEG create a multifaceted hydrogel system with improved moisture retention, holding significant promise for applications in biomedical and cosmetic fields (Lv et al., 2019).

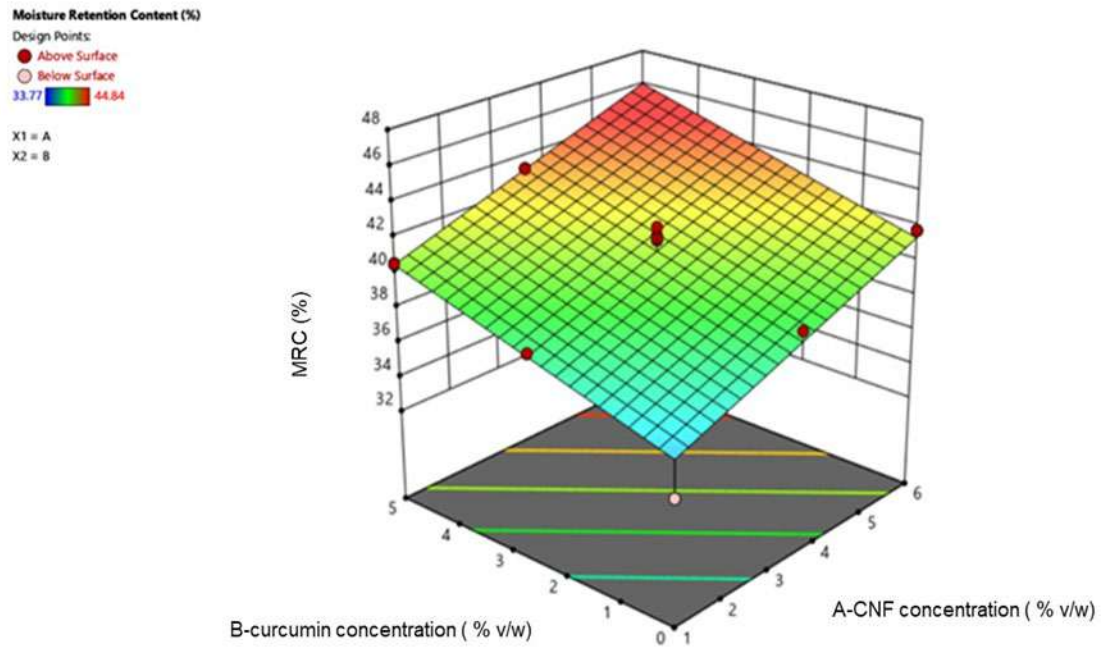


Figure 1: The surface plot showed the effect of CNF and curcumin concentrations on the MRC of hydrogel.

Swelling Behaviors of Hydrogel

The hydrogels, upon absorbing water, underwent swelling, displaying characteristics akin to biological tissue. This behavior is crucial in biomedical applications, particularly in wound care, where the hydrogel's ability to absorb and retain liquids on the wound surface is a key determinant of its effectiveness as a dressing material (Rahmi et al., 2020). The swelling capacity of a hydrogel is indicative of its ability to accommodate and retain moisture, a critical feature for promoting wound healing and maintaining a conducive environment for tissue repair (Lv et al., 2019). In the case of the optimized PVA-PEG/CNF-curcumin hydrogel formulation, the observed swelling capacity of 26.44% underscores its potential as a promising dressing material. This swelling capability indicates that the hydrogel can efficiently absorb and retain a significant amount of liquid, making it well-suited for applications where moisture retention on the wound surface is essential (Lv et al., 2019).

The swelling capacity of the PVA-PEG/CNF-curcumin hydrogel, reaching 26.44%, indicated a significant improvement and potential for biomedical applications. The incorporation of PEG into the hydrogel formulation played a crucial role in enhancing its swelling properties. PEG is a hydrophilic polymer known for its water-absorbing capabilities. By introducing PEG into the PVA-PEG hydrogel matrix, additional hydrophilic sites are provided, promoting increased water absorption. The intermolecular interactions between PEG and water molecules, facilitated by hydrogen bonding, contribute to the hydrogel's ability to swell and retain moisture efficiently (Cui et al., 2021). This synergistic effect results in a hydrogel with a substantial swelling capacity, making it well-suited for applications where controlled hydration and moisture retention are vital, such as in wound dressings (Ahmed et al., 2018).

Furthermore, the integration of CNF into the hydrogel formulation contributes to its swelling behavior. CNF, with its high surface area and hydrophilic nature, creates a network within the hydrogel matrix that enhances water absorption. The porous structure formed by CNF provides ample spaces for water molecules to be absorbed, leading to increased swelling capacity (Butylina et al., 2016, 2020). The combination of CNF and PEG creates a dual mechanism for water absorption, optimizing the hydrogel's ability to swell while maintaining structural integrity. The role of curcumin in the hydrogel formulation also adds to its swelling potential. Curcumin, being hydrophilic, contributes to the overall water-absorbing capabilities of the hydrogel. Additionally, the interactions between curcumin and the polymer chains further influence the hydrogel's structure, making it conducive to water absorption and retention (Alven et al., 2020). The collective impact of PEG, CNF, and curcumin creates a multifaceted hydrogel system with a notable swelling capacity, showcasing its potential for applications in wound care and other biomedical fields.

Hydrogel Membrane Porosity

The porosity of a hydrogel, indicating the extent of voids or empty spaces within its structure, stands as a vital parameter in the realm of wound dressings (Altaf et al., 2021). Its significance lies in its direct impact on the hydrogel's capacity to absorb and retain crucial fluids like wound exudate and water, pivotal for establishing a conducive, moist wound environment conducive to healing. An appropriate level of porosity allows the hydrogel to efficiently absorb these fluids, preventing leakage and maintaining the necessary equilibrium between moisture management and fluid retention (Altaf et al., 2021). Furthermore, porosity facilitates the exchange of oxygen and nutrients, which are crucial for tissue regeneration, and supports the removal of waste materials from the wound site (Rahmi et al., 2020). Hence, maintaining an optimal level of porosity emerges as a critical factor to ensure the hydrogel effectively plays its role in the context of wound care.

The porosity of the PVA-PEG/CNF-curcumin hydrogel was 48%, indicates a notable increase resulting from the incorporation of PEG, CNF, and curcumin into the PVA-based hydrogel matrix. Porosity is a critical parameter as it reflects the proportion of empty spaces or voids within the hydrogel structure, influencing its ability to absorb and retain fluids (Altaf et al., 2021). In this case, the inclusion of PEG, known for its hydrophilic nature, likely contributes to enhance water absorption capacity, leading to increase porosity (Ahmed et al., 2018). Additionally, CNF, with its high surface area and hydrophilicity, may play a role in creating a porous network within the hydrogel, further contributing to the observed increase in porosity (Butylina et al., 2016, 2020). Curcumin, being hydrophilic as well, may also contribute to the hydrogel's porous structure, although its specific role in porosity enhancement may depend on its interaction with other components (Alven et al., 2020).

In wound dressings, the optimal porosity range of 30% to 40% is often recommended (Altaf et al., 2021). This range is considered suitable for promoting a moist wound environment, ensuring effective fluid absorption, and facilitating gas exchange for tissue regeneration. Consequently, while the observed porosity of 48% in the PVA-PEG/CNF-curcumin hydrogel surpasses the typical range for hydrogels utilized in wound dressings, this higher porosity level presents potential advantages, including heightened fluid absorption and improved gas exchange (Baghaie et al., 2017). These attributes may prove beneficial in specific wound healing scenarios. However, it's also crucial to balance these advantages with the need for structural integrity and mechanical stability, as excessively high porosity might compromise the hydrogel's ability to maintain its form and adhere to the wound surface (Altaf et al., 2021).

WVTR of Hydrogels

The Water Vapor Transmission Rate (WVTR) of hydrogels is a critical parameter, particularly in the context of wound dressings. WVTR measures the ability of a material, such as a hydrogel, to allow the passage of water vapor through its structure. This property is crucial for wound dressings as it directly influences the regulation of the wound environment (Altaf et al., 2021). An optimal WVTR is essential to strike a balance between preventing excessive moisture buildup, which could lead to maceration and bacterial growth, and promoting sufficient moisture to facilitate the healing process (Baghaie et al., 2017). Hydrogels with an appropriate WVTR offer a breathable and permeable barrier that allows for effective gas exchange, ensuring the removal of excess moisture and promoting a conducive environment for tissue regeneration. In wound care, maintaining an ideal WVTR is thus paramount for supporting the healing process, preventing complications, and enhancing overall wound management (Altaf et al., 2021; Lin et al., 2019).

The WVTR of the produced PVA-PEG/CNF-curcumin hydrogel, measuring at 76.73 g/m²h, reflected the material's ability to allow the passage of water vapor. This substantial WVTR suggested that the incorporation of PEG, CNF, and curcumin into the PVA-based hydrogel has significantly enhanced its moisture permeability. PEG, being a hydrophilic polymer, likely contributed to increased water vapor transmission by promoting the absorption and movement of moisture through the hydrogel matrix. This enhanced hydrophilicity facilitates efficient water vapor transfer, leading to the observed higher WVTR (Ahmed et al., 2018; Cui et al., 2021).

The addition of CNF further augmented the WVTR of the hydrogel. The high surface area and hydrophilic nature of CNF create a network within the hydrogel, potentially forming pathways for water vapor diffusion. This network structure, coupled with the hydrophilic interactions between CNF and water molecules, enhances the overall permeability of the hydrogel to water vapor (Butylina et al., 2016; Carating et al., 2019). Moreover, the incorporation of curcumin, known for its hydrophilic properties, may contribute to the hydrogel's increased WVTR by influencing the interactions between water molecules and the polymer matrix, further facilitating moisture transmission (Alven et al., 2020; Miah et al., 2017).

This elevated WVTR is advantageous for enhancement moisture management by facilitating the efficient removal of excess water vapor from the wound site. This is essential in preventing the accumulation of moisture, which, if not properly controlled, can lead to complications such as maceration and bacterial growth. Moreover, an elevated WVTR supports a breathable environment, enabling the exchange of gases such as oxygen and carbon dioxide. This is vital for tissue regeneration, as it ensures an optimal oxygen supply to the wound area, fostering a conducive milieu for the healing process (Altaf et al., 2021; Carating et al., 2019). The combination of PEG, CNF, and curcumin has synergistically influenced the WVTR, making the PVA-PEG/CNF-curcumin hydrogel a promising candidate for applications where controlled moisture permeability is essential.

Anti-Bacterial Activity

The PVA-PEG/CNF-curcumin hydrogel showed robust resistance against gram-positive bacteria, specifically *B. subtilis.* However, the hydrogels showed comparatively lower resistance when tested against gram-negative bacteria of *E. coli.* This distinction in antibacterial efficacy between gram-positive and gram-negative bacteria underscores the specific impact of the hydrogel formulation on different bacterial types. The observed antibacterial properties suggest that the integration of curcumin, combined with the structural support provided by PVA-PEG/CNF, contributed to the hydrogel's ability to combat certain bacterial strains effectively.

A more pronounced and larger inhibitory zone on the *B. subtilis* plate compared to the *E. coli* plate were observed. This observation suggested that the developed PVA-PEG/CNF-curcumin hydrogels exhibited a stronger inhibitory effect against *B. subtilis* than against *E. coli.* The size of the inhibition zone serves as a visual representation of the antibacterial activity, with a clearer and larger zone correlating with more potent inhibitory effects (Alven et al., 2020; Miah et al., 2017). The PVA-PEG/CNF-curcumin hydrogel exhibited an inhibition zone measuring 16.0 ± 0.5 cm against *B. subtilis,* whereas no inhibition zone was observed for *E. coli*.

The antibacterial activity of curcumin within the PVA-PEG/CNF-curcumin hydrogel against *B. subtilis* can be attributed to the inherent properties of curcumin, a natural polyphenolic compound known for its antimicrobial effects. Curcumin has demonstrated antimicrobial activity against a broad spectrum of bacteria, including gram-positive strains like *B. subtilis*. Its mechanism of action involves disrupting bacterial cell membranes, interfering with cellular processes, and inducing oxidative stress, leading to the inhibition of bacterial growth. The incorporation of curcumin into the hydrogel matrix enhances its antibacterial properties, creating an environment where the release of curcumin molecules actively hinders the proliferation of *B. Subtilis* (Alven et al., 2020; Miah et al., 2017). The observed inhibition zone against *B. subtilis* reflected the successful deployment of curcumin's antibacterial potential within the hydrogel.

On the other hand, the absence of an inhibition zone against *E. coli* may be attributed to the intrinsic differences in the cell wall structure and membrane properties between gram-positive and gram-negative bacteria. Gram-negative bacteria possess an outer membrane that acts as a barrier, making them less susceptible to certain antimicrobial agents (Altaf et al., 2021). Additionally, the hydrogel's components, such as PVA-PEG and CNF, may interact differently with gram-negative bacteria, affecting the release or efficacy of curcumin in hindering their growth. The lack of resistance against *E. coli* might also be influenced by the specific concentration of curcumin within the hydrogel, as gram-negative bacteria often require higher concentrations of antibacterial agents for effective inhibition (Alven et al., 2020; Miah et al., 2017).

Furthermore, the variable responses against different bacterial strains highlight the selective nature of the PVA-PEG/CNF-curcumin hydrogel's antibacterial activity. While curcumin contributes significantly to the inhibition of gram-positive bacteria like *B. subtilis*, the observed lack of resistance against *E. coli* emphasizes the need for a nuanced understanding of the interactions between hydrogel components and bacterial species. Table 3 shows the summary of the antibacterial activity of the hydrogel compared to the controls. The chlorohexidine (0.05%), chlorohexidine gluconate (0.05%), water and curcumin were used as controls.

Table 3: Antibacterial activity data of hydrogel.

Compound	Inhibition zone diameter (mm)	
	E. coli	*B. subtilis*
Hydrogel (6% CNF, 5% curcumin concentration)	NA	16.5 ±0.5
Control 1 (Chlorohexidine 0.05%)	NA	17.0 ± 0.5
Control 2 (Chlorohexidine Gluconate 0.05%)	15.0 ± 0.5	16.0 ± 0.5
Water	NA	NA
Curcumin (5% Concentration)	NA	6.0 ± 0.5

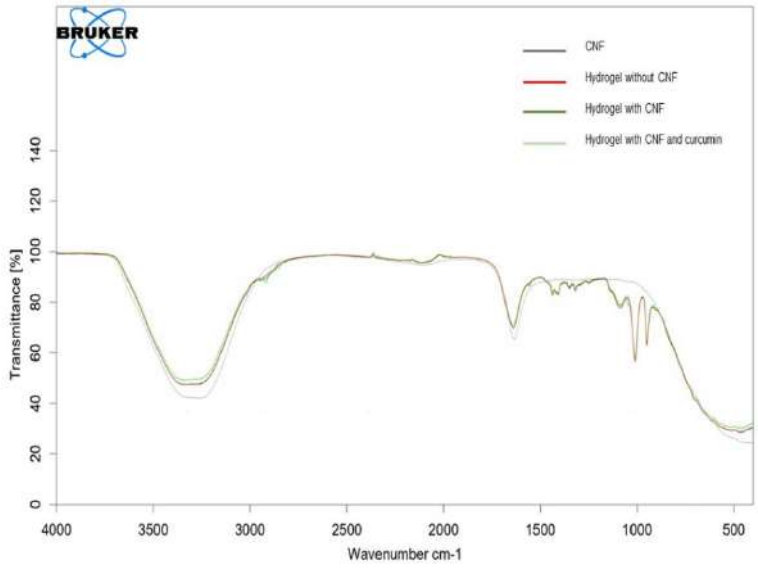
FTIR Analysis

The CNF exhibited peaks at 3262.62 cm^{-1}, indicating the presence of free hydroxyl groups. Additionally, for the hydrogel membranes, both with and without CNF, distinct peaks at 3321.96 cm^{-1} and 3320.04 cm^{-1}, respectively, were observed, signifying the existence of free hydroxyl groups. When the hydrogel membrane contained a 5% (v/w) concentration of curcumin, a robust peak at 3333.33 cm^{-1} was evident, indicating the presence of hydroxyl groups. This observation suggests that the introduction of curcumin into the hydrogel matrix results in a distinct alteration in the FTIR spectra, specifically in the hydroxyl group region, potentially signifying interactions between curcumin and the hydrogel components.

As indicated by Figure 2, the hydroxyl groups of PVA and PEG, engaged in hydrogen bonding interactions, exhibit -OH bond vibrations within the range of 1100-1450 cm^{-1} (Bacha, 2022). Notable peaks observed at approximately 1438 cm^{-1} and 2916 cm^{-1} signify the stretching vibrations of -CH and CH_2, respectively, with PVA being the primary contributor to these vibrations (Li et al., 2019). Additionally, the presence of both C=O and C=C bonds, inherent in the primary components of PVA, PEG, and LG, is evidenced by peaks at 1600 and 1800 cm^{-1} (Saleem & Saeed, 2020). It is important to highlight that the broadest band observed in each spectrum, spanning between 3200 and 3400 cm^{-1}, results from the stretching vibration of -OH (hydroxyl) bonds present in PVA and PEG (Bialik-Was et al., 2021; Cui et al., 2021).

As indicated by Figure 2, the inclusion of cellulose in the hydrogel is confirmed by the emergence of a distinct peak at approximately 1011 cm^{-1}, indicating the C-O and C-H stretching vibration (Yang et al., 2021). Notably, several significant peaks are detected at lower wavenumbers. The $-CH_2-$ group within the aliphatic PVA chain gives rise to C-H bonds, as affirmed by deformation vibrations in the 1430–1440 cm^{-1} region (de Lima et al., 2020).

Figure 2 depicted the Fourier transform infrared (FTIR) images of hydrogels.



Conclusion

This study aimed to develop a polyvinyl alcohol-polyethylene glycol (PVA-PEG) hydrogel reinforced with cellulose nanofibers (CNF) derived from oil palm empty fruit bunches (OPEFB), incorporating with curcumin as an antibacterial agent potentially to be used as wound dressings. The produced PVA-PEG/CNF-curcumin hydrogel showed the significant enhancements in MRC, WVTR, swelling capacity and hydrogel membrane porosity in the optimized hydrogel composition of 6% (v/w) CNF and 5% (v/w) curcumin. The presence of curcumin in the produced hydrogel showed significantly to the inhibition of gram-positive bacteria, *B. subtilis* only.

Authors Contributions

All authors collaboratively contributed to every aspect of the work presented in the manuscript. N. H. S. J. assisted in the laboratory work, analyzed the data and revised the manuscript. A. A. assisted in the laboratory work. D. N. J. was involved in supervision, planning, and editing the paper. S. I. S. S. involved in supervision and provided input and guidelines for the laboratory work. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare that the financial support received from the Ministry of Education Malaysia under grant FRGS19-091-0700 may be perceived as a potential competing interest.

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REVIEW ARTICLE

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Prospects of Artificial Intelligence in the Improvement of Healthcare Professions: A Review

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ABSTRACT

In 1956, the development of engineering science led to the birth of the first intelligent machines. This has led to the term Artificial Intelligence (AI) coined by a scientist named John McCarthy. The basic purpose of AI is to minimise human cognitive function. Advanced computer technology allows humans to do comparative critical thinking and simulate intelligent behaviour by producing intelligent modelling to solve boost and uplift cracking problems, imaging knowledge, and making a decision. Consequently, rapid analytical technique progress, powered by the increasing data availability in healthcare, has directed a paradigm shift in the healthcare system, especially in the analysis of medical imaging in the disease of oncology by detection of brain tumours. It helps the diagnosis of cancer stages based on the abnormal cell growth in the brain. AI is also important in diagnosis and treatment in other medical departments like dermatology, nephrology, ophthalmology, pathology, pulmonary medicine, endocrinology, gastroenterology, and neurology. In recent years, AI has played a key role in pharmacy, drug delivery, drug discovery, drug formulation development, hospital pharmacy, and poly-pharmacology. The term AI has a broad range of applications in medicine, medical statistics, medical diagnosis, human biology, pharmacy, clinical, and robotics. Automated selective medication uses the scientific task approach of pharmacists and is only possible by the use of AI. Algorithmic tasks reserved by using AI automation and such type of AI demonstration are better than pharmacists in comparison. In general terms of AI, the minimal intervention of humans implies intelligent behaviour through computer models. The invention of robots is deemed the starting point of the AI journey. It started with the introduction of robotic biosynthetic machines utilised to support medical personnel. In the meantime, an AI is capable of analysing complex clinical and medical data where a potentially significant data set relationship can be used for treatment and predicting outcomes in the case study and diagnosis.

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Introduction

Artificial Intelligence (AI) in the Healthcare Profession

The use of AI in medicine (Hamet & Tremblay, 2017) can be categorised into physical and virtual use. The physical use of AI is represented by the utilization of robotic machines to assist elderly patients and attending surgeons. Meanwhile, the virtual use of AI entails the informatics approach from a level of in-depth learning management information to control the health management system under the umbrella of physician treatment decision guidance and electronic health records. The mainstream area of AI covers different fields like cardiology, oncology, urology, and radiology. In the context of pharmacy, pharmacists should lead in designing, implementing, and evaluating ongoing AI-related technologies and applications directly affecting medication use and task processes. AI plays a unique role in new drug delivery and discovery by target nano-robots. AI also impacts ethical complexities and society of these wider applications of economic value, proof of medical utility, and interdisciplinary development strategies.

Ancient Calculation &Computation

The natural evolution process in the general computational methods is based on the natural selection mechanism and survival of the fittest in solving real-world problems. Genetic algorithms are used widely in ancient computational techniques. The scientist John Holland 1975 proposed a class of optimization algorithms and stochastic search based on the evolution of natural biological diversity (Holland et al., 1975). That work solves many problem solutions at hand, and the solution of the next generation will evolve. In this way, the population arrives at satisfactory solutions. The best-fit solutions added to the population and eliminated the inferior ones. In this way, by repeating the better element, repeated improvement will produce population survival and generate new problem solutions. The search for information in making medical decisions often requires big and complex searchers; for example, cell identification specialists decide whether the cell is malignant or not and provide a clear diagnosis of the malignancy. Genetic algorithms exploit the natural evolution mechanism to search efficiently in a given space. Through the application, they perform different tasks like diagnosis, medical imaging, prognosis, signal processing scheduling, and planning. The genetic algorithms principles are utilised to predict lung cancer (Jefferson et al., 1997), critically ill patients, melanoma, and the outcome of warfarin (Narayanan & Lucas, 1993), while the computerised analysis of mammography (Chan et al., 1998) and magnetic resonance imaging MRI segmentation calculation of brain tumours also count the efficacy of strategies treatment. It was also helpful for computerised analysis of 2-D images for cancer diagnosis (Handels et al., 1999).

Hybrid Intelligent Systems

Each AI technique has its strengths and weaknesses. Learning is mainly concerned with the neural network, fuzzy logic with imprecision, and ancient computation with optimization and search. The useful edge of these technologies is the combination in a complementary manner to evolve the hybrid intelligent system. These hybrid systems boost knowledge extraction from raw data and accommodate common sense use like a human reasoning mechanism. It impacts the adaption of rapidly changing and unknown circumstances. Examples of hybrid systems include fuzzy systems for designing Artificial neural networks (ANNs), genetic architecture neural networks, and genetic algorithms for automatic training. In this light, the hybrid system explores many diversified clinical scenarios such as tumour diagnosis, digital mammogram (Verma & Zakos, 2001) for microcalcification, coronary artery stenos as the diagnosis, calculation anaesthesia depth (Allen & Smith, 2001), and viability assessment of myocardial infection (Behloul *et al*., 2001).

Processing Of Natural Language

The machine understands the genetic data and images of Electrophysiological Data, and machine learning (ML) algorithms can directly perform quality control processes. Sometimes, large amounts of information, like clinical laboratory reports, physical examinations, operative notes, and hospital discharge summaries, are incomprehensive and unstructured for a computer program. Under this context, natural language processing extracts useful information from the narrative text, and it helps make many clinical decisions (Kantor, 2001). Natural Language Processing has two main pathways, i.e. 1- Classification 2- text processing.

Using historical databases-based text processing (Afzal *et al*., 2017), Natural Language processing identifies a series of keywords related to diseases in clinical notes. The keyword subsets are selected by monitoring their effects on the distribution of normal and abnormal cases. The approved standard keyword enriches the structured data-making to support the clinical decision. The pipeline Natural Language Processing has been developed for the assistance of clinical decisions, arrangement of treatment alerts, adverse effect monitoring, and so on. Natural Language Processing, introduced for reading chest reports of X-rays, would assist the system of antibiotics in indicating to the physician the need for therapy like anti-infective and also used in the auto-monitoring of laboratory-based adverse effects (Miller *et*

al., 2017).

Machine Learning &Deep Learning in A New Era

An advanced and modern technique like deep learning is an extension of a classical neural network (Devunooru *et al.*, 2021). Some think that deep learning with many layers is a neural network, as shown in Figure 1, which is not feasible with a neural network. The data work of deep learning can explore more complex non-linear patterns. The other reason for deep learning's popularity is due to the complexity of data and the increase in volume. According to the literature survey, the application of deep learning has been done nearly two times in recent years. The main use of deep learning in the medical radiology department depicts that images are high-volume and naturally complex (Altman, 2017).

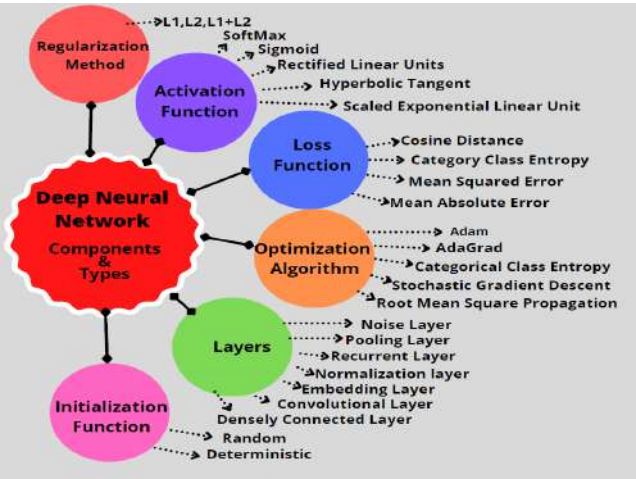

Figure 1: Medical AI (Development Stages) in Clinical Integration

Background of AI in Medicine

Promising applications of AI were identified in the middle of the twentieth century when scientists proposed and developed many clinical decisions to support the system (Miller., 1994). These approaches were very successful in 1970 (Shortliffe, 2012), for instance, in the field of ECG interpretation disease diagnoses (De Dombal *et al*., 1972), selection of appropriate treatment clinical reasoning interpretations (Barnett et al., 1987), and assisting the physician in the complex cases in generating diagnostic hypotheses as shown in Figure 2. However, the rule-based system is very expensive to build and can be fragile. Moreover, they require human authorization and decision rules, like a textbook (Roberts et al., 2017).

Higher-order interaction encoding is a difficult task in different segments of knowledge authorised by different experts, and the system performance is narrow due to the comprehensiveness of prior knowledge of medicine. The implementation of the system includes probabilistic and deterministic reasoning narrowed down to the clinical context, which prioritises the recommended therapy and diagnostic hypothesis (Deo, 2015).

Figure 2: AI Applications in different healthcare professions

The first-generation AI system emphasises expert medical knowledge and the rules of robotic decisions. The advancement in AI research in recent years has mainly focused on the method of machine learning accounting for data identification and complex interactions. It depends on the task intent to solve the primary algorithm of machine learning, which can be bifurcated into supervised and unsupervised categories. In the first category of the supervised method of machine learning work by gathering a large number of training cases (for example, fundus photographs) and desired label output (such as in the presence and absence of a doctor) and analysis of all patterns of labelled *Paris* of input and output, the advantage of the algorithm to calculate correct one output for a given input on new cases (Yu & Snyder, 2016).

Supervised machine learning algorithms are designed to identify the parameter of optimal and in models to reduce the deviation between their assumption of training cases. The observed associations in the case study are generalised to the case but not included in dataset training. The model test set can be evaluated by regression, classification, characterization of similar outcome labels, and most tasks supervised by the model of machine learning. The category of unsupervised learning links to the underlying pattern in unlabelled data for finding original data from sub-cluster, identifying data outliers, or for data production representation of low dimension. It is noted that the identification representation of low-dimension for labelled instances is achieved effectively in a supervised way. Moreover, machine learning-enabled AI application facilitates previously unorganised pattern discovery in data without a specific task for each task to

account for complex interaction in the input features.

Building framework machine learning provides a proffered framework for AI utilities (Roberts et al., 2017). Deep learning applications have mostly been driven by AI In recent years, which helps in the training of artificial neural networks and is a large source of labelled data. From the year 2012, image task classification has been improved by deep learning. Deep feed-forward network of neural enhanced by the deep residual neural network by permission of skip connections, preventing the model performance saturation (Goodfellow *et al.*, 2016). The latest neural networks contain more than 100 layers where multiple layers in the neural network model produce complex relations in input and output. However, more data is needed, and the latest architectural design requires more computation time to achieve optimal performance (Gill, 2017).

In designing neuronal mathematical operation and method regularization by different layers, such convolution layers are very beneficial for temporal relations. In contrast, the circular connection of recurrent layers uses model temporal events. The model performance increases many functions of initialization and activations (Jha & Topol, 2016), where the combination of components handles the neural data and enables a neural network with or without depending on temporal or spatial. Figure 3 presents an automated diagnosis of medical imaging, one of the most successful medical AI applications. Image-based diagnosis is front and centre in many medical specialities like dermatology, pathology, cardiology, ophthalmology, and radiology (Jha & Topol, 2016).

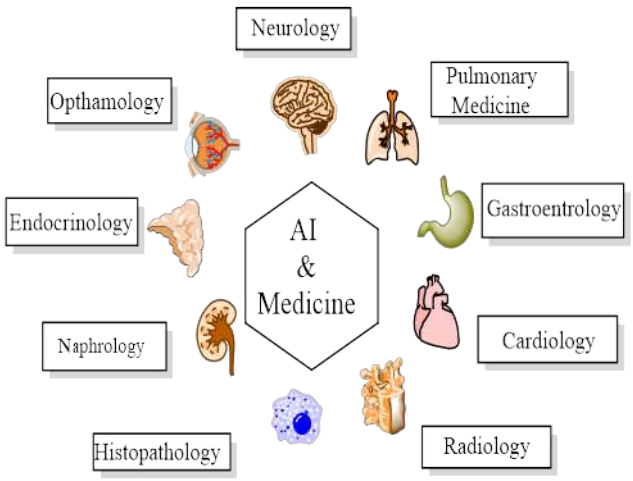

Figure 3: AI Importance in Medical Treatment

AI& Magnetic Resonance Imaging (MRI) Segmentation

MRT for tumour segmentation is an example of modern AI technology used in tumour analysis (Deshmukh & Jadhav, 2014). Different tumour segmentation method of brain MRI image has different advantages and disadvantages. The recent segmentation method is done through various efficient and suitable parameters for quantitative analysis (Liew, 2018). MRI allows human body image structure extraction for information about the patient (Gordillo *et al*., 2013). This advanced computerised technology helps specialists obtain physicians' information and measure tumour growth (Mukherjee, 2017). MRI imaging technology is also used to explore brain anatomy and injury at high-resolution quality with a large amount of data (Dogra *et al*., 2020).

A technician does not easily analyse a large amount of data and manually extracts the region which is affected (Dignum, 2018). Hence, different steps for segmentation are applied for a detailed description of anatomical regional segmentation in MRI. The development of modern techniques and technology allows images to be captured and analysed more precisely and accurately to resolve the degree of complexity (Myronenko, 2018). For the accurate and high-resolution imaging of brain disease diagnosis, the technique of MRI is delicate to the characteristics of the disease (Xuan & Liao, 2007). For the investigation of the processing of the medical image from the patient, the image set was collected. Enhancement techniques can increase the quality of the image. The method of image segmentation is important and accurate in the procedure of imaging processing (Pereira *et al*., 2016). The extraction of focal factures from segmented and enhanced images is the final step of image processing linked to post-imaging processing.MRI imaging is a non-invasive technique of imaging in medical science for intensive discussion.MRI of the brain and tumour segmentation, The fully convolutional neural network (FCNN) and conditional random field (CRF) are both combined; the only difference in groups and similarity index can be measured by global criterion and graph partition problem (Wadhwa *et al*., 2019). The segment image calculation is based on the method utilization. MRI images of the brain tumor are helpful in the diagnosis and stage of the tumor.

For the analysis of brain tumours, the MRI graph cut method is very common due to the processing and analysis of tumour images (Salem *et al*., n.d.). This is obtained from the results of experimentation. In the early step of Mathematical Morphological Reconstruction (MMR) by use of a computer for the diagnosis of a tumour (Fischl *et al*., 2002). The removal of artefacts and noise in the pre-processing stage of image segmentation helps verify the feature of texture and statistical findings to determine whether the brain tumour is benign or malignant (Figure: 4). Therefore, the MRI is a good technique for tumour diagnosis (Tustison *et al*., 2015).

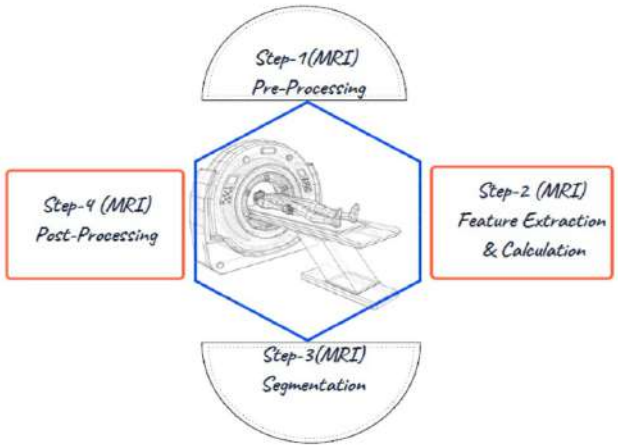

Figure 4: View of Al &Magnetic Resonance Imaging Segmentation

Drug Discovery and Artificial Intelligence

In pharmaceutical chemistry, Klopman (1984) introduced the structure-activity relationship (SAR) study in organic molecules (Klopman, 1984). It is based on the computer-automated structure evaluation, where the KNL code helps in reorganising the structure of the molecules. The coding routine of the molecules is based on a linear coding routine and is further recognised automatically by analysing biophores along with tabulation. The actual structure of the molecule is responsible for the molecule's statistical and biological activity. This method was applied to the tumour cell to identify aromatic hydrocarbon of polycyclic (Ketoxime carbamate-pesticides activity and tumour cell study of N-nitrosamine in rats. This was followed by Cherksaov, who generated the idea of small chains of peptide preparation with the broad-spectrum activity of antibiotics to gather information accumulated in chemical biology. Array technology uses peptides to form the unique composition of a peptide chain that is very active and is designed randomly by two libraries of a peptide containing 9-amino acid.

Agatonovic-Kustrin & Beresford (2000) conducted random sampling and prepared one million virtual peptides in the models, and their activity was observed successfully. It was found that these peptides are highly effective against top candidates and have the property of multidrug resistance along with better antibiotic activity than four commonly used antibiotics. Moreover, it is more effective as compared to antimicrobial peptides that are an advanced candidate in clinical and more powerful against bacterial strain *Staphylococcus aureus* infection when tested in in-vivo models of the animal.

Other studies, like Aliper *et al.* (2016), proposed novel approaches for utilising the deep neural network for the estimation of several drug pharmacological activities. 678 drug samples were used, and A549, MCF-7, and PC-3 were used in the cell line. Researchers obtained training on deep neural networks to study the therapeutic use of several drugs using gene expression data. The study reported that the deep neural networks offered high accuracy in classifying drugs into different therapeutic categories. It also reflected the advantages of data gained from human cell line experimentation.

AI Importance in Pharmacy

Previously, a pharmacist's responsibility is to ensure that prescriptions (Vyas *et al*., 2018) indicate the right amount of medicine, specifically when multiple medications are dispensed and ensure no drug-drug interactions between the different types of medicine (Vyas *et al*., 2018). These scenarios have changed dramatically in the last five years. Technology advances have increased doctors' trust in robots and AI in handling big data. Subsequently, companies and institutes employ robots (Vyas *et al*., 2018) to perform tasks previously performed only by humans.

Pharmaceutical companies isolate a large number of molecules with the potential to combat specific kinds of diseases(Khanna *et al*., 2020). However, as such, they have no tools at their disposal for identification. The heavy cost and long period required for drug development and production by pharmaceutical companies. AI allows pharmaceutical companies to reduce the time and cost of drug development increase the return on investment, and it may affect the end-user cost (Khanna *et al*., 2020); (Liu *et al*., 2017). As shown in Figure 5, the main advantage of AI is that it is faster, more accurate and superior in analysing data as compared to humans, and AI can analyse big data that normally do not fit conventional computers. AI has been mostly used in areas of research such as gene mutation, where big data is used to obtain important information (Ulfa *et al*., 2019 and Vyas *et al*., 2018).

Figure 5 Al Advantages &Disadvantages in the healthcare profession

AI Tools & Pharmacy Profession

Different AI tools have been utilised in the pharmaceutical industry to meet the current needs. These AI tools have presented encouraging outcomes (Knebel & Greiner,

2003) and gained popularity in the industry.

Oncology by Supercomputer Waston

IBM, a renowned computer company, has designed a supercomputer named Waston (Rouse, 2017). It combines AI and advanced sophisticated software to answer complex questions. For instance, in cancer treatment, Waston (Bambauer, 2017) assists oncologists in making better decisions on cancer treatment plans. It works based on patient clinical information from expertise, big data networks, and treatment options (Ross & Swetlitz, 2017). It is capable of analysing both the context and the meaning of any data type presented in both properly structured and unstructured clinical notes and reports. Waston gathered data on critical patient information and executed it in the write-up in English format, which provided a true treatment plan for the patient (Khatib & Ahmed, 2020). It also collaborates critical attributes from the patient file for clinical research, external research, and big data after determining the most suitable treatment plan for the patients (Boyd & Chaffee, 2019), as shown in Figure 6. Waston supercomputer also has a big array of information from literature by MSK, 290 medical-related journals, nearly 200 textbooks, and twelve million text pages (Bambauer, 2017).

Figure 6: Computer Base Tools Used in AI & Drug Discovery

Robot Pharmacy

The main aim of robotic applications in the field of pharmacy is to enhance patients through identifying and prescribing medications. Recent reports by UCSF Medical Center documented the use of robotic technology to prepare approximately 3.5 million medication doses without error. This proves that modern robotic technology provides better and more accurate medication delivery compared to humans. Robotics help prepare medicine for cancer treatment and assist in the preparation of injections and oral preparations. Nurses and pharmacists in modern medical centres, such as UCSF, can optimise their expertise by mainly focusing on patient care and working with physicians (Khatib & Ahmed, 2020).

Conclusion

The progress in Al in the field of healthcare, made possible by the large-scale technology development has shown considerable progress. The application of AI, specifically in clinical, pharmaceutical, imaging technologies, clinical and drug discovery, is still in the primary stage of implementation and validation. AI has not yet been explored fully due to the diversity in healthcare professions, and very limited assessment cases have been identified. While medicine, pharmacy, drug discovery, and imaging technologies require more practical outcomes and more testing, studies have found that such an intervention helps medical practitioners identify connections and sources of the anticipated outcomes. AI is a critical technological area in the advancement of the healthcare system where healthcare professionals can integrate AI and human deliberation in all medical fields for better identification and decision-making. The advantages of AI in healthcare should be considered to steer its future growth.

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Authors' contribution

T.A.K: Guidance & supervision and contributed final draft preparations. M.U.M & S.N.A.B: Contributed reference management and writing of introduction. M.M.A: Contributed to figure development. M.A.N: Contributed to reviewing & editing. All authors have read and agreed.

Conflict of Interest

The authors declare no conflict of interest.

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